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Tail-extension following the termination codon is critical for release of the nascent chain from membrane-bound ribosomes in a reticulocyte lysate cell-free system

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ABSTRACT

Nascent chain release from membrane-bound ribosomes by the termination codon was investigated using a cell-free translation system from rabbit supplemented with rough microsomal membrane vesicles. Chain release was extremely slow when mRNA ended with only the termination codon. Tail extension after the termination codon enhanced the release of the nascent chain. Release reached plateau levels with tail extension of 10 bases. This requirement was observed with all termination codons: TAA, TGA and TAG. Rapid release was also achieved by puromycin even in the absence of the extension. Efficient translation termination cannot be achieved in the presence of only a termination codon on the mRNA. Tail extension might be required for correct positioning of the termination codon in the ribosome and/or efficient recognition by release factors.

1. Introduction

Cell-free translation systems are utilized for various objectives, such as the production of cytotoxic proteins [1]; incorporation of non-natural amino acids into polypeptide chains [2]; and as experimental systems for protein folding [3], including cotranslational folding [4], post-translational protein localization to mitochondria and peroxisomes [5], and studies of protein conformations [6]. Cell-free systems supplemented with vesicles derived from rough endoplasmic reticulum membrane (RM) provide an ideal tool for the biochemical analysis of cotranslational translocation across the membrane. These systems can reproduce complex processes for membrane insertion of proteins by membrane-bound ribosomes. The RM vesicles are prepared from dog pancreas through differential centrifugation and density fractionation. In this system, newly synthesized polypeptide chains with signal peptides or signal-anchor sequences are cotranslationally targeted to RM vesicles and transported to the lumenal side of the membrane or inserted into the membrane. N-linked glycosylation is also reproduced. Among various cell-free systems, the reticulocyte system provides an efficient system for these processes because it contains various cytosolic factors for protein transport, signal recognition particles, and hsp70 chaperons. Synthesized polypeptide chains are almost completely targeted to and inserted into the RM. The cell-free experiments have revealed various dynamic aspects of nascent polypeptide chains on the RM, e.g., charges of nascent chains moving through the translocation channel tentatively arrest movement [7,8], and hydrophobic segments and positive charges cooperate on the membrane to induce the transmembrane orientation of the protein [9]. In some experiment, we utilized RNA lacking termination codon, by which the synthesized nascent chain is retained in the ribosome peptidyl transferase center (PTC) as peptidyl-tRNA. Using this system, we demonstrated various intermediates of membrane insertion and nascent chain folding [10,11]. Cell-free experiments have also revealed the dynamic interactions between ribosome and protein translocation channels [12,13] and novel modes of membrane protein insertion into the endoplasmic reticulum membrane [14,15].

Membrane-bound ribosomes provide a convenient system for easily monitoring nascent chain release from the ribosome; when a potential glycosylation site is incorporated near the termination codon, the site is accessible to the glycosylation enzyme in the lumen only after release of the polypeptide chain from the ribosome. In our ongoing studies of the function of membrane-bound ribosomes, we found that nascent chain release is regulated not only by the presence of the termination codon but that it also depends on the context of the termination codon. Here we examined the effects of the context of the termination codons on the efficiency of their function in membrane-bound ribosomes.

Abbreviations: PTC, peptidyl transferase center; RM, rough microsomal membranes; RNC, ribosome nascent chain complex.

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2. Materials and methods

2.1. Materials

RM were prepared from dog pancreas and treated with EDTA and then with *Staphylococcus aureus* nuclease (Roche) to remove endogenous mRNAs, as described previously [16]. Puromycin (Sigma) and DNA-manipulating enzymes (Takara and Toyobo) were obtained from the indicated sources.

2.2. Construction of model proteins

A model polypeptide was derived from rat serum albumin as described previously [9]. The cDNA fragment of the model protein was subcloned between the XbaI and ApaI sites of pRcCMV. The cloned DNA fragment contained the Kozak sequence at the 5′-region. The glycosylation sites, Asn^{67}-Ser-Thr and Asn^{180}-Ser-Thr, were included. Several residues around the 2nd site were optimized for efficient glycosylation by the oligosaccharyl transferase in the lumen.

2.3. Cell-free transcription

Template DNA for cell-free transcription was generated by polymerase chain reaction using the following primers. The forward primer had a sequence at the 50-base upstream of the T7-RNA polymerase promoter of pRcCMV: GCAGAGCTCTCTGGCTAACT.

Reverse primers, including the termination codon and various tail-extensions, were used as follows. To generate the ribosome nascent chain complex (RNC), the termination codon and the tail-extensions were omitted. Three nucleotides corresponding to the termination codon, TAA, are underlined, and were changed for other termination codons where indicated.

GGTGTAAGCAGCAGTGCTATT for RNC
GGATATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 13
ATATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 11
ATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 9
TTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 7
TGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 6
GTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 5
TATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 4
ATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 3
TATTAGGTGTAAGCAGCAGTGCTATT for TAA + 2
ATTAGGTGTAAGCAGCAGTGCTATT for TAA + 1
TTAGGTGTAAGCAGCAGTGCTATT for TAA + 0

Polymerase chain reaction was performed using KOD-Plus DNA polymerase (Toyobo) according to the supplier's manual. The reaction cycle was as follows: initial denaturing at 95 °C for 2 min; cycle reaction, including denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. The amplified DNA fragment was purified by agarose gel electrophoresis, band excision, and column purification using the DNA Gel band purification system (Qiagen). The transcription mixture of 50 μl included 50 units of T7 RNA Polymerase (Takara), 40 mM Tris–HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 0.5 mM 4NTPs, RNase Inhibitor (Promega), and 1 μg DNA fragment, and was incubated at 37 °C for 2 h. The RNA products were subjected to phenol/chloroform extraction, then precipitated with ethanol precipitation, dissolved in 20 μl of water, and stored until use at −20 °C.

2.4. Preparation of reticulocyte lysate

The reticulocyte lysate was prepared from male New Zealand White rabbits as previously described [17]. All procedures were performed in accordance with the guidelines established by the University of Hyogo for the care and use of experimental animals. Blood from the phenyl hydrazine-treated rabbits was immediately mixed with heparin solution (Mochida) and transferred to an ice-cold 50-ml centrifuge tube, then centrifuged using a swing rotor centrifuge at 2000*g* at 4 °C for 5 min (3500 rpm, HITACHI, T4SS31 rotor). The supernatant, including the white fluffy layer on the precipitate, was removed and the pellet was resuspended up to a 50-ml total volume with buffer A (0.14 M NaCl, 5 mM KCl, 7.5 mM $Mg[OAc]_2$). The cells were subjected to the same washing step for two more times, then vigorously mixed with an equal volume of ice-cold water for 2 min. The lysed cell-suspension was centrifuged at 20,000*g* (14,000 rpm, HITACHI, R18A rotor) at 4 °C for 10 min. The supernatant was treated with 100 U/ml *S aureus* nuclease in the presence of 1 mM $CaCl_2$ and the reaction was terminated with 2 mM EGTA. The nuclease-treated lysate was divided into 50 μl aliquots, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.5. Cell-free translation in reticulocyte lysate

Cell-free translation of in vitro-synthesized mRNAs was performed essentially as described previously [18], except that translation reactions with RM contained castanospermine to prevent heterogeneity of the products due to trimming of the core sugar chain. The translation reaction included 100 mM potassium acetate (KOAc), 1.2 mM magnesium acetate ($Mg[OAc]_2$), 32% reticulocyte lysate, castanospermine (20 μg/ml), and 15.5 kBq/μl [^{35}S]-EXPRESS protein-labeling mix (Perkin Elmer). Translation was performed at 30 °C for the indicated time period in either the absence or presence of RM. The translation reaction mixtures were treated by RNase in sample buffer and subjected to SDS–polyacrylamide gel electrophoresis. Radio-labeled polypeptide chains were visualized with an imaging analyzer (BAS1800, Fuji Film), and quantified using Image Gauge software (Fuji Film). In the presence of RM, the synthesized nascent chains were almost completely targeted to membrane vesicles and glycosylated. The translocation percentage was estimated as the percentage of the diglycosylated product among monoglycosylated and diglycosylated products.

3. Results and discussion

3.1. Function of the termination codon depends on tail-extension

To examine the effect of tail-extension on the termination codon function, we generated two classes of mRNA. One class possessed no tail-extension and the open reading frame ended just at the termination codons (Fig. 1A, +0). Others possessed a 13-base tail-extension (Fig. 1A, +13) downstream of the termination codon. As a control, one mRNA ended at the open reading frame and possessed no termination codon. In this situation, the nascent chain is retained in the ribosome as peptidyl-tRNA and forms the static ribosome-nascent chain complex (RNC) (Fig. 1B, RNC). The model protein consisted of a rat serum albumin backbone of 186 residues, an N-terminal signal peptide, and artificial potential glycosylation sites (Figs. 1A and 2). The glycosylation sites were optimized for the glycosylation reaction. When the model protein is synthesized in the presence of RM, the N-terminal signal peptide initiates translocation of the nascent chain through the translocation channel in the microsomal membranes. The signal peptide is cleaved off during elongation. The upstream glycosylation site (Asn^{67}) is translocated in the earlier phase of synthesis and glycosylated irrespective of the release of the nascent chain from ribosome (Fig. 1B, center). This glycosylation is a decisive indication of translocation initiation. On the other hand, the downstream second glycosylation

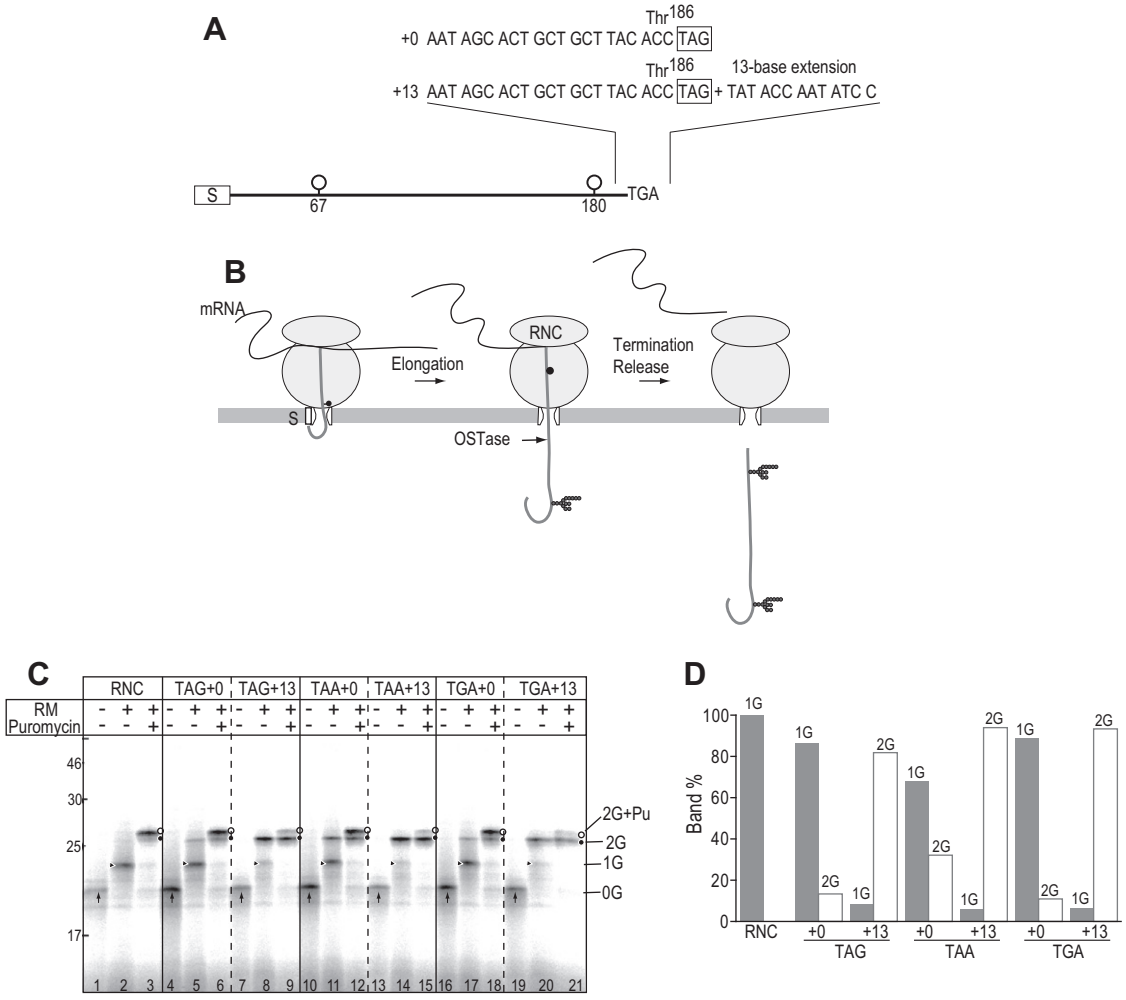

Fig. 1. Tail-extension affected nascent chain release from the ribosomes. (A) Model mRNAs possessed no tail-extension (+0) or a 13-base (+13) extension after the termination codon. The encoded model protein included the signal peptide (S) at the N-terminus and potential glycosylation sites (open circles) at the indicated positions of the rat serum albumin backbone. (B) Assessment of polypeptide chain release from membrane-bound ribosomes. When the model protein was synthesized in the presence of RM, the nascent chain ribosome complex (RNC) is targeted to the RM and the N-terminal signal peptide is cleaved by signal peptidase in the ER lumen and the former glycosylation site (Asn^{67}) is glycosylated (indicated by the fork) by oligosaccharyl transferase (OSTase). Once the polypeptide chain is released from the ribosome, it is fully translocated and the downstream site (Asn^{180}) is glycosylated. Diglycosylation indicates the chain release from ribosome. When the mRNA includes no termination codon, the nascent chain is statically retained in the ribosome as peptidyl tRNA to form the ribosome nascent chain complex (RNC). (C) Membrane translocation and chain release assay of model proteins. Each mRNA possessed one of the termination codons (TAG, TAA and TGA). The model proteins were translated in a cell-free system in the absence or presence of RM for 15 min. An aliquot of the translation product was treated with puromycin to fully release the nascent chain. The puromycin adduct of the diglycosylated forms (2G + Pu, open circles) is slightly larger than the normally released diglycosylated form (2G, dots). Monoglycosylated forms (triangles) and unglycosylated forms (arrows) are indicated. (D) Percent of diglycosylated (2G) and monoglycosylated (1G) forms in the absence of puromycin strongly depended on the context of the mRNA. In the case of RNC, the polypeptide chain was not released and remained in the monoglycosylated form. Release from the ribosome by the termination codons was affected by the tail-extension.

site (Asn^{180}) is glycosylated only when the nascent chain is released from the ribosome. When the nascent chain is retained in the ribosome, the downstream site is retained in the ribosome and is not accessible to oligosaccharyl transferase. Thus, the glycosylation status is a sensitive indicator of translation termination; diglycosylation is a direct indication of the release of the nascent chain from the ribosome.mRNAs with or without the 13-base tail-extensions were generated by in vitro transcription using DNA templates produced by polymerase chain reaction as described in the methods. The templates included the T7-RNA polymerase promoter, the open reading frame, and the designed tail-extension sequences. They were translated in the reticulocyte lysate cell-free system and subjected to SDS–PAGE analysis. In all cases, products were treated with RNaseA to degrade the tRNA moiety, which might bind to the C-terminus of the nascent chain. When translated in the absence of the RM, the products showed the same molecular weight corresponding to the polypeptide backbone (20 kDa) (Fig. 1C, upward arrows).

When translated in the presence of the RM, the products were monoglycosylated or diglycosylated. The status of glycosylation depended on the tail-extensions (Fig. 1C). In the case of the truncated mRNA that possessed no termination codon (Fig. 1C, RNC), the nascent polypeptide chain was retained in the ribosome as RNC and remained in the monoglycosylated form (Fig. 1C, lane 2). The monoglycosylated form was converted to the diglycosylated form by puromycin (Fig. 1C, lane 3). In mRNAs with the termination codon and no tail-extension, the products were mainly in the monoglycosylated form (Fig. 1C, lanes 5, 11 and 17; Fig. 1D), indicating that those termination codons did not function efficiently. The monoglycosylated forms were quantitatively converted to the diglycosylated form by puromycin. The puromycin-induced diglycosylated form was slightly larger than the normally released

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                10                  20                  30                  40                  50                  60
ATG AAG TGG GTA ACC TTT CTC CTC CTC CTC TTC ATC TCC GGT TCT GCC TTT TCC AGG GGT
 M   K   W   V   T   F   L   L   L   L   F   I   S   G   S   A   F   S   R   G 20

                70                  80                  90                 100                 110                 120
GTG TTT CGC CGA GAA GCA CAC AAG AGT GAG ATC GCC CAT CGG TTT AAG GAC TTG GGA GAA
 V   F   R   R   E   A   H   K   S   E   I   A   H   R   F   K   D   L   G   E 40

               130                 140                 150                 160                 170                 180
CAG CAT TTC AAA GGC CTA GTC CTG ATT GCC TTT TCC CAG TAT CTC CAG AAA TGC CCA TAT
 Q   H   F   K   G   L   V   L   I   A   F   S   Q   Y   L   Q   K   C   P   Y 60

               190                 200                 210                 220                 230                 240
GAA GAG CAT ATC AAA TTG AAT AGC ACT GTA ACA GAC TTT GCA AAA ACA TGT GTC GCT GAT
 E   E   H   I   K   L   N   S   T   V   T   D   F   A   K   T   C   V   A   D 80

               250                 260                 270                 280                 290                 300
GAG AAT GCC GAA AAC TGT GAC AAG TCC ATT CAC ACT CTC TTC GGA GAC AAG TTA TGC GCC
 E   N   A   E   N   C   D   K   S   I   H   T   L   F   G   D   K   L   C   A 100

               310                 320                 330                 340                 350                 360
ATT CCA AAG CTT CGC GAC AAC TAC GGT GAA CTG GCT GAC TGC TGT GCA AAA CAA GAG CCC
 I   P   K   L   R   D   N   Y   G   E   L   A   D   C   C   A   K   Q   E   P 120

               370                 380                 390                 400                 410                 420
GAA AGA AAC GAG TGT TTC CTG CAG CAC AAG GAT GAC AAC CCC AAC CTG CCA CCC TTC CAG
 E   R   N   E   C   F   L   Q   H   K   D   D   N   P   N   L   P   P   F   Q 140

               430                 440                 450                 460                 470                 480
AGG CCG GAG GCT GAG GCC ATG TGC ACC TCC TTC CAG GAG AAC CCT ACC AGC TTT CTG GGA
 R   P   E   A   E   A   M   C   T   S   F   Q   E   N   P   T   S   F   L   G 160

               490                 500                 510                 520                 530                 540
CAC TAT TTG CAT GAA GTT GCT AGC AGC GCT AGT ACT TTC TAT GCC CCA GCT CAG CAG AAT
 H   Y   L   H   E   V   A   S   S   A   S   T   F   Y   A   P   A   Q   Q   N 180

               550                 560
AGC ACT GCT GCT TAC ACC TAG
 S   T   A   A   Y   T   *
```

Fig. 2. Sequences of nucleotides and amino acids of the model protein. The model protein possesses the signal peptide (underlined) at the N-terminus and potential glycosylation sites (Asn^{67} and Asn^{180}). Glycosylation sites and flanking sequences were optimized (double underlines). For the transcription templates, the termination codon (asterisk) was deleted or exchanged for other termination codons.

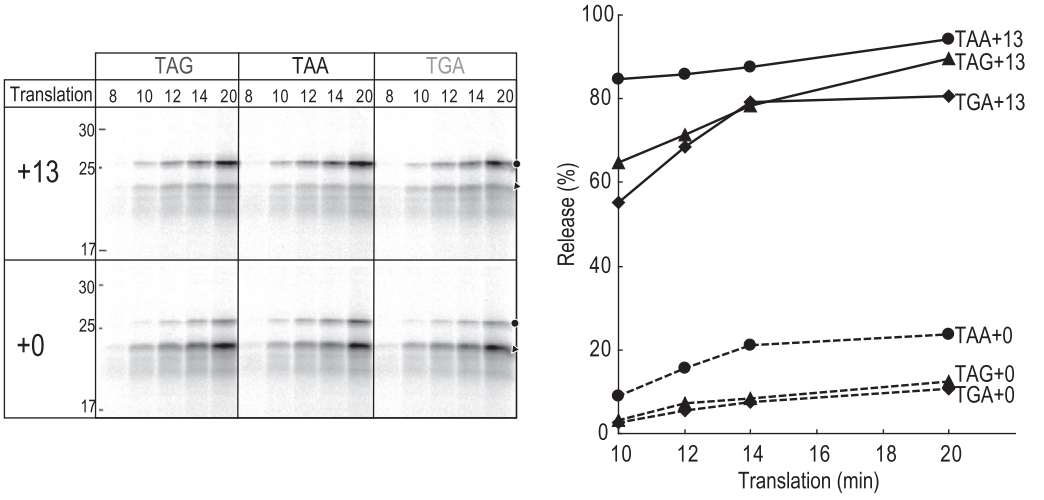

Fig. 3. Time-course of synthesis and release of the nascent chain mRNAs with one of the termination codons and the indicated tail-extensions (+0 or +13) were translated in the presence of RM. The aliquots were sampled at the indicated time after the translation initiation. The products were directly subjected to SDS–PAGE. The monoglycosylated (triangles) and the diglycosylated (dots) forms were quantified and the percent of the diglycosylated form was calculated as the percent release.

diglycosylated form (Fig. 1C, lane 6, 12 and 18; open circles and dots). The nascent chain retained at the PTC of the ribosome was released by puromycin at the A-site depending on the peptidyl transferase activity. Puromycin covalently attached to the C-terminus. The addition of puromycin increased the molecular weight. The appearance of the puromycin adducts directly indicated that those polypeptides remained as peptidyl tRNA, even in the presence of the termination codons.

In contrast to the +0 mRNAs, +13 mRNAs resulted in the diglycosylated form as major products (Fig. 1C, lanes 8, 14 and 20). Puromycin treatment led to the conversion of the small amount of the residual monoglycosylated form to the diglycosylated form (Fig. 1C, lanes 9, 15 and 21). Taken together, these findings indicate that our experimental system can be used to monitor the release of nascent polypeptide chains from membrane-bound ribosomes and demonstrate that nascent chain release from the membrane-bound ribosomes by the termination codon strongly depends on the tail-extension.

3.2. Length of tail-extension is critical for nascent chain release from ribosomes

To examine the release of the nascent chain as soon as it is synthesized, we performed a time-course experiment (Fig. 3). mRNAs (with or without tail-extensions) were translated in the presence of the RM and aliquots were sampled at short time periods. The full-length nascent chain became detectable after 10 min translation. In the case of +13 mRNAs, the products were largely diglycosylated immediately after synthesis. The residual monoglycosylated forms were also rapidly converted to diglycosylated forms. On the other hand, the products translated from +0 mRNA were very

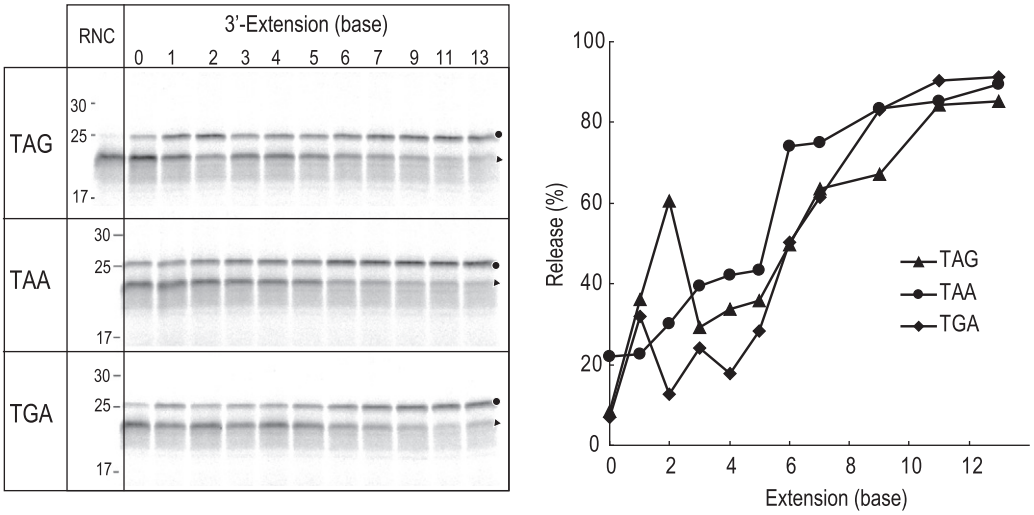

Fig. 4. Effect of the tail-extension length on nascent chain release from the ribosomes mRNAs including various tail-extensions (0–13 bases) were translated in the presence of RM for 15 min. The products were directly subjected to SDS–PAGE. Percent diglycosylation was quantified and plotted. As the tail-extension became longer, the release efficiency increased.

slowly diglycosylated. These findings also indicated that tail-extension determines the efficiency of the termination codon function.

We next examined the effect of the length of tail-extension on release of the nascent chain (Fig. 4). Various extensions were added to the termination codon and translated in the presence of RM for 15 min. The release efficiency strongly depended on the length of the extensions.

3.3. Impressions

Our findings clearly demonstrated that the functional efficiency of the termination codon on membrane-bound ribosomes depends on the tail-extension of the mRNA. To date, various artificial RNAs are subjected to cell-free translation. We demonstrated here that the tail-extension downstream of the termination codon is critical for nascent chain release from the ribosomes. If the coding regions of the mRNAs end with only the termination codon, ribosomes stack at the ends, which could impede their reuse for translation. Retention of the nascent chain in the ribosome would inhibit folding of the nascent chain, whereas tail-extensions lead to enhanced production and activity.

The requirement for tail-extension of the mRNA suggests two possibilities. First, when the peptidyl-tRNA and the corresponding codon of the mRNA move to the P-site of the ribosome, the next termination codon lacking the tail might be unstable at the A-site and might thus fluctuate. It does not correctly fit the PTC and therefore cannot induce an efficient reaction of the release factor. The release factors might not even efficiently access the termination codon at the A-site. Peptidyl transferase activity itself on the C-terminus is not affected by the tail-extension. Even in the case of non-stop mRNA, the nascent chain can be efficiently released from PTC by puromycin. The effect of the tail-extension is likely due to the combined actions of the release factors. Second, release factors might recognize not only the termination codon itself, but also some extension sequences of the termination codon.

Here we also demonstrated that this cell-free experimental system modeling protein translocation across membrane provides a sensitive assay of nascent chain release from membrane-bound ribosomes synthesizing authentic long polypeptide chains. This system could be useful for examining ribosomal function and the ribosome-translocation channel complex.

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References

[1] Y. Xun, P. Tremouilhac, C. Carraher, C. Gelhaus, K. Ozawa, G. Otting, N.E. Dixon, M. Leippe, J. Grötzinger, A.J. Dingley, A.V. Kralicek, Cell-free synthesis and combinatorial selective 15N-labeling of the cytotoxic protein amoebapore A from Entamoeba histolytica, Protein Expr. Purif. 68 (2009) 22–27.
[2] H. Taira, M. Fukushima, T. Hohsaka, M. Sisido, Four-base codon-mediated incorporation of non-natural amino acids into proteins in a eukaryotic cell-free translation system, J. Biosci. Bioeng. 99 (2005) 473–476.
[3] Y. Matsumura, L. Rooney, W.R. Skach, In vitro methods for CFTR biogenesis, Methods Mol. Biol. 741 (2011) 233–253.
[4] M. Kowarik, S. Küng, B. Martoglio, A. Helenius, Protein folding during cotranslational translocation in the endoplasmic reticulum, Mol. Cell 10 (2002) 769–778.
[5] N. Miyata, K. Hosoi, S. Mukai, Y. Fujiki, In vitro import of peroxisome-targeting signal type 2 (PTS2) receptor Pex7p into peroxisomes, Biochim. Biophys. Acta 1793 (2009) 860–870.
[6] D. Kajihara, R. Abe, I. Iijima, C. Komiyama, M. Sisido, T. Hohsaka, FRET analysis of protein conformational change through position-specific incorporation of fluorescent amino acids, Nat. Methods 3 (2006) 923–929.
[7] M. Yamagishi, H. Fujita, F. Morimoto, Y. Kida, M. Sakaguchi, A sugar chain at a specific position in the nascent polypeptide chain induces forward movement during translocation through the translocon, J. Biochem. 149 (2011) 591–600.
[8] H. Fujita, M. Yamagishi, Y. Kida, M. Sakaguchi, Positive charges on the translocating polypeptide chain arrest movement through the translocon, J. Cell Sci. 124 (2011) 4184–4193.
[9] H. Fujita, Y. Kida, M. Hagiwara, F. Morimoto, M. Sakaguchi, Positive charges of translocating polypeptide chain retrieve an upstream marginal hydrophobic segment from the endoplasmic reticulum lumen to the translocon, Mol. Biol. Cell 21 (2010) 2045–2056.
[10] Y. Kida, C. Kume, M. Hirano, M. Sakaguchi, Environmental transition of signal-anchor sequences during membrane insertion via the endoplasmic reticulum translocon, Mol. Biol. Cell 21 (2010) 418–429.
[11] Y. Kida, F. Morimoto, M. Sakaguchi, Two translocating hydrophilic segments of a nascent chain span the ER membrane during multispanning protein topogenesis, J. Cell Biol. 179 (2007) 1441–1452.
[12] B. Hou, P.J. Lin, A.E. Johnson, Membrane protein TM segments are retained at the translocon during integration until the nascent chain cues FRET-detected release into bulk lipid, Mol. Cell 48 (2012) 398–408.
[13] P.K. Devaraneni, B. Conti, Y. Matsumura, Z. Yang, A.E. Johnson, W. Skach, Stepwise insertion and inversion of a type II signal anchor sequence in the ribosome-Sec61 translocon complex, Cell 146 (2011) 134–147.

[14] K. Ota, M. Sakaguchi, G. von Heijne, N. Hamasaki, K. Mihara, Forced transmembrane orientation of hydrophilic polypeptide segments in multispanning membrane proteins, Mol. Cell 2 (1998) 495–503.
[15] K. Ojemalm, K.K. Halling, I. Nilsson, G. von Heijne, Orientational preferences of neighboring helices can drive ER insertion of a marginally hydrophobic transmembrane helix, Mol. Cell 45 (2012) 529–540.
[16] P. Walter, G. Blobel, Preparation of microsomal membranes for co-translational protein translocation, Methods Enzymol. 96 (1983) 84–93.
[17] R.J. Jackson, T. Hunt, Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA, Methods Enzymol. 96 (1983) 50–74.
[18] M. Sakaguchi, N. Hachiya, K. Mihara, T. Omura, Mitochondrial porin can be translocated across both endoplasmic reticulum and mitochondrial membranes, J. Biochem. 112 (1992) 243–248.