Biochimica et Biophysica Acta, 1065 (1991) 130–134

ADONIS 000527369100237Y

BBAMEM 75246

GTP is required for the integration of a fragment of the *Neurospora crassa* H^+-ATPase into homologous microsomal vesicles

Randolph Addison

Department of Biochemistry, University of Tennessee, Health Science Center, Memphis, TN (U.S.A.)

(Received 31 December 1990)

Key words: ATPase, H^+-; GTPase; (*N. crassa*)

The integration of a fragment of the *Neurospora crassa* plasma membrane H^+-ATPase was examined to determine if insertion of the fragment into homologous microsomal vesicles is obligatorily dependent on a nucleoside triphosphate. RNA transcripts that encoded the amino terminal 344 amino acids of the *Neurospora crassa* plasma membrane H^+-ATPase(pma^+_{344}) were translated in a *N. crassa in vitro* system. The pma^+_{344} was integrated post-translationally into homologous microsomal vesicles independent of the associated ribosomes and dependent on the presence of GTP or guanylyl imidodiphosphate, a nonhydrolyzable analogue of GTP. ATP or analogues thereof did not support the integration of pma^+_{344} into nRM post-translationally. These results were interpreted to suggest that a GTPase plays an essential role in the integration of the amino terminal portion of the pma^+ into the endoplasmic reticulum.

Introduction

The *Neurospora crassa* H^+-ATPase is an electrogenic, proton translocating ATPase (pma^+) of the aspartylphosphoryl-enzyme intermediate family of cation-motive ATPase [1]. The pma^+ is predicted to have at least 10 transmembrane spanning segments with domains alternately exposed to the cytoplasmic and the exoplasmic sides of the membrane [2]. It is not synthesized with a transient amino-terminal extension nor is it glycosylated by homologous microsomal vesicles [3]. The molecular mechanisms by which polytopic integral membrane protein(IMP) is integrated asymmetrically into the membrane are not clearly understood. Various models suggest several possible mechanisms which would establish the asymmetrical arrangement of an IMP. One postulates that IMPs are integrated into the membrane by a series of translocation-initiation and stop-translocation events. Each is initiated by a topogenic sequence that is decoded by proteinaceous effector(s) [4]. Another postulates that integration is initiated by a signal sequence which is in a loop structure with other transmembrane spanning segments forming pairs of antiparallel α-helices. The actual insertion of the transmembrane spanning segments into the membrane is not assisted by proteinaceous component(s) but is driven by the free energy changes resulting from the transfer of the hydrophobic segments from a hydrophilic milieu to a hydrophobic milieu. No stop-transfer sequence being required [5]. Using genetically engineered IMPs, it was observed, however, that a stretch of amino acid residues could function as a signal or a stop-transfer sequence depending on their position within the polypeptide chain [6,7]. Stop-transfer sequences apparently are used for generating the asymmetrical arrangment of an IMP. It is, however, a controverisal issue whether proteinaceous effectors are required to decode these topogenic sequences [5,8].

Of the several IMPs which have been studied, two different classes can be distinguished: some IMPs are synthesized as larger precursors with transient amino-terminal signal sequence [9]; some are not [10,11]. The

Abbreviations: pma^+, plasma membrane H^+-ATPase; pma^+_{344}, a truncated pma^+ containing the 344 amino acids from the amino terminal; nRM, microsomal vesicles derived from *N. crassa* rough endoplasmic reticulum; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NTP, nucleoside triphosphate; GMPPNP, guanylyl imidodiphosphate; IMP, integral membrane protein.

Correspondence: R. Addison, The University of Tennessee-Memphis, The Health Science Center, Department of Biochemistry, 800 Madison Avenue, Memphis, TN 38163, U.S.A.

IMPs are integrated into heterologous microsomal vesicles by two empirically distinguishable mechanisms: One is dependent on the signal recognition particle (SRP) [12]; another, for example cytochrome b_5 [13] and some small polypeptides [14], is independent of the SRP.

Because of the relative simplicity of pma^+, it may be the evolutionary precursor of the more complex cation-motive ATPases [1], and because of the physiological significance of these cation-motive ATPases, the present study was undertaken for the purpose of gaining insight into the mechanisms for the integration of the pma^+ into microsomal vesicles in an homologous, reconstituted *in vitro* system. RNA transcripts for the pma^+ and for the pma^+_{344} were translated in a *N. crassa in vitro* system. Both were integrated co- and post-translationally into microsomal vesicles [3]. The pma^+ became post-translationally incompetent to integrate into microsomal vesicles after desalting the translation mixture. Whereas, after desalting the translation mixture containing pma^+_{344}, the pma^+_{344} retained competence for integration into the microsomal vesicles post-translationally. The integration, however, of the pma^+_{344} post-translationally into the microsomal vesicles was dependent on GTP or on GMPPNP, a nonhydrolyzable analogue of GTP.

Experimental procedures

Desalting of the in vitro translation mixture. Translations of the RNA transcripts were as described [3]. After incubation, protein synthesis was stopped by adding emetine to 1 mM and incubating for 5 min at 16 °C. Afterwards, the mixture was applied to a column containing Sephacryl S-200 (Pharmacia) with a bed volume 10-times the applied sample volume. The column was equilibrated with Buffer A (0.25 M sucrose, 40 mM K^+-Hepes (pH 7.5), 120 mM KOAc, 3 mM $Mg(OAc)_2$, 50 units/ml of trasylol, 5 mM DTT, and 0.5 μg/ml each of pepstatin A, leupeptin, chymostatin, and antipain). The sample was applied to the column and eluted with Buffer A. The collected void volume, representing approximately twice the applied sample volume, was adjusted to 1 mM emetine before the addition of other components (see figure legends). For some studies, the translation mixture was centrifuged in an airfuge (Beckman) for 10 min at 25 psi, in rotor type A-100/30, before desalting.

Other methods. The preparation of the *Neurospora crassa* translation extract and the microsomal vesicles (nRM) were as described [15,16]. Methods for fractionation of the translation mixture [3], digestion with proteinase K [15], resolution of samples by sodium dodecylsulfate-polyacrylamide gel electrophoresis [3], and generation of RNA transcripts for the pma^+ and the pma^+_{344} [3] were as described.

Materials. The sources of most of the materials have been previously described [15,16]. Guanylyl imidodiphosphate were from Boehringer Mannheim Biochemicals. All other reagents were of the highest commercially available grade.

Results

The plasmid pPSM8 was linearized with the restriction endonuclease *Nde*I prior to transcription with SP6 RNA polymerase to generate RNA transcripts for the pma^+. The pma^+ has at least 10 putative transmembrane spanning segments (Fig. 1) [2]. pPSM8 was also linearized with the restriction endonuclease *Nco*I prior to transcription by the polymerase to generate RNA transcripts encoding the amino terminal 344 amino acids of the pma^+(pma^+_{344}): The RNA transcripts for pma^+_{344} have no termination codon. The translated product contains the first three transmembrane spanning segments and 24 of the 32 amino acids of the fourth transmembrane spanning segment (Fig. 1). As demonstrated previously, the translation of the RNA transcripts for the pma^+ and the pma^+_{344} can be separated from the integration of the products into microsomal vesicles (nRM) [3]. This should make it easier to delineate the role of various factors required for the integration of the pma^+ into nRM.

The post-translational integration of the pma^+ into nRM is depicted in Fig. 2. For these experiments, the translation mixture was separated into soluble (S) and pelleted (P) fractions by first adding a 5-fold volume of 0.1 M Na_2CO_3 (pH 11.5) to the mixture and centrifuging the resultant mixture through a sucrose-Na_2CO_3 (pH 11.5) cushion. In the presence of Na_2CO_3 (pH 11.5) microsomal vesicles are converted to open sheets, permitting the extraction of the lumenal contents as well as peripherally associated proteins from the cytoplasmic and exoplasmic sides of the membrane sheets. The pma^+ and the pma^+_{344} were found associated with nRM after extraction of the microsomal vesicles by 4 M urea, 25 mM EDTA, or 0.5 M KOAc [3]. Puromycin had no effect on the post-translational integration of pma^+ into nRM (Fig. 2). To determine if a nucleoside

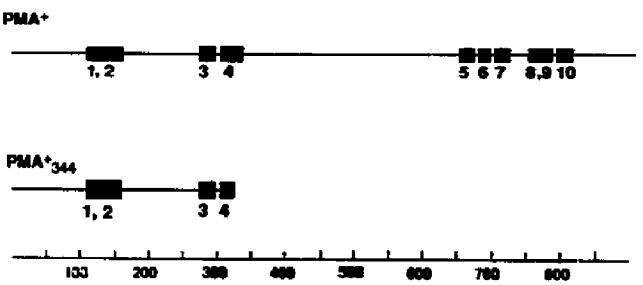

Fig. 1. Schematic diagrams of proteins by pSPM8. The black boxes represent putative transmembrane spanning segments. The amino acids sequence number is indicated along the horizontal axis.

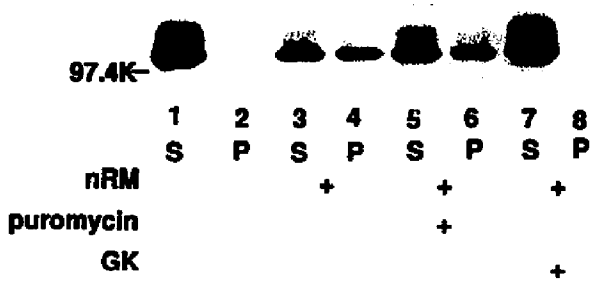

Fig. 2. Integration of pma$^+$ into nRM post-translationally. The RNA transcripts for the pma$^+$ were translated in the *N. crassa in vitro* system in a final volume of 200-μl at 20 ng/μl as described [15]. After adding emetine (1 mM) to stop protein synthesis, the mixture was centrifuged in an airfuge at 25 psi for 10 min. The supernatant was aliquoted and assayed either in the absence or in the presence of nRM at 3 A_{280} units/μl. Samples were incubated with puromycin (1 mM) or glycerol kinase (*E. coli*) (0.5 units/μl) (GK) for 10 min at 18°C before adding nRM. The samples were fractionated into soluble (S) and microsomal membranes (P) fractions by centrifugation through sucrose-0.1 M Na_2CO_3 (pH 11.5) cushions and prepared for analysis by SDS-PAGE as outlined in Experimental procedures. The resultant gel was prepared for fluorography as described [15]. The molecular weight marker is phosphorylase b with M_r = 97.4K.

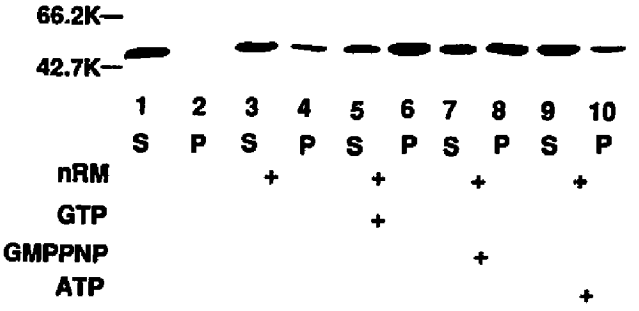

Fig. 3. Nucleoside triphosphate requirements for the integration of pma$^+_{344}$ into nRM post-translationally. The RNA transcripts for the pma$^+_{344}$ were translated in the *N. crassa in vitro* system in a final volume of 200 μl at 40 ng/μl as described [15]. After adding emetine (1 mM) to stop protein synthesis, the resultant mixture was desalted by passage through a column containing Sephacryl S-200. The void volume (300 μl) was aliquoted into 15-μl fractions. Each was adjusted to 30 μl and incubated at 18°C for 20 min in the presence of 40 mM K^+-Hepes (pH 7.5), 120 mM KOAc, 3 mM $Mg(OAc)_2$, 250 mM sucrose, 5 mM DTT, 2 units of trasylol/μl and 0.2 ng/μl each of chymostatin, antipain, leupeptin and pepstatin A. nRM were added to 3 A_{280} units/μl with the following: 1 mM GTP (lanes 5 and 6); 1 mM GMPPNP (lanes 7 and 8); 1 mM ATP (lanes 9 and 10). The molecular weight markers are bovine serum albumin and ovalbumin with M_r of 66.2K and 42.7K, respectively.

triphosphate plays an essential role in the post-translational integration of pma$^+$ into nRM, the translation mixture was treated with glycerol kinase prior to the addition of nRM. Glycerol kinase (*Escherichia coli*) uses only ATP as a substrate in the phosphorylation of glycerol [17]. As demonstrated in Fig. 2, the pma$^+$ was not integrated post-translationally into nRM (lanes 7 and 8), suggesting a requirement for ATP. Since this crude mixture most likely contains nucleosidediphosphate kinases [18], it is difficult to say which NTP was used for the pma$^+$ integration into nRM. To determine which NTP may be required, the translation mixture containing the pma$^+$ was desalted by passage over a column with Sephacryl S-200. The addition of GTP, ATP, or a combination thereof, did not cause the integration of the pma$^+$ into nRM (data not shown). In contrast, when similar experiments were conducted with the pma$^+_{344}$, this truncated form of pma$^+$ retained its ability to integrate into nRM post-translationally (Fig. 3). When nRM were added to the desalted mixture, in the absence of exogenous NTP, only 2% of the pma$^+_{344}$ co-sedimented with nRM (lanes 3 and 4), in the presence of 0.1 M Na_2CO_3 (pH 11.5). When GTP was added to the desalted mixture (lanes 5 and 6), the amount that co-sedimented with nRM in the presence of 0.1 M Na_2CO_3 (pH 11.5) was increased to 95%; in the presence of GMPPNP, to 75% (lanes 7 and 8); ATP had no effect (lanes 9 and 10). UTP and ITP were also inefficient as substrates in promoting the integration of pma$^+_{344}$ into nRM (data not shown). Since GTP and GMPPNP were effective in promoting the integration of pma$^+_{344}$ into nRM, other analogues of GTP were assayed for their effectiveness in this assay. Results from these experiments are depicted in Fig. 4. GDP (lanes 7 and 8) and GMP (lanes 9 and 10) were ineffective as substrates in catalysing the integration of the pma$^+_{344}$ into nRM; whereas, dGTP (lanes 11 and 12) was slightly better than GMPPNP in this assay (lanes 5 and 6).

The concentrations of GTP used in the experiments outlined in Figs. 3 and 4 are significantly greater than the concentration of GTP in the *in vitro* system. If this phenomenon is physiologically relevant, lower concentrations of GTP should give an identical response.

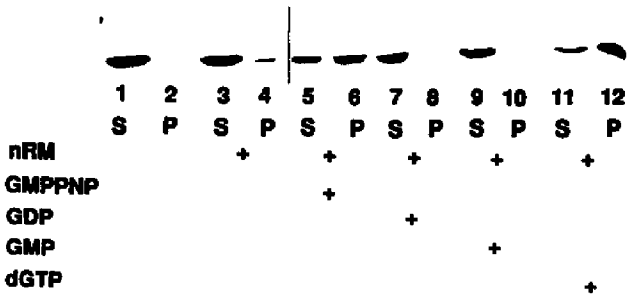

Fig. 4. The effects of GTP analogues on the integration of pma$^+_{344}$ into nRM post-translationally. Translation of transcripts for the pma$^+_{344}$, stopping protein synthesis, desalting, aliquoting and assaying the resultant fractions were identical as those outlined in the legend to Fig. 3. 1 mM GMPPNP was present during incubation with nRM (lanes 5 and 6); 1 mM GDP (lanes 7 and 8); 1 mM GMP (lanes 9 and 10); 1 mM dGTP (lanes 11 and 12). The various fractions were fractionated into soluble (S) and membranes (P) fractions by centrifugation through sucrose-0.1 M Na_2CO_3 (pH 11.5) cushions and were analyzed by SDS-PAGE, using 12.5%(w/v) acrylamide gel. The resultant gel was prepared for fluorography.

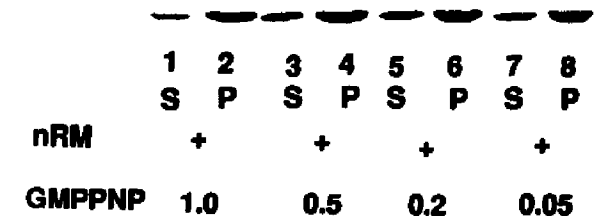

Fig. 5. The effects of various concentrations of GMPPNP on the integration of pma^{+}_{344} into nRM post-translationally. Experimental details are outlined in the legend to Fig. 3. Incubation was conducted in the presence of nRM at 3 A_{280} units/μl, with GMPPNP at the following concentrations: 1 mM (lanes 1 and 2); 0.5 mM (lanes 3 and 4); 0.2 mM (lanes 5 and 6); and 0.05 mM (lanes 7 and 8). The various fractions were analyzed by SDS-PAGE, using 12.5%(w/v) acrylamide gel. The gel was prepared for fluorography.

Since GTPases may be presence in this desalted, complex mixture, the nonhydrolyzable analogue of GTP, GMPPNP, was used to determine the minimal concentration required to promote the integration of pma^{+}_{344} into nRM. The translation mixture with pma^{+}_{344} was desalted; nRM were added to the resultant mixture and pma^{+}_{344} was assay for its ability to integrate into nRM as a function of various concentrations of GMPPNP. These results are depicted in Fig. 5. A concentration as low as 50 μM (lanes 7 and 8) caused approximately 80% of the pma^{+}_{344} to co-sediment with nRM. Similarly, 200 μM of GTP was just as effective as 1 mM of GTP (data not shown). The reduced efficiency of lower concentrations of GTP may have been caused by the presence of GTPases in this complex mixture.

The integration of a truncated opsin into canine pancreatic microsomal vesicles demonstrated a requirement for a tight ribosome membrane junction. The formation of which required GTP, or an analogue thereof [19]. To determine if ribosomes are essential for the pma^{+}_{344} integration into homologous microsomal vesicles, the tRNA analogue puromycin was added to the translation mixture with pma^{+}_{344}. As demonstrated [3], puromycin causes the release of about 90% of the pma^{+}_{344} from the ribosomes. The pma^{+}_{344} was treated with puromycin, and the resultant mixture was centrifuged in an airfuge (Beckman) at 25 psi for 10 min. The postribosomal supernatant was desalted by passage over a column containing Sephacryl S-200. The efficiency of puromycin treated pma^{+}_{344} to integrate into nRM as a function of GTP was not diminished (data not shown), demonstrating that the association of the pma^{+}_{344} with the ribosomes is not obligatory for its integration into nRM.

Discussion

It is demonstrated that after completion of protein synthesis the pma^{+} and the pma^{+}_{344} are integrated into nRM. This demonstrates that the amino terminal portion of pma^{+} contains a noncleavable signal sequence. Furthermore, the integration of the pma^{+}_{344} into homologous microsomal vesicles showed an obligatory dependence on the presence of GTP, or its nonhydrolyzable analogue GMPPNP. This truncated form of pma^{+} do not contain the ATP-binding site [2]. Since ATP did not promote the integration of this truncated ATPase into nRM, these results would imply that it is improbable that the pma^{+}_{344} is binding GTP and promoting its own integration into nRM. This suggests that the integration of the pma^{+}_{344} into microsomal vesicles required component(s) extrinsic to the pma^{+}_{344}. These results, therefore, were interpreted to suggest that a GTPase(s) plays an obligatory role during the integration of pma^{+}_{344} into microsomal vesicles.

The integration of the pma^{+}_{344} into nRM post-translationally required the presence of GTP. GDP, GMP, UTP, ITP, ATP and analogues thereof were ineffective as substrates in catalysing the integration of the pma^{+}_{344} into the microsomal vesicles. dGTP was almost as effective as GTP. Since GMPPNP, the nonhydrolyzable analogue of GTP, was just as effective as GTP, GTP hydrolysis was not obligatory: This suggests that the energy required for the integration of pma^{+}_{344} into nRM is derived from the binding of GTP to a putative GTPase, not from GTP hydrolysis. Since the only difference between GDP and GTP is the presence of the γ-phosphoryl moiety on GTP, this suggests that this moiety plays an essential role in the integration of pma^{+}_{344} into the membranes. This group could be essential for inducing a change in the GTPase to shift the reaction equilibrium to favor the integration of pma^{+}_{344} into nRM [20]. The location of this GTPase(s) and its role in the integration of pma^{+}_{344} into the microsomal membranes remain to be determined. It has been demonstrated that the translocation and the integration of proteins into canine pancreatic microsomal vesicles require a GTPase [21,22]. The α-subunit of the signal recognition particle (SRP)-docking protein (SRP-receptor) is a GTPase [23] and is required for the displacement of SRP from the ribosome-nascent polypeptide complex. Studies are currently being conducted to determine if analogoue components exist in *N. crassa*.

Desalting the translation mixture abolished the post-translational integration of the pma^{+} into nRM [3]. The addition of S-100, with or without ATP/GTP, to the desalted mixture was ineffective in restoring integration competence to pma^{+} (unpublished observations), suggesting that the process was irreversible. The pma^{+}, apparently, is maintained in an integration-competent form that may require NTP, or other small molecules. The removal of these could cause the pma^{+} to acquire a conformation that was no longer competent to integrate into nRM post-translationally. In support of this notion, it was demonstrated previously that the trypsin

cleavage patterns of the integration-competent and integration-incompetent forms were different [3], demonstrating that the pma^+ in the desalted mixture had acquired a different tertiary, or quaternary, structure. Whether the conversion of the pma^+ to an altered conformation is the only reason for its integration-incompetence remains to be determined. Studies from several laboratories, however, suggest that the conformation of proteins play an essential role in their ability to be translocated across the lipid bilayer [24,25], and that ATP is essential in maintaining precursors in translocation-competent conformations [26]. The pma^+_{344}, however, retained its ability to integrate post-translationally into nRM after desalting the translation mixture. This was independent of putative ATP reaction(s) that may be required to stabilize integration competent conformational states and of its association with the ribosomes, demonstrating also that the integration of pma^+_{344} into the microsomal vesicles was independent of an obligatory link with its translation.

Acknowledgments

This work was supported in part by a National Institutes of Health grants PHS GMP5678 to R.A. and by PHS GMP7155 to Günter Blobel. Some of this work was done while R.A. was in The Laboratory of Cell Biology at The Rockefeller University, New York, NY.

References

1 Mitchell, P. (1981) in Oxygen, Fuels, and Living Matter (Semenza, G., ed.), Part 1, pp. 1–160, John Wiley and Sons Ltd., Chichester.
2 Addison, R. (1986) J. Biol. Chem. 261, 14896–14901.
3 Addison, R. (1990) Biochim. Biophys. Acta 1030, 127–133.
4 Blobel, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496–1500.
5 Mueckler, M. and Lodish, H.F. (1986) Cell 44, 629–637.
6 Yost, C.S., Hedgpeth, J. and Lingappa, V.R. (1983) Cell 34, 759–766.
7 Lipp, J., Flint, N., Haeuptle, M.-T. and Dobberstein, B. (1989) J. Cell Biol. 109, 2013–2022.
8 Gilmore, R. and Blobel, G. (1985) Cell 42, 497–505.
9 Lingappa, V.R., Katz, F.N., Lodish, H.F. and Blobel, G. (1978) J. Biol. Chem. 253, 8667–8670.
10 Mostov, K.E., Defoor, P., Fleischer, S. and Blobel, G. (1981) Nature 292, 87–88.
11 Markoff, L., Lin, B.-C., Sueda, M.M. and Lai, C.-J. (1984) Mol. Cell Biol. 4, 8–16.
12 Anderson, D.J., Mostov, K.M. and Blobel, G. (1983) Proc. Natl. Acad. Sci. USA 80, 7249–7253.
13 Takagaki, Y., Rhadakrishnam, R., Wirtz, K.W.A. and Khorana, H.G. (1983) J. Biol. Chem. 258, 9136–9142.
14 Zimmermann, R. and Meyer, D.I. (1986) Trends Biochem. Sci. 11, 512–515.
15 Addison, R. (1987) J. Biol. Chem. 262, 17031–17037.
16 Addison, R. (1988) J. Biol. Chem. 263, 14281–14287.
17 Thorner, J.W. and Paulus, H. (1973) in The Enzymes (Boyer, P.D., ed.), Vol. 8, pp. 487–508, Academic Press, New York.
18 Palmieri, R., Yue, R.H., Jacobs, H.K., Maland, L., Wu, L. and Kuby, S.A. (1973) J. Biol. Chem. 248, 4486–4499.
19 Hoffman, K.E. and Gilmore, R. (1988) J. Biol. Chem. 263, 4381–4385.
20 Stryer, L. and Bourne, H.R. (1986) Annu. Rev. Cell Biol. 2, 391–419.
21 Connolly, T. and Gilmore, R. (1986) J. Cell Biol. 103, 2253–2261.
22 Wilson, C., Connolly, T., Morrison, T. and Gilmore, R. (1988) J. Cell Biol. 107, 69–77.
23 Connolly, T. and Gilmore, R. (1989) Cell 57, 599–610.
24 Eilers, M. and Schatz, G. (1986) Nature 322, 228–232.
25 Randall, L.L. and Hardy, S.J.S. (1986) Cell 46, 921–928.
26 Rothman, J.E. and Kornberg, R.D. (1986) Nature 322, 209–210.