Monoribosomal Attachment to Messenger Ribonucleic Acid in Sodium Fluoride-Treated Rabbit Reticulocytes

By MICHAEL GERAGHTY, MARILYN GALLER, FRED SCHIFFMAN AND MICHAEL FREEDMAN
*Department of Medicine, New York University School of Medicine,
550 First Avenue, New York, N.Y. 10016, U.S.A.*

(Received 15 March 1973)

Mild proteolysis with Pronase selectively dissociates ribosomes not attached to mRNA into subunits; ribosomes attached to mRNA remain intact. A portion of monoribosomes from reticulocytes incubated with NaF resisted proteolytic dissociation. Recovery of mRNA from monoribosomes of NaF-treated reticulocytes therefore may be explained by persistent attachment of some monoribosomes to mRNA.

Treatment of intact rabbit reticulocytes with NaF results in reversible disaggregation of polyribosomes to single ribosomes and cessation of haemoglobin synthesis (Marks *et al.*, 1965; Conconi *et al.*, 1966). Recently it was shown that after treatment with NaF the mRNA initially associated with polyribosomes may be recovered quantitatively in the monoribosome region (Terada *et al.*, 1972). These investigators suggested that, since NaF appears to inhibit initiation of globin chain synthesis, the free mRNA might be rendered inactive by inhibitory factors and yet still be conserved in association with monoribosomes. An alternative explanation, however, is that after NaF treatment a portion of the monoribosomes are still attached to mRNA. The present study was undertaken in order to investigate these two possibilities.

Experimental

Incubation of cells and isolation of ribosomes. Reticulocyte-rich blood was collected in heparin by cardiac puncture of phenylhydrazine-treated rabbits. The cells were washed with an iso-osmotic salt solution and incubated with glucose, amino acids, buffered salt solution and iron-transferrin, as previously described (Rabinovitz *et al.*, 1969). Incubations were performed with and without 10mM-NaF for 1 h at 37°C. In a separate procedure cells were incubated first with 0.1M-butan-1-ol for 20 min at 37°C (Freedman *et al.*, 1967). These reticulocytes were then washed three times with iso-osmotic salt solution and reincubated in the complete medium with and without NaF for 1 h at 37°C. The total volume of each incubation mixture was 8.0 ml in a 25 ml Erlenmeyer flask, with each flask containing 1.0 ml of reticulocytes.

Incubations were stopped by placing the flasks on ice. The cells were centrifuged at 600g and the super-

natant was removed. The cells were washed and lysed and the coarse particulate fraction was removed, as previously described (Rabinovitz *et al.*, 1969). Ribosomes were removed from the supernatant haemoglobin by centrifugation of the lysate in a Beckman 40 rotor at 100000g (r_{av} , 5.9 cm) for 3 h at 4°C over a cushion of 30% (w/w) sucrose in standard buffer (10mM-Tris-HCl buffer, pH 7.4, containing 10mM-KCl and 1.5mM-MgCl₂). The resulting adherent pellet was rinsed gently with standard buffer and gently resuspended in the same buffer.

Analysis of ribosomes and isolation of monoribosomes. The ribosomal suspension or cleared lysate was layered on 36 ml of a linear 15–30% (w/w) sucrose gradient in standard buffer. After centrifugation in a Beckman SW27 swinging-bucket rotor at 4°C for 3 h at 82500g (r_{av} , 11.8 cm) the gradient was pumped through a flow-through cell of a Beckman Kintrec VII spectrophotometer to measure absorbance at 260 nm. The area of the sucrose density gradient containing the monoribosome fraction was collected and centrifuged at 100000g at 4°C in the Beckman 40 rotor for 3 h. The pellet was rinsed with standard buffer and resuspended in 2.0 ml of the same buffer. The suspended ribosomes were then divided into two equal portions and the concentration of ribosomes was determined at 260 nm by using an extinction coefficient of 11.2 units per mg/ml (Ts'o & Vinograd, 1961).

Proteolytic dissociation of monoribosomes. Suspended monoribosomes (1 mg/ml) were exposed either to 0.2 mg of Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) at 4°C for 1 h or to an equal volume of standard buffer. After proteolysis, the solutions were centrifuged at 600g for 10 min and any pellet was discarded. The suspensions were then layered on a sucrose density gradient, as described above, and centrifuged at 4°C for 16 h at 67650g (r_{av} , 11.8 cm). The gradients were then pumped through the spectrophotometer and absorbance at 260 nm was recorded.

Fig. 1. Effect of Pronase on monoribosomes isolated from reticulocytes incubated in the presence of 10 mM-NaF for 1 h

Incubation and isolation of monoribosomes and sucrose-density-gradient analysis of ribosomes were performed as described in the text under 'Experimental'. (a) Ribosomes from control cells. (b) Ribosomes from cells incubated with 10 mM-NaF; the stippled area represents the area of the sucrose density gradient from which monoribosomes were isolated. (c) Proteolysis of the monoribosomes from NaF-treated cells: —, without Pronase; ----, with Pronase (0.2 mg per mg of ribosomes/ml) at 4°C for 1 h.

Fig. 2. Effect of Pronase on the monoribosomes isolated from cells treated sequentially with 0.1 M-butan-1-ol and 10 mM-NaF

Incubation of cells, isolation of monoribosomes and sucrose-density-gradient analysis were performed as described in the text under 'Experimental'. (a) Ribosomes from cells that had been incubated for 20 min with 0.1 M-butan-1-ol. (b) Ribosomes from cells that had been incubated with 0.1 M-butan-1-ol, washed and reincubated with 10 mM-NaF for 1 h; the stippled area represents the area of the sucrose density gradient from which monoribosomes were isolated. (c) Proteolysis of the monoribosomes from cells treated with butan-1-ol and NaF: —, without Pronase; ----, with Pronase (0.2 mg per mg of ribosomes/ml) at 4°C for 1 h.

Results

In intact reticulocytes most of the ribosomes are present as polyribosomes (Fig. 1a). After exposure of intact reticulocytes to 10 mM-NaF a large portion of the polyribosomes disaggregated to form monoribosomes (Fig. 1b). The monoribosomes were collected from the shaded area as designated in Fig. 1(b) and centrifuged again. The pellet was resuspended in standard buffer and divided into two portions. One portion received Pronase, the other buffer. Fig. 1(c) shows that a significant proportion of these monoribosomes resisted proteolytic dissociation into 40S and 60S subunits.

After exposure to butan-1-ol (Fig. 2a) and subse-

quent treatment with NaF (Fig. 2b), there were no obvious polyribosome peaks. The monoribosomes were collected as designated by the shaded area in Fig. 2(b) and centrifuged again. The pellet was resuspended and divided into two portions. One portion received Pronase, the other buffer. Fig. 2(c) shows that all of the monoribosomes from these cells have been dissociated into ribosomal subunits by proteolysis. When cells were treated with butan-1-ol and then reincubated in the complete medium without NaF, there was complete regeneration to a control pattern identical with that shown in Fig. 1(a). Monoribosomes isolated from this reincubation gradient showed that a significant portion resisted proteolytic

dissociation into subunits, confirming results obtained in previous studies (Malkin & Rich, 1967; Velez *et al.*, 1971; Freedman *et al.*, 1972).

Discussion

Mild low-temperature proteolysis with Pronase selectively dissociates ribosomes not attached to mRNA into 40S and 60S subunits (Velez *et al.*, 1971). Ribosomes attached to either intact or fragmented mRNA are completely resistant and remain intact (Velez *et al.*, 1971; Freedman *et al.*, 1972). Monoribosomes attached to mRNA resist proteolytic dissociation as well as polyribosomes do (Freedman *et al.*, 1972). This protective effect of mRNA attachment is independent of the presence of nascent chains on the ribosomes (Freedman *et al.*, 1972). In the present study we have exposed monoribosomes derived from NaF-treated reticulocytes to proteolysis and shown that a significant proportion remains intact. This suggests that these resistant ribosomes are still attached to mRNA. On the other hand, when reticulocytes were first treated with butan-1-ol to obtain monoribosomes not attached to mRNA (Freedman *et al.*, 1967; Velez *et al.*, 1971), the alcohol was removed and the cells were reincubated with NaF, all of the resultant monoribosomes were vulnerable to the proteolytic effects of Pronase. Reincubation of butan-1-ol-treated cells under identical conditions, but without NaF, resulted in a regeneration to the normal polyribosome pattern with a proportion of the monoribosomes resistant to proteolysis. The demonstration of non-attachment of ribosomes to mRNA in cells treated with both butan-1-ol and NaF confirms the previously postulated role of NaF in inhibition of ribosome attachment to mRNA (initiation of globin chain synthesis) (Marks *et al.*, 1965; Conconi *et al.*, 1966; Colombo *et al.*, 1968; Vesco & Colombo, 1970).

In cells treated only with NaF, failure to demonstrate complete non-attachment of ribosomes to mRNA is consistent with at least two explanations. It may simply represent failure to completely inhibit initiation of globin chain synthesis. Alternatively, it has been suggested that in addition to inhibition of attachment of ribosomes to mRNA (Baglioni *et al.*, 1972) NaF also blocks a step common to both initiation and elongation: the activation of ribosome-bound methionyl-tRNA_F at the initiator codon

(Thach & Thach, 1971). According to this theory, any ribosomes beyond the initiator codon would complete translation and be released from mRNA in the presence of NaF. Those at the initiator codon would remain attached to mRNA. Our findings are compatible with either explanation.

These results show that in cells treated directly with NaF a proportion of the monoribosomes remains attached to mRNA. This might explain the conversion of mRNA in this fraction, as previously described by Terada *et al.* (1972). In any event it is not possible to draw any conclusion about the state of free mRNA storage in intact cells unless it is simultaneously demonstrated that those ribosomes are indeed not attached to mRNA. Sequential treatment with butan-1-ol and NaF might prove useful in this regard.

These studies were supported by National Institutes of Health Hematology Training Grant AM05282 and National Institutes of Health Research Grant AM13532. M. F. is a recipient of a Special Fellowship (AM49358) from the National Institutes of Health.

- Baglioni, C., Jacobs-Lorena, M. & Meade, H. (1972) *Biochim. Biophys. Acta* **277**, 188–197
- Colombo, B., Vesco, C. & Baglioni, C. (1968) *Proc. Nat. Acad. Sci. U.S.* **61**, 651–658
- Conconi, F. M., Bank, A. & Marks, P. A. (1966) *J. Mol. Biol.* **19**, 525–540
- Freedman, M. L., Hori, M. & Rabinovitz, M. (1967) *Science* **157**, 323–325
- Freedman, M. L., Velez, R. & Mucha, J. (1972) *Exp. Cell Res.* **72**, 431–435
- Malkin, L. I. & Rich, A. (1967) *J. Mol. Biol.* **26**, 329–346
- Marks, P. A., Burka, E. R., Conconi, F., Perl, W. & Rifkind, R. A. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 1437–1443
- Rabinovitz, M., Freedman, M. L., Fisher, J. M. & Maxwell, C. R. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 567–578
- Terada, M., Metafora, S., Banks, J., Dow, L. W., Banks, A. & Marks, P. A. (1972) *Biochem. Biophys. Res. Commun.* **47**, 766–774
- Thach, S. S. & Thach, R. E. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1791–1795
- Ts'o, P. O. P. & Vinograd, J. (1961) *Biochim. Biophys. Acta* **49**, 113–129
- Velez, R., Farrell, N. L. & Freedman, M. L. (1971) *Biochim. Biophys. Acta* **228**, 719–727
- Vesco, C. & Colombo, B. (1970) *J. Mol. Biol.* **47**, 335–352