# Two *Dictyostelium* ribosomal proteins act as RNases for specific classes of mRNAs

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Phosphorylation of ribosomal protein S6 leads to the stabilization of pre-spore specific mRNAs during development of *Dictyostelium discoideum*. The purification of S6 kinase has allowed the identification of protein S11 as the mRNase specific for pre-spore mRNAs. Methylation of ribosomal protein S31 leads to the destabilization of ribosomal protein mRNAs. The purification of S31 methyltransferase has allowed the identification of protein S29 as the mRNAse specific for ribosomal protein mRNAs.

Key words: S6 kinase, S31 methyltransferase.

## INTRODUCTION

During development of *Dictyostelium discoideum* the synthesis of several classes of proteins is controlled at the post-transcriptional level. mRNAs specific for pre-spore cells are synthesized at maximum rates in growing and pre-aggregative cells, but accumulate only at the time of formation of tight cell aggregates, when their half-lives rise from 6 min to 6 h. Their stabilization is induced by the phosphorylation of ribosomal protein S6 [1–5]. The mRNAs for ribosomal proteins (rpmRNAs) left over from growth are excluded from polyribosomes in the first half of development [7] because they are sequestered by 40 S subunits, in which protein S24 is methylated [8], and are then rapidly degraded [7] by 40 S subunits possessing the methylated form of protein S31 [8].

The addition of the mammalian membrane lipid peroxidation product 4-hydroxynonenal (4-HNE) to growing cells causes a sequential increase of adenylate cyclase activity, of the intracellular concentration of cAMP, of cAMP-dependent protein kinase (PKA) activity, of S6 phosphorylation and of S31 methylation (G. Mangiarotti, unpublished work). The presence of significant amounts of S6 kinase and S31 methyltransferase in growing cells treated with 4-HNE has allowed us to purify the two enzymes. The ability to phosphorylate and methylate purified proteins S6 and S31 has allowed us to establish that S11 is an mRNAse that acts only on pre-spore specific mRNAs and is inhibited by phosphorylation of S6 and that S29 is an mRNAse specific for rpmRNAs and that it is activated by methylation of S31.

## EXPERIMENTAL

### Materials

Fast Flow SP (FFSP), Fast Flow Q (FFQ), and Activated CH Sepharose 4B media for chromatography, as well as columns, were obtained from Pharmacia (Freiburg, Germany).

The 20-residue peptide $S6_{228–249}$ [9] was synthesized by Research Genetics (Huntsville, AL, U.S.A.). [$^{32}$P]ATP (3000 Ci/mmol) and 5-methyltetrahydropteroylpolyglutamate [5-$CH_3$-$H_4$PTE$Glu_3$ (1000 Ci/mmol)] were purchased from Amersham.

### Solutions for chromatography of S6 kinase

Buffer A contained 15 mM pyrophosphate, pH 6.8, 5 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM benzaminidine. Buffer B contained 1 mM $KH_2PO_4/K_2HPO_4$, pH 6.8, at 4 °C. Buffer C contained 1 mM triethanolamine, pH 7.4. Buffer D contained 20 mM triethanolamine, pH 7.4, 1 mM EDTA, 50 mM NaF, 1 mM DDT, 1 mM benzaminidine, and 0.1 % Triton X-100.

Coupling of the HPLC-purified $S6_{228–249}$ peptide (22 mg) to activated CH Sepharose 4B resin (0.33 g) was carried out following the manufacturer's instructions.

### Assay of S6 kinase

Samples of S6 kinase preparations were incubated for 20 min at 23 °C with 1 mg of 40 S ribosomal subunits derived from growing cells and therefore unphosphorylated [5] and 20 mCi [$^{32}$P]ATP in 0.5 ml of 20 mM Tris/HCl, pH 7.0, 5 mM $MgCl_2$, 50 mM $NH_4Cl$ at 23 °C. Ribosomal proteins were then extracted and separated by two-dimensional gel electrophoresis as described [10].

For the first dimension, proteins were separated in cylindrical gels of polyacrylamide. The gel composition was 8.0 M urea, 57 mM Bis-Tris/acetate (pH 4.5), 4 % (w/v) acrylamide and 0.1 % (w/v) methylenebisacrylamide. The gel mixture was de-aereted for at least 15 min and polymerized by adding 1 $\mu$l of *N*,*N*,*N*′,*N*′-tetramethylethyldiamine (TEMED) and 5 $\mu$l of 10 % (w/v) ammonium persulphate/ml of gel solution. The gel was overlayed with 6.6 M urea, 57 mM Bis-Tris/acetate (pH 4.5). Samples, 50–200 $\mu$l (0.5 cm × 13–15 cm gels) or 30–80 $\mu$l (0.28 cm × 13 cm gels) were applied and overlayed with 3.5 M urea, 10 mM Bis-Tris/acetate (pH 4.0). The length of the run was chosen such that the tracking dye, basic fucsin [0.1 % (w/v) in 10 mM Bis-Tris/acetate, pH 4.0 and 10 % (w/v) glycerol] migrated within 1.5–2 cm from the bottom of the gel. The gels were run at constant current (1 mA/gel for 12–15 h). For the second dimension, gel slabs were formed in a model 220 vertical dual slab gel (Bio-Rad). Slabs of 10 % (w/v) polyacrylamide were prepared (0.3 cm × 10 cm × 15 cm). The gel consisted of 143 mM Bis-Tris/HCl (pH 6.75), 10 % acrylamide and 0.6 % methylenebisacrylamide. Freshly weighed acrylamide and methylenebisacrylamide were dissolved, de-aerated for 20 min and polymerized with 0.8 $\mu$l of TEMED and 1.8–2 $\mu$l of 10 %

Abbreviations used: CM, carboxymethyl; 5-$CH_3$-$H_4$PTE$Glu_3$, 5-methyltetrahydropteroylpolyglutamate; DTT, dithiothreitol; 4-HNE, 4-hydroxynonenal; PKA, cAMP-dependent protein kinase; RNP, ribonucleoprotein; rpmRNA, ribosomal protein mRNA.

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**Table 1 Summary of S6 kinase purification**

| Step | Volume (ml) | Total Units | Protein (mg) | Units/mg | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| 1. Lysate | 10 | 1200 | 5 | 240 | 100 | 1 |
| 2. FFSP | 5 | 800 | 1.5 | 533 | 80 | 2.2 |
| 3. FFQ | 7 | 600 | 0.3 | 2000 | 60 | 8.3 |
| 4. Sepharose 4B | 3 | 400 | 0.03 | 12.000 | 40 | 50 |

(w/v) ammonium persulphate/ml of gel solution. The gels were overlayed with 143 mM Bis-Tris/HCl, pH 6.75. After a polymerization period of at least 2 h the overlay was removed. A stacking gel solution [7 M urea, 0.143 M Bis-Tris/HCl, pH 6.75, 4 % (w/v) acrylamide, 0.1 % methylenebisacrylamide and 0.2 % (w/v) SDS] was poured to a height of 0.7 cm and the freshly extruded cylindrical gels from the first dimension run were embedded immediately. Polymerization was effected with 2 $\mu$l of TEMED and 8 $\mu$l of 10 % (w/v) ammonium persulphate/ml of gel mixture. The upper electrode chamber was filled with 70 mM each of Bis-Tris and Mes (pH 6.4), 0.015 M mercaptoacetic acid and 0.2 % SDS. To the lower chamber sufficient 0.029 M Bis-Tris/HCl (pH 6.75) was added in order to keep the slab gels cool during electrophoresis. The tracking dye was Bromophenol Blue [0.1 % (w/v) in 0.028 M Bis-Tris/HCl, pH 6.75, and 10 % (w/v) glycerol]. A constant current was applied towards the anode at 20 mA/slab for 30 min and then increased to 30 mA/slab. The gel was dried and scanned with a BioRad Phosphoimager to locate the phosphoserine residues [11] and to quantify their $^{32}$P content. One unit of the enzyme was defined as the activity capable of adding 1000 c.p.m. of $^{32}$P to protein S6 in 30 min.

## Purification of S6 kinase

$10^9$ cells growing in the presence of 10 $\mu$g/ml 4-HNE were collected by centrifugation at 5000 ***g*** for 5 min at 4 °C and lysed in 10 ml of lysis buffer [10] containing 20 mM PMSF. The lysate was loaded on to a 20 ml FFSP column. Proteins were eluted by applying a 200 ml linear gradient from 0–0.5 M NaCl in buffer A. The kinase eluted between 0.18 and 0.30 NaCl, after the main peak of protein. The eluate was diluted with 3 vol. of buffer B containing 20 mM PMSF and rapidly loaded on to a 20 ml FFQ Sepharose column pre-equilibrated in buffer C. A 250 ml linear gradient from 0–0.5 M NaCl in buffer C was applied to the column at a flow rate of 5 ml/min. The main peak of activity was eluted between 0.25 and 0.42 M NaCl. The FFQ pool (10 ml) was adjusted to 20 mM in PMSF, diluted with 4 vol. of buffer D and loaded overnight on to a 1 ml S6 peptide–Sepharose 4B column. The following day the column was washed until $A_{280}$ returned to baseline and proteins were then eluted with a 30 ml linear gradient from 0–1.5 M NaCl in buffer D. S6 kinase eluted between 0.70 and 1.1 M NaCl. The purity of the final enzyme fraction was shown by subjecting 10 $\mu$g of protein to two-dimensional gel electrophoresis. A single spot was detected by silver staining.

A summary of the purification procedure is shown in Table 1.

## Assay of S31 methyltransferase

Samples of S31 methyltransferase preparations were incubated for 20 min at 23 °C with 1 mg of 40 S ribosomal subunits derived from growing cells and therefore unmethylated [8] and 0.5 mCi [$^3$H]5-$CH_3$-$H_4$PTEGlu$_3$ in 0.5 ml of potassium phosphate, pH 7.0, 5 mM homocysteine, 0.2 mM 2-mercaptoethanol. Ribosomal proteins were then extracted and separated by two-dimensional gel electrophoresis. Protein S31 was eluted from the gel as described in [11] and its $^3$H content was determined in a Kontron counter. One unit of the enzyme was defined as the activity capable of introducing 1000 $^3$H c.p.m. into protein S31.

## Purification of S31 methyltransferase

$2 \times 10^9$ cells growing in the presence of 10 $\mu$g/ml 4-HNE were collected by centrifugation at 8000 ***g*** for 20 min at 4 °C and lysed. The lysate was diluted with 5 vol. of ice-cold 0.05 M potassium phosphate, pH 7.9 and 20 mM PMSF and the mixture was stirred overnight at 4 °C. It was then centrifuged at 8000 ***g*** for 20 min and the precipitate was discarded.

### Ammonium sulphate fractionation

Ammonium sulphate, 535 g/l, was added and the mixture was stirred for 1 h, then centrifuged at 20000 ***g*** for 40 min. The precipitate, which contained the enzyme, was suspended in 100 ml of 0.05 M phosphate buffer, pH 7.0, and dialysed against three changes of the same buffer, 500 ml each, over a period of 18 h. Insoluble material was removed by centrifugation at 15000 ***g*** for 30 min.

### Heat denaturation

The enzyme solution was heated rapidly to 55 °C with stirring and kept at this temperature for 5 min. After rapid cooling to below 10 °C, denatured proteins were removed by centrifugation at 8000 ***g*** for 10 min.

### Acid treatment

The supernatant solution, containing the enzyme, was brought to pH 4.5 by the addition of glacial acetic acid with rapid stirring and immediately centrifuged at 10000 ***g*** for 10 min. The precipitate was discarded. The supernatant solution was adjusted to pH 7.0 with 1 M NaOH and the enzyme was precipitated by the addition of ammonium sulphate to 535 g/l. The mixture was stirred for 1 h, the precipitate was collected by centrifugation at 37000 ***g*** for 20 min, dissolved in 20 ml of 0.03 M phosphate buffer, pH 7.4, and dialysed against two changes of the same buffer, 500 ml each, over a period of 12 h.

### DEAE-cellulose chromatography

DEAE-cellulose (Whatman DE52) was equilibrated with 0.02 M potassium phosphate, pH 7.4, and allowed to settle in a glass tube to form a 1.0 cm × 12 cm gravity-packed column. After washing the column with 100 ml of buffer, the enzyme was applied. The column was developed first with 100 ml of 0.02 M potassium phosphate buffer, pH 7.4, then with a linear gradient of KCl in this buffer, prepared from 200 ml of buffer and 200 ml of buffer containing 0.02 M KCl. Enzyme was eluted immediately after non-absorbed protein.

### Carboxymethyl (CM)-cellulose chromatography

The enzyme solution was concentrated 4-fold by mixing it with dry coarse Sephadex G-25, 36 g/100 ml, and centrifugal filtration at 2500 ***g*** for 20 min. The concentrated enzyme was dialysed against 2 litres of 0.015 M potassium phosphate buffer, pH 5.9, for 10 h, and passed on to a CM-cellulose column equilibrated with 0.02 M potassium phosphate buffer, pH 5.9. The column (1.0 cm × 12 cm) was washed with 100 ml of equilibrating buffer, and proteins were eluted with a linear gradient of KCl prepared with 100 ml of 0.02 M potassium phosphate, pH 5.9, and 100 ml

**Table 2 Summary of S31 methyltransferase purification procedure**

| Step | Volume (ml) | Total Units | Protein (mg) | Units/mg | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| 1. Lysate | 20 | 700 | 10 | 70 | 100 | 1 |
| 2. Heat denaturation | 100 | 600 | 3.3 | 182 | 86 | 2.6 |
| 3. Acid treatment | 20 | 550 | 1.5 | 370 | 78 | 5.3 |
| 4. DEAE-Cellulose | 10 | 480 | 0.5 | 980 | 68 | 14 |
| 5. CM-Cellulose | 5 | 350 | 0.2 | 1750 | 50 | 20.7 |
| 6. Sephadex G-100 | 3 | 300 | 0.1 | 3000 | 43 | 42.9 |

of the same buffer containing 0.075 M KCl. The enzyme appeared in the eluate after most of the protein had been removed from the column, with the peak of activity at 0.03 M KCl.

### Chromatography on Sephadex G-100

The enzyme solution from the previous step was concentrated by precipitation with ammonium sulphate, 535 g/l. After 1 h of stirring, precipitated protein was collected by centrifugation at 37000 ***g*** for 20 min and dissolved in 2 ml of 0.10 M potassium phosphate buffer, pH 7.0. The solution was placed on a 1 cm × 12 cm column of Sephadex G-100 equilibrated with this buffer, and was eluted with the same buffer. Protein emerged from the column in two incompletely separated peaks. Enzyme activity was associated with the minor peak, which eluted between 70 and 90 ml. The purity of the final enzyme fraction was shown by subjecting 10 $\mu$g of protein to two-dimensional gel electrophoresis. A single spot was detected by silver staining (results not shown).

A summary of the purification procedure is shown in Table 2.

## Cloned genes

Genes GM5, GM7, GM27 and GM55b were described in [12], gene D14 was described in [2] and genes RB1, RB2, RB3 and RB4 were described in [8].

## RESULTS

### Properties of S6 kinase

Contrary to the corresponding mammalian enzyme, *Dictyostelium* S6 kinase is activated by PKA [8]. The sedimentation coefficient of the protein ($5.2 \times 10^{-13}$) is consistent with a molecular mass of about 75000 for a globular protein. To characterize the enzyme activity, it was tested under different conditions. The S6 kinase activity requires $Mg^{2+}$ (optimum concentration 20 mM) and $NH_4^+$ (optimum concentration 50 mM). The optimum pH of the reaction is 7.2 and the optimum temperature 30 °C. S6 kinase is inactive in other proteins subject to phosphorylation (histones, myosin, glycogen synthase, phosphorylase, fructose kinase, pyruvate kinase, fructose-1,6-biphosphatase). The purified enzyme is stable when stored at −20 °C.

### Ribosomal protein S11 is an mRNase and its activity is inhibited by the phosphorylated form of ribosomal protein S6

Proteins S6 and S11 were eluted according to [11] from a two-dimensional gel, in which the protein complement of 2 mg of 40 S ribosomal subunits had been separated by electrophoresis. An aliquot of isolated protein S6 was then phosphorylated with S6 kinase in the presence of PKA, cAMP and ATP. To remove the components of the reaction, protein S6 was re-purified by two-dimensional gel electrophoresis. Mixtures containing either 10 $\mu$g of S11 and 10 $\mu$g of S6, or 10 $\mu$g of S11 and 10 $\mu$g of phosphorylated S6, together with samples of four pre-spore specific mRNAs, one pre-stalk specific mRNA and four rpmRNAs were prepared in 50 mM Tris/HCl, pH 7.2, 10 mM $MgCl_2$, 50 mM $NH_4Cl$. The mixtures were incubated at 23 °C for 5 min and the sizes of the various mRNAs were then determined by Northern blot analysis (Figure 1). In the presence of unphosphorylated S6, rpmRNAs and pre-stalk specific mRNA remained intact but all four pre-spore specific mRNAs were highly segmented. mRNA degradation must be attributed to an RNase activity of protein S11, since both this protein and protein S6 were pure and were the only protein present in the reaction mixture.

On the contrary, in the presence of phosphorylated S6, all mRNAs remained intact. The inhibition of S11 RNase must be attributed directly to the phosphorylation of protein S6, and not to an RNase inhibitor present in the S6 preparation, since an S6 phosphatase has been isolated, which eliminates the possibility that protein S6 de-stabilizes; pre-spore specific mRNAs [13].

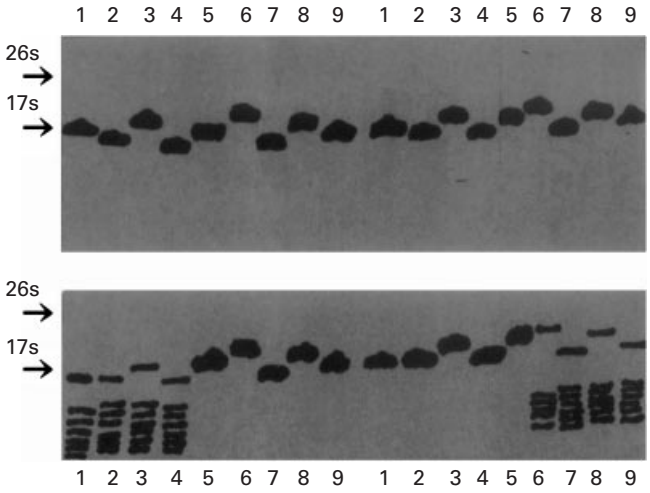


**Figure 1 Characterization of mRNase activities**

Nine mRNAs were used as substrates: four pre-spore specific, GM5 (1), GM7 (2), GM27 (3), GM55b (4); one pre-stalk specific, D14 (5); four rpmRNAs, Rb1 (6), Rb2 (7), Rb4 (8) and Rb5 (9). Upper left panel, mRNAs were incubated with 10 $\mu$g of protein S11 in the presence of 10 $\mu$g of phosphorylated protein S6. Lower left panel, mRNAs were incubated with 10 $\mu$g of protein S11 in the presence of 10 $\mu$g of unphosphorylated protein S6. Upper right panel, mRNAs were incubated with 10 $\mu$g of protein S29 in the presence of 10 $\mu$g of unmethylated protein S31. Lower right panel, mRNAs were incubated with 10 $\mu$g of protein S29 in the presence of 10 $\mu$g of methylated protein S31. After 5 min of incubation, the RNAs were extracted and analysed by Northern blot analysis. 26 S and 17 S ribosomal RNAs were used as size markers.

### Properties of S31 methyltransferase

The sedimentation coefficient of the S31 protein ($3.7 \times 10^{-13}$) is consistent with a molecular mass of 54000 for a globular protein. To characterize the enzyme activity, it was tested under different conditions. The enzyme activity requires $Mg^{2+}$ (optimum concentration 10 mM) and $K^+$ (optimum concentration 20 mM). The optimum pH of the reaction is 7.4 and the optimum temperature 32 °C. The purified enzyme is stable at −20 °C.

### Ribosomal protein S29 is an mRNase activated by the methylated form of ribosomal protein S31

The protein complement of 2 mg of 40 S ribosomal subunits was separated by two-dimensional gel electrophoresis and proteins S29 and S31 were eluted according to [11]. An aliquot of isolated protein S31 was methylated by incubation at 23 °C for 20 min with S31 methyltransferase in 0.01 M 5-$CH_3$-$H_4$PTE$Glu_3$, 10 mM $MgCl_2$, 20 mM potassium phosphate pH 7.0, 5 mM homocysteine, and 0.2 mM 2-mercaptoethanol. To remove the components of the reaction mixture, protein S31 was repurified by two-dimensional gel electrophoresis.

The same series of nine mRNAs that were used to demonstrate that ribosomal protein S11 is a pre-spore specific mRNase were incubated for 5 min at 23 °C in 50 mM Tris/HCl, pH 7.1, 10 mM $MgCl_2$, 50 mM $NH_4Cl$ in the presence of 10 $\mu$g of protein S29 plus 10 $\mu$g of the methylated or unmethylated form of protein S31. The sizes of the various mRNAs were then determined by Northern blot analysis. In the presence of unmethylated S31, all the mRNAs remained intact, whereas in the presence of methylated S31, pre-spore and pre-stalk mRNAs were unaffected but rpmRNAs were highly segmented (Figure 1). The degradation of rpmRNAs must be attributed to an RNase activity of protein S29, since both S29 and S31 were pure and were the only proteins present in the reaction mixture.

### S6 kinase, S31 methyltransferase, S11 RNase and S29 RNase are the only proteins that exert the ascribed functions

To establish whether other cellular proteins were capable of exerting the activities ascribed to the four ribosomal proteins described in the present study cytosolic proteins were prepared as described in [10] and ribosomal proteins were eluted from a two-dimensional gel as described in [11] in two steps: first, proteins S6, S11, S29 and S31 were eluted and discarded, then the remaining proteins were eluted and collected. Ribonucleoproteins (RNP) particles were prepared as described in [10]. Neither cytosolic proteins, nor the bulk of ribosomal proteins (depleted of proteins S6, S11, S29 and S31), nor RNP particles were capable of phosphorylating protein S6, of methylating protein S29, or of degrading pre-spore specific mRNAs or rpmRNAs when tested in the assays described above and in [10] (results not shown).

## DISCUSSION

Many mRNases have been identified in prokaryotes and eukaryotes [14] and these are mainly 3′–5′ exonucleases and endonucleases. Although a number of mRNases are found in association with polyribosomes, none have been found with mRNP or ribosome complexes.

The two endonucleases described in the present study are two proteins of 40 S ribosomal subunits. Their activity is regulated by the phosphorylation of protein S6, which inhibits S11 mRNases and leads to the stabilization of pre-spore specific mRNAs, and by the methylation of protein S31, which activates S29 mRNase and leads to the rapid decay of rpmRNAs. The effect of phosphorylation of protein S6 and of methylation of protein S31 on the stability of two different classes of mRNAs suggested the existence of two ribosomal proteins with mRNase activity. It seemed likely that the pre-spore specific mRNase should be a neighbour of protein S6 in 40 S ribosomal subunit in three-dimensional organization, in order to be subject to the influence of a change of conformation of this protein; the same was true for the rpmRNAs RNase and protein S31. In *Dictyostelium discoideum*, nothing is known about the organization of proteins in ribosomes. Therefore, in searching for the putative mRNases, we had to purify and test *in vitro* each protein of 40 S ribosomal subunit.

It is surprising that the mRNase activities of proteins S11 and S29 are still influenced by proteins S6 and S31 in the *in vitro* assay, when they are no longer part of a ribosomal subunit. It is likely that proteins S6 and S11 and proteins S29 and S31 have a specific affinity for each other, since *in vitro* they form complexes with sedimentation coefficients of $7.2 \times 10^{-13}$ and $5 \times 10^{-13}$ respectively. Alternatively, the two couples of proteins may have a specific affinity for a given sequence of pre-spore specific mRNAs or rpmRNAs respectively. The existence of specific sequences, which act as the cis-elements of the mRNA stability control, is also implied by the specificity of action of the S11 and S29 mRNases.

Results to be published elsewhere [16] show that a class of germination-specific mRNAs are rapidly degraded in emerging amoebae by the RNase activity of ribosomal protein S12, which is activated by methylation of ribosomal protein S27. Thus, post-transcriptional control of gene expression appears to be exerted by covalent modifications of several ribosomal proteins which act on the RNase activity of coupled ribosomal proteins. The involvement of different ribosomal proteins in post-transcriptional control ensures that different classes of mRNAs are differentially controlled.

When we first discovered the ribosome cycle [15], it was interpreted as a consequence of the mechanism of initiation and termination of protein synthesis. The data reported here show that its significance is much broader. Separation of 40 S from 60 S subunits at the end of protein synthesis is required because while traversing the 5′UTR of mRNAs the subunits have to exert several regulatory functions through the covalent modification and interaction with some of their own proteins.

The work was supported by Italian CNR (Progetto finalizzato di Ingegneria Genetica), Compagnia di S.Paolo and Regione Piemonte. We thank Dr D. Hayes for critical reading of the manuscript.

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Received 22 April 2002/7 August 2002; accepted 23 October 2002
Published as BJ Immediate Publication 23 October 2002, DOI 10.1042/BJ20020638