**The EMBO Journal** vol.3 no.1 pp.95–100, 1984

# Differential kinetics of changes in the state of phosphorylation of ribosomal protein S6 and in the rate of protein synthesis in MPC 11 cells during tonicity shifts

**Joachim Kruppa* and Michael J. Clemens[1]**

Institute of Physiological Chemistry, Department of Molecular Biology, University of Hamburg, D-2000 Hamburg 13, FRG, and [1]Cancer Research Campaign Mammalian Protein Synthesis and Interferon Research Group, Department of Biochemistry, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK

*To whom reprint requests should be sent

*Communicated by F. Cohen*

**Mouse myeloma (MPC 11) cells respond rapidly to hypertonic conditions by shutting down protein synthesis at the level of polypeptide chain initiation. Translational activity recovers equally quickly upon a return to isotonicity. Disaggregation and reformation of polysomes occur in parallel to the changes in protein synthesis. Ribosomal protein S6 becomes dephosphorylated under hypertonic conditions and rephosphorylated when isotonic conditions are restored. The kinetics with which these changes occur are, however, too slow to account for the changes in protein synthesis. Treatment of the cells with a low concentration of cycloheximide allows reformation of polysomes under hypertonic conditions; conversely, puromycin prevents the restoration of polysomes which otherwise occurs on return to isotonicity. Neither inhibitor prevents the changes in S6 phosphorylation resulting from the tonicity shifts. We conclude that the overall extent of phosphorylation of S6 neither regulates nor is determined by the rate of protein synthesis and is not obligatorily related to the proportion of ribosomes in polysomes.**

*Key words:* S6 phosphorylation kinetics/protein synthesis/hyperosmolarity/protein synthesis inhibitors/S6 phosphopeptide patterns

## Introduction

Ribosomal protein S6 of eukaryotic cells is remarkable for its ability to exist in a variety of states of phosphorylation and for the variations in the extent of its phosphorylation in response to a wider range of stimuli. S6 phosphorylation (Wool, 1979; Traugh, 1981) changes in systems such as liver (Gressner and Wool, 1974a), HeLa cells (Lastick and McConkey, 1980), 3T3 cells (Thomas *et al.*, 1979), MPC 11 cells (Kruppa and Martini, 1978), *Xenopus laevis* oocytes (Nielsen *et al.*, 1982; Kruppa *et al.*, 1983) and tomato cells (Scharf and Nover, 1982) in response to alterations of the external milieu of the cells. Serum (Wool, 1979; Traugh, 1981; Gressner and Wool, 1974a), growth factors (Lastick and McConkey, 1980; Thomas *et al.*, 1982), mitogens (Wettenhall and Howlett, 1979), and hormones (Gressner and Wool, 1974a; Lastick and McConkey, 1980; Nielsen *et al.*, 1982; Wettenhall *et al.*, 1982; Kruppa *et al.*, 1983; Thomas *et al.*, 1982) rapidly enhance S6 phosphorylation and also induce an increase in the protein synthetic activity of their target tissues. Rapid stimulation of S6 phosphorylation and a rise in the rate of protein synthesis were also observed after fertilization of sea urchin eggs (Ballinger and Hunt, 1982). In spite of the failure of attempts to detect functional differences between ribosomes with different states of S6 phosphorylation (Leader *et al.*, 1981; Eil and Wool, 1973; Krystosek *et al.*, 1974) it has recently been suggested that S6 phosphorylation is important in determining the rate of protein synthesis (Nielsen *et al.*, 1982; Thomas *et al.*, 1982; Duncan and McConkey, 1982b) and that 40S ribosomal subunits containing phosphorylated S6 form initiation complexes and enter polysomes more efficiently than those with non-phosphorylated S6 (Thomas *et al.*, 1982; Duncan and McConkey, 1982a). However, a close inspection of the time-dependent increase of S6 phosphorylation in relation to changes in translational activity revealed that highly phosphorylated S6 is not required for maintaining maximal protein synthesis activity (Nielsen *et al.*, 1981). To reconcile these observations, highly phosphorylated S6 was postulated to be necessary for the activation of the systems at an early step of the initiation process, namely for the recruitment of mRNA (Duncan and McConkey, 1982a).

Serum factor depletion (Meedel and Levine, 1978), heat shock (Scharf and Nover, 1982) and hyperosmolarity (Kruppa and Martini, 1978; Martini and Kruppa, 1979) on the other hand reduce protein synthetic activity by affecting initiation rates as well as reducing the fraction of mRNAs in polysomes. These treatments lead in addition to a massive dephosphorylation of protein S6 (Kruppa and Martini, 1978; Scharf and Nover, 1982). In the case of hyperosmolarity we have previously shown that S6 phosphorylation and protein synthesis of MPC 11 cells decrease considerably after raising the tonicity of the growth medium to 120 mM excess NaCl for ~1 h (Martini and Kruppa, 1979). Since the inhibition of protein synthesis can be easily reversed by readjusting the culture medium to isotonic conditions in this system (Saborio *et al.*, 1974) we followed the time-course of changes of S6 phosphorylation in relation to translational activity by measuring polysomal content and relative rates of protein synthesis. In these salt shift experiments we succeeded in dissociating the changes in S6 phosphorylation from changes in both the translational activity of the cells and the proportion of ribosomes in polysomes.

## Results and Discussion

### *Changes in S6 phosphorylation and in translational activity during hypertonic treatment and recovery*

After stimulation for 1 h in fresh growth medium, MPC 11 cells contained ribosomes with multiply phosphorylated forms of S6 protein. Under optimal separation conditions, up to six individual spots can be visually distinguished in the S6 region of a Coomassie Blue stained two-dimensional polyacrylamide gel pattern (Figure 1A). The degree of phosphate incorporation increases from the S6b to the S6f derivative, whereas S6a is not phosphorylated at all (Figures 1C and D). When cells are exposed to 120 mM excess NaCl in the growth medium S6 phosphorylation is decreased after 15 min, as is

© IRL Press Limited, Oxford, England.

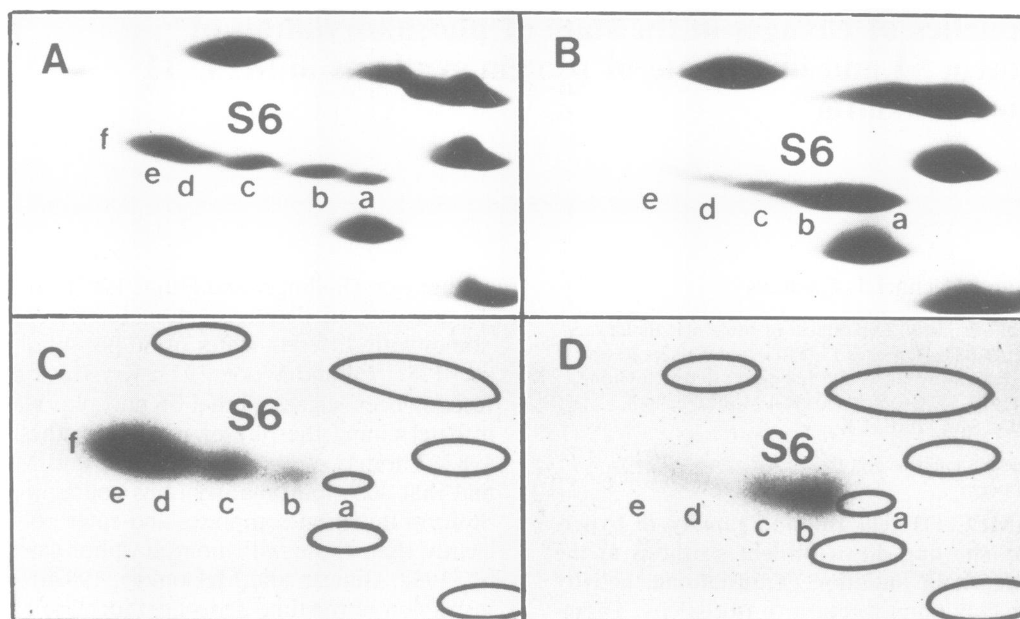


**Fig. 1.** Separation of phosphorylated species of ribosomal protein S6 from MPC 11 cells incubated in isotonic and hypertonic medium. MPC 11 cells were labeled with [$^{32}$P]orthophosphate (2 $\mu$Ci/ml) overnight. Phosphorylation of the S6 protein was stimulated for 1 h by addition of fresh medium containing a serum mixture. One half of the culture was then made hypertonic for 15 min by addition of 120 mM excess NaCl. Cells were harvested, ribosomal proteins prepared, and analysed. **A** and **B** show the S6 region of two-dimensional gels of ribosomal proteins from control and salt treated cells, respectively; **C** and **D** are the autoradiograms projected on a sketch of the corresponding S6 regions.

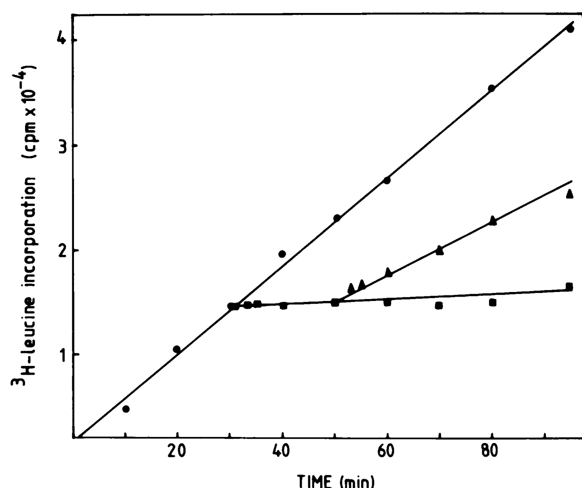


**Fig. 2.** Kinetics of protein biosynthesis in MPC 11 cells during tonicity shifts. MPC 11 cells were incubated with [$^3$H]leucine as described (●). 30 min after addition of the labeled amino acid, the osmolarity was raised in two thirds of the cell suspension by addition of 120 mM excess NaCl (■). Incubation was continued for 20 min and an aliquot of the hypertonic sample was then readjusted to isotonic conditions by dilution with salt-free medium (▲). Samples were taken for the estimation of protein labeling as described in Materials and methods.

obvious from the smaller number of S6 derivatives observed by staining (Figure 1B) and also from the $^{32}$P-labeling pattern (Figure 1D).

Incorporation of [$^3$H]leucine was almost instantaneously blocked upon raising the tonicity of the growth medium by 120 mM excess NaCl (Figure 2). Similarly, cellular protein synthesis resumed very rapidly when medium osmolarity was readjusted to isotonic conditions (Figure 2). Disaggregation of polysomes under hypertonic conditions and reformation of polysomes after readjustment to isotonic conditions (Figure 3) took place in parallel with the changes in amino acid incorporation. Establishment of hypertonic conditions caused a loss of polysomes (compare Figures 3B and D) with a time of half maximal change of 3 min (Figure 3A). Polysomes reformed with a similar time of half maximal change (Figure 3A) when isotonic conditions (compare Figures 3D and E) were restored.

These rapid changes in protein synthesis and polysomal content were in contrast to the slower processes of dephosphorylation and rephosphorylation of S6 seen in the same population of cells (Figure 4). A 3 – 4 times longer period was needed for the corresponding changes in the S6 phosphorylation state, so that it took 10 min for the highly modified species S6d to f to decline from 37% to 19% of the total S6 (Figure 4B) and 14 min for S6a to increase from 23% to 43% of the total (Figure 4A). At 5 min after NaCl addition when protein synthesis had come to a halt (Figure 2) and the amount of polysomes was considerably reduced (Figures 3A and D), multiply phosphorylated S6 species were still quite prominent in the corresponding two-dimensional gel pattern (Figure 5B). The degree of phosphorylation subsequently decreased, but under the conditions used did not lead to complete dephosphorylation of S6, because even after 65 min in the presence of 120 mM excess NaCl, 37% (Figure 4B) of total S6 was still in the mono- and diphosphorylated forms in the cells (Figure 5E). After readjustment of the medium to isotonic conditions a slow but steady enhancement of S6 rephosphorylation was observed (Figure 4 and Figures 5F – I). S6 phosphorylation occurred with a time of half maximal change of 16 min (Figure 4B) which is slower by a factor of 5 than polysome reformation (Figure 3A). The increase in translational activity of MPC 11 cells preceded the enhanced phosphate incorporation into S6. At ~45 min after changing the medium osmolarity to isotonic conditions, the distribution of S6 species and the polysomal profiles were indistinguishable from those of untreated cells (compare Figures 5A

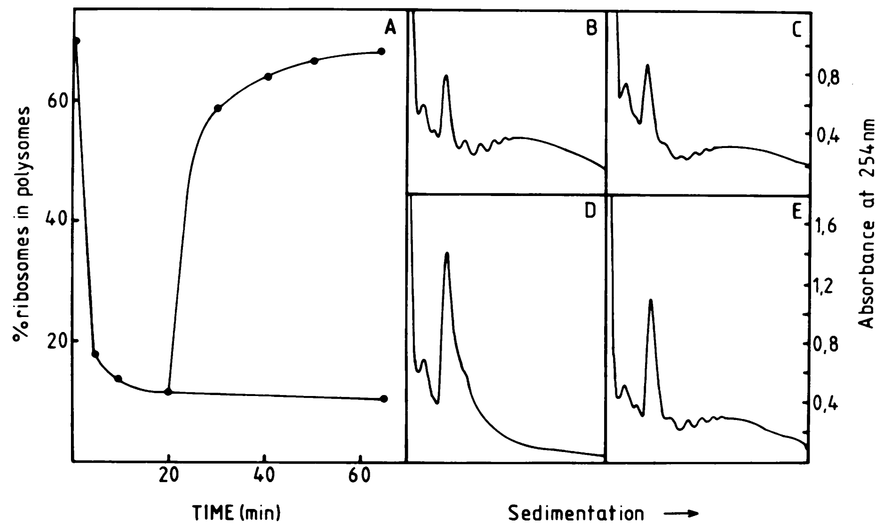


**Fig. 3.** Quantitative changes in ribosome distribution during tonicity shifts. MPC 11 cells were prelabeled with [$^{35}$S]methionine overnight. Cells were preincubated for 1 h in fresh growth medium and then subjected to a cycle of tonicity shifts from isotonic to hypertonic (for 20 min) and back to isotonic (for 45 min) conditions. Aliquots of the cell extracts were loaded on to 20–50% sucrose gradients containing buffer D and centrifuged as detailed in Materials and methods. **Panel A**: relative distribution of ribosomes in polysomes quantified by the procedure of Martin (1973). Ribosomal profiles are shown from cells: (**B**) just before addition of 120 mM excess NaCl (control); (**C**) after hypertonic shock for 20 min and a recovery period of 45 min under isotonic conditions; (**D**) after hypertonic shock for 5 min; and (**E**) after hypertonic shock for 20 min and a recovery period of 10 min under isotonic conditions. The other three ribosomal profiles of salt treated cells looked very similar to **panel D**.

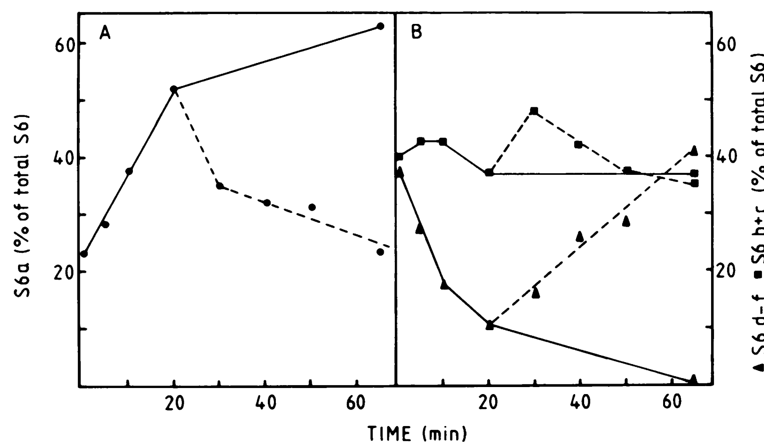


**Fig. 4.** Quantitative changes in phosphorylation of S6 species during tonicity shifts. MPC 11 cells prelabeled with [$^{35}$S]methionine were subjected to a cycle of shifts in tonicity (same experiment as in Figure 3). Ribosomes were prepared and the ribosomal proteins separated by two-dimensional electrophoresis, as shown in Figure 5. The S6 patterns were quantified by excising each spot and determining the $^{35}$S radioactivity after oxidation of the gel slices with $H_2O_2$. S6a corresponds to the unphosphorylated S6 species (**A**). S6b and c, the mono- and diphosphorylated forms respectively, remained nearly constant during the tonicity shifts (**B**). S6d to f are more highly phosphorylated S6 species (**B**). The values represent the % distribution of the S6 derivatives in each particular sample. (——), 120 mM excess NaCl from time 0; (- - -), restoration of isotonic conditions at 20 min.

and I, and Figures 3B and C).

*Tryptic phosphopeptide patterns of S6 from control and salt-treated cells*

Trypsinization of unfractionated S6 protein, extracted from the total ribosome population of serum-stimulated cells and separated by two-dimensional gel electrophoresis, gave rise to a complex phosphopeptide profile when the digest was chromatographed on a DEAE-Sephadex A25 column (Figure 6A). This reflects the complex composition of the S6 sample (Figure 1A). The corresponding S6 protein isolated after hypertonic shock showed substantially less label in phosphopeptide peaks eluting at higher ionic strength (Figure 6B). This suggests that due to the increase in medium osmolarity specific serine sites have been partially or completely dephosphorylated. All phosphate groups of S6 protein are linked to serine residues (insets of Figure 6).

*Lack of correlation between the phosphorylation state of S6 and the distribution of ribosomes in polysomes*

The foregoing experiments suggested that changes in overall S6 phosphorylation cannot be the cause of the changes in protein synthetic rate or extent of polysome aggregation because they occurred too slowly. Nevertheless, it was possible that they were the result of the altered translational activity, as could be the case if inactive ribosomes were preferentially dephosphorylated for example (Thomas *et al.*, 1982). To test this idea, experiments with cycloheximide and puromycin were performed to evaluate the interrelationship between protein synthesis, polysomal content, and extent of

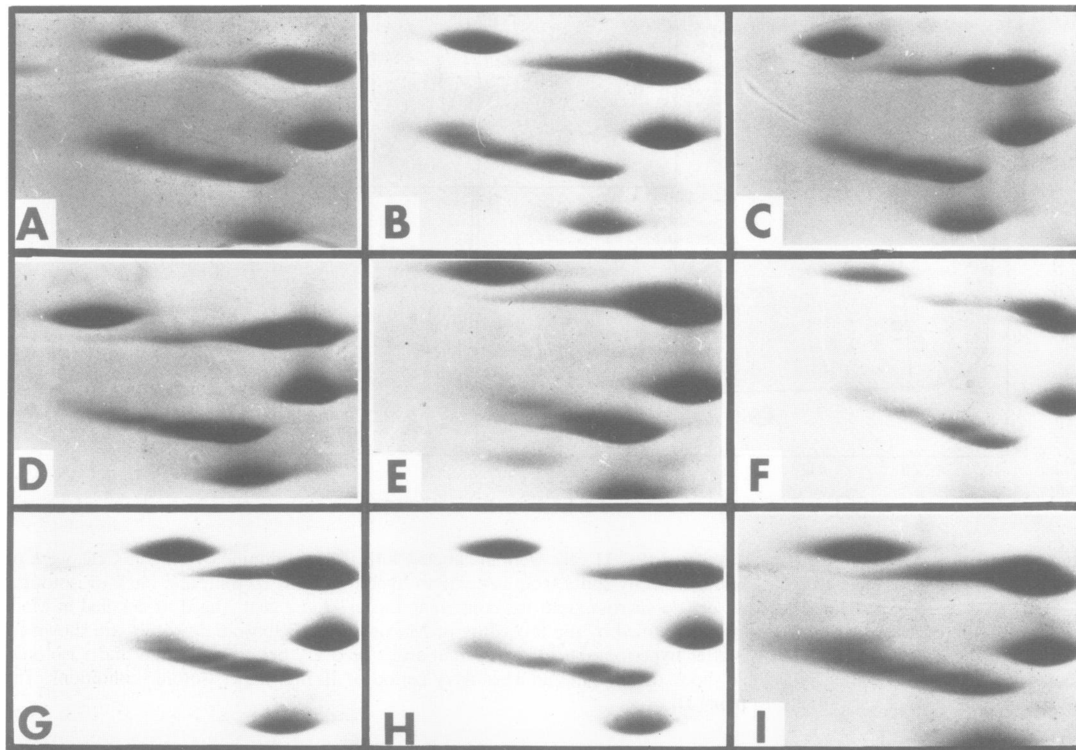


**Fig. 5.** Changes in the phosphorylation state of S6 in MPC 11 cells during tonicity shifts. Ribosomal proteins of all MPC 11 cell samples (same experiment as in Figure 3) were prepared and analysed on two-dimensional gels. Coomassie blue-stained S6 regions of the ribosomal patterns are shown. **Panel A**: pattern of control cells; **panels B – E**: patterns of cells treated with 120 mM excess NaCl for 5, 10, 20 and 65 min; **panels F – I**: patterns of cells after readjustment to isotonic conditions for 10, 20, 30 and 45 min.

S6 phosphorylation in MPC 11 cells. A cycloheximide concentration of 0.1 μg/ml was experimentally found to slow down the elongation rate to such a degree that the reduced initiation rate, due to 75 mM excess NaCl, was no longer rate-limiting for protein synthesis. This caused the cells to maintain a high proportion of polysomes even under the hypertonic conditions (Table I, Exp. 1). Cycloheximide itself had a pronounced stimulatory effect on S6 phosphorylation (Lastick and McConkey, 1980; Gressner and Wool, 1974b) as well as on polysome formation in both control and hypertonic cells (Table I). S6 phosphorylation was nevertheless reduced in cycloheximide-treated cells in the presence of 75 mM excess NaCl as shown by the lower proportions of the most highly phosphorylated S6 species, although the polysome content in these cells exceeded that of the control (Table I). Cellular protein synthesis was decreased in the hypertonic medium in the absence of cycloheximide to 53% of the control value. Cycloheximide, in the presence or absence of excess salt, reduced translation *in vivo* to 30% of the control. Under these conditions, protein synthesis, polysome content and the degree of S6 phosphorylation are obviously not correlated in any simple way.

Puromycin inhibits polysome formation by prematurely releasing nascent peptide chains from ribosomes, blocking protein synthesis completely, and affecting S6 phosphorylation only moderately (Lastick and McConkey, 1980). Using this inhibitor it was therefore possible to dissociate the effects of restoring cells to isotonicity on S6 phosphorylation from that on polysome formation. In the absence of puromycin, readjusting the hypertonic growth medium to isotonic conditions resulted in an increase in polysomal content and in an enhancement of S6 phosphorylation, measured after 40 min

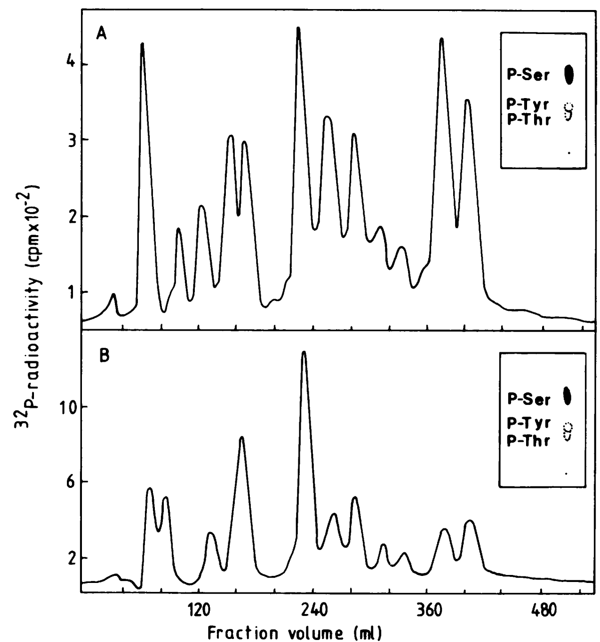


**Fig. 6.** Tryptic phosphopeptide patterns of S6 protein. Ribosomal protein S6 was separated by two-dimensional gel electrophoresis, localized by Coomassie blue staining and digested by trypsin after equilibrating the excised gel slice in 50 mM $NH_4HCO_3$, pH 8.6. The extracted phosphopeptides were separated on a DEAE-Sephadex A25 column and $^{32}P$ radioactivity was determined in the eluate by Cerenkov counting of each fraction. **(A)** shows the phosphopeptide pattern of S6 from control cells; **(B)** is the corresponding pattern from salt-treated cells. The distribution of S6 derivatives in both samples is shown in Figure 1.

**Table I.** Quantitative changes in the phosphorylation state of S6 and in polysomal content following treatment with inhibitors of protein synthesis

| Treatment | % of [$^{35}$S]methionine-labeled S6 | | | % of ribosomes in polysomes |
|---|---|---|---|---|
| | S6a | S6b and c | S6d to f | |
| Exp. 1: isotonic (control) | 8 | 28 | 58 | 70 |
| hypertonic | 22 | 48 | 30 | 42 |
| hypertonic + cycloheximide | 11 | 36 | 49 | 76 |
| cycloheximide | 6 | 16 | 77 | 87 |
| Exp. 2: hypertonic (20 min) | 48 | 52 | – | 15 |
| hypertonic (60 min) | 51 | 49 | – | 13 |
| hypertonic/isotonic | 22 | 48 | 30 | 68 |
| hypertonic/isotonic + puromycin | 30 | 50 | 20 | 10 |

Exp. 1: cells were incubated with or without 0.1 μg/ml cycloheximide and 75 mM excess NaCl for 1 h before preparation of total ribosomes from each sample. Exp. 2: cells were incubated for 20 min with 120 mM excess NaCl. Portions were then returned to isotonic conditions with 1 volume NaCl-free medium and incubated in the presence of 0.2 mM puromycin for a further 40 min. Samples were processed and the S6 patterns were quantified by determining the $^{35}$S radioactivity of the corresponding gel slices after $H_2O_2$ oxidation. The proportions of ribosomes in polysomes were determined by planimetry of profiles of analytical sucrose gradients in buffer D as detailed in Materials and methods.

(Table I, Exp. 2). Rephosphorylation also occurred in the presence of puromycin under isotonic conditions although polysome formation (Exp. 2 of Table I) and protein synthesis (data not shown) were extensively inhibited. Again, the state of S6 phosphorylation does not correlate with either of these parameters. Thus the experiments with both translational inhibitors corroborate the results obtained from the analysis of the kinetics of the responses to the tonicity shift.

Taken together, our observations suggest that the extent of protein synthesis, the proportion of polysomes, and the degree of S6 phosphorylation seem not to be closely interrelated in MPC 11 cells. The results indicate the importance of careful kinetic studies in order to recognize the early divergence between the three parameters. In studies in which such kinetic measurements have also been done, translational activity frequently does not change in parallel with the phosphorylation state of S6 (Ballinger and Hunt, 1982). In MPC 11 cells, protein synthesis is rapidly reinitiated and polysomes are almost completely reformed before rephosphorylation of S6 rises substantially (Figure 2–4). These data do not support the hypothesis (Thomas *et al.*, 1982; Duncan and McConkey, 1982b) that highly phosphorylated S6 is an important prerequisite for activating the translational machinery of cells by recruiting mRNAs. The subtle control of S6 kinase and/or phosphatase activities by intracellular signals (Pouyssegur *et al.*, 1982) and the precise roles of individual phosphorylated species (Nielsen *et al.*, 1982) of this ribosomal protein require further characterization before the significance of much of the data in this field can be fully appreciated.

## Materials and methods

*Materials*

Joklik modified Minimal Essential Medium was obtained from Seromed. Horse and fetal calf serum were purchased from Boehringer Mannheim. Cycloheximide, puromycin, Triton X-100, and cytochrome c were from Sigma. Acrylamide, N,N′-methylenebisacrylamide, Coomassie brilliant blue, and bovine serum albumin (BSA) were obtained from Serva. Trypsin (TPCK-treated) was purchased from Worthington. All other chemicals were of analytical grade and were obtained from Merck. Soluene 350 and dimilume 30 were products of Packard Instrument. L-[$^{35}$S]Methionine (1245 Ci/mmol), L-[$^{3}$H]leucine (60 Ci/mmol), and [$^{32}$P]orthphosphate (carrier-free) were from Amersham Buchler.

*Buffers*

Buffer A: phosphate buffered saline, pH 7.4 (Dulbecco and Vogt, 1954). Buffer B: 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4. Buffer C: 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4. Buffer D: 100 mM KCl, 5 mM $MgAc_2$, and 0.1 mM sodium cacodylate, pH 5.5. Buffer E: 800 mM KCl, 15 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6 (Martin, 1973).

*Growth and treatments of cells*

Mouse myeloma (MPC 11) cells were propagated in spinner cultures in Joklik modified Minimal Essential Medium supplemented with a mixture of 5.5% (v/v) horse and 2% (v/v) fetal calf serum. Suspension cultures reached a density of 1 x $10^6$ cells/ml overnight and were diluted routinely each morning with 1 volume of fresh medium containing serum. In order to maximize the extent of S6 phosphorylation, cells were concentrated by centrifugation, resuspended in fresh growth medium containing serum to a density of 1 x $10^6$ cells/ml, and incubated at 37°C for 1 h. The growth medium was made hypertonic by raising the NaCl concentration and readjusted to isotonic conditions by diluting with an equal volume of NaCl-free growth medium at the times indicated in the figure and table legends. Cells were harvested by pouring the cell suspension on to crushed frozen buffer A and centrifuging at 700 *g* for 5 min.

*Labeling of cells*

Cells were labeled overnight with 2 μCi/ml of [$^{35}$S]methionine in Joklik medium in which the unlabeled methionine concentration was reduced by 50%. For $^{32}$P labeling, cells were resuspended in fresh growth medium supplemented with serum, 2 μCi/ml [$^{32}$P]orthophosphate was added and incubation was continued at 37°C overnight.

*Preparation of ribosomes and extraction of ribosomal proteins*

Cells were washed with ice-cold buffer A and resuspended in the hypotonic buffer B containing 1% Triton X-100. Nuclei were removed by centrifugation (700 *g* for 5 min). An aliquot of the cell extract was layered on 20–50% (w/v) sucrose gradients in buffer D and centrifuged in a Beckman SW41 rotor at 40 000 r.p.m. for 1.5 h at 4°C. The gradients were fractionated using a Buchler autodensiflow and the ribosomal profiles were determined with an ISCO UA5 monitor equipped with a continuous flow cell. Ribosomes and polysomes of the main fraction were sedimented through a 2 ml 2 M sucrose cushion containing buffer C in a Beckman Ti 50 rotor at 45 000 r.p.m. overnight at 4°C.

The amount of ribosomes in polysomes was quantified by planimetry of the analytical gradient profiles mentioned above or by a modification of Martin's procedure (Martin, 1973). Aliquots of cell extracts were treated with micrococcal nuclease (10 μg/ml), layered on 10–30% sucrose gradients in buffer E and centrifuged for 1.5 h in a Beckman SW 65 rotor at 65 000 r.p.m. and 4°C. The relative distribution of messenger-free 40S and 60S subunits and messenger-bound 80S particles derived from polysomes were determined by planimetry of the gradient profiles.

Ribosomal pellets were extracted with 66% acetic acid as previously described (Hardy *et al.*, 1969). RNA was removed by centrifugation in a Beckman JA 21 rotor at 16 000 r.p.m. for 10 min at 4°C. The supernatant was precipitated by addition of 5 volumes of acetone (Barritault *et al.*, 1976). The precipitate was washed once with acetone and dissolved in sample buffer. Protein concentration was determined (Hartree, 1972) using BSA as a standard.

*Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins*

Ribosomal proteins were separated by two-dimensional gel electrophoresis (Lastick and McConkey, 1976). Separation in the first dimension was on 4% (w/v) polyacrylamide disc gels (0.33 x 13 cm), which were run at 120 V for 20–24 h with cytochrome c as a marker (Leader, 1980). Electrophoresis in the second dimension was on a 15% (w/v) polyacrylamide slab gel (21 x 17 x 0.2 cm) at 15 mA (constant current) for ~22 h. Gels were stained with Coomassie brilliant blue, destained, sealed into a plastic bag, and exposed to Kodak XAR-5 X-ray film, using the Dupont intensifying screen Cronex Quanta II. Radioactivity of individual $^{35}$S-labelled S6 derivatives was quantified by incubating gel slices in 0.3 ml of 30% $H_2O_2$ for 15 h at 50°C and then adding dimilume aqueous scintillation fluid.

*Kinetics of protein synthesis*

MPC 11 cells were resuspended at a density of 1 x $10^6$ cells/ml in fresh medium which was buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.6) and supplemented with the serum mixture. After 1 h of incubation at 37°C [$^3$H]leucine was added (2 $\mu$Ci/ml final concentration). Aliquots of 1 ml were removed at the times indicated in the figure and pipetted onto 1 ml of 10% trichloroacetic acid (TCA). A hot TCA treatment (100°C, 5 min) was carried out and the precipitate was finally washed with ethanol before the pellets were solubilized in 0.5 ml soluene and counted after addition of 10 ml dimilume. The osmolarity of the growth medium was changed at the times indicated in the figure legends.

*Trypsin digestion of phosphoprotein S6*

Radioactive spots corresponding to protein S6 were excised from stained two-dimensional gels. Gel slices were equilibrated in 50 mM $NH_4HCO_3$ by several changes of the buffer solution. After equilibration trypsin (TPCK-treated) was added to a final concentration of 50 $\mu$g/ml in 50 mM $NH_4HCO_3$ (pH 8.6) and the digestion was carried out at 37°C under vigorous stirring for 16 h. The stirring bar completely homogenized the gel slices thus facilitating the extraction of S6 protein and peptides. Gel particles were removed by centrifugation and radioactivity was determined in the supernatant fraction. Incubation with a fresh trypsin solution was repeated until >90% of the counts had been extracted from the gel particles. The combined tryptic digests were diluted with 0.3 volume of water and loaded on a DEAE-Sephadex A25 column (1 x 30 cm), which was equilibrated in 50 mM $NH_4HCO_3$ (starting buffer). After sample application the column was washed with 10 ml of starting buffer and then eluted with a linear gradient of 50 mM to 500 mM $NH_4HCO_3$ (pH 8.6, each 300 ml). Fractions of 2 ml were collected and radioactivity of the fractions was measured by Cerenkov counting. The salt gradient was monitored by measuring the conductivity in the eluate. $^{32}$P radioactivity in each phosphopeptide was quantified after combining the corresponding fractions. Recovery of radioactivity from the DEAE-Sephadex A25 columns was 80–90% of the input counts.

## Acknowledgements

The skilfull technical assistance of Mr. A. Bauche and Ms. C. Behn is gratefully acknowledged. M.J.C. holds a Career Development Award from the Cancer Research Campaign (UK). This work was supported by a grant of the Deutsche Forschungsgemeinschaft to J.K.

## References

Ballinger,D.G. and Hunt,T. (1982) *Dev. Biol.*, **87**, 277-285.

Barritault,D., Expert-Bezancon,A., Guerin,M.-F. and Hayes,D. (1976) *Eur. J. Biochem.*, **63**, 131-135.

Dulbecco,R. and Vogt,M. (1954) *J. Exp. Med.*, **99**, 167-182.

Duncan,R. and McConkey,E.H. (1982a) *Eur. J. Biochem.*, **123**, 535-538.

Duncan,R. and McConkey,E.H. (1982b) *Eur. J. Biochem.*, **123**, 539-544.

Eil,C. and Wool,I.G. (1973) *J. Biol. Chem.*, **248**, 5130-5136.

Gressner,A.M. and Wool,I.G. (1974a) *J. Biol. Chem.*, **249**, 6917-6925.

Gressner,A.M. and Wool,I.G. (1974b) *Biochem. Biophys. Res. Commun.*, **60**, 1482-1490.

Hardy,S.J.S., Kurland,C.G., Voynow,P. and Mora,G. (1969) *Biochemistry (Wash.)*, **8**, 2897-2905.

Hartree,E.F. (1972) *Anal. Biochem.*, **48**, 422-426.

Kruppa,J. and Martini,O.H.W. (1978) *Biochem. Biophys. Res. Commun.*, **85**, 428-435.

Kruppa,J., Darmer,D., Kalthoff,H. and Richter,D. (1983) *Eur. J. Biochem.*, **129**, 537-542.

Krystosek,A., Bitte,L.F., Cawthon,M.L. and Kabat,D. (1974) in Nomura, M., Tissieres,A. and Lengyel,P. (eds.), *Ribosomes*, Cold Spring Harbor Laboratory Press, NY, pp. 855-870.

Lastick,S.M. and McConkey,E.H. (1976) *J. Biol. Chem.*, **251**, 2867-2875.

Lastick,S.M. and McConkey,E.H. (1980) *Biochem. Biophys. Res. Commun.*, **95**, 917-923.

Leader,D.P. (1980) *J. Biochem. Biophys. Methods*, **3**, 247-248.

Leader,D.P., Thomas,A. and Voorma,H.O. (1981) *Biochim. Biophys. Acta*, **656**, 69-75.

Martin,T.E. (1973) *Exp. Cell Res.*, **80**, 496-498.

Martini,O.H.W. and Kruppa,J. (1979) *Eur. J. Biochem.*, **95**, 349-358.

Meedel,T.H. and Levine,E.M. (1978) *J. Cell Physiol.*, **94**, 229-242.

Nielsen,P.J., Duncan,R. and McConkey,E.H. (1981) *Eur. J. Biochem.*, **120**, 523-527.

Nielsen,P.J., Thomas,G. and Maller,J.L. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2937-2941.

Pouyssegur,J., Chambard,J.C., Franchi,A., Paris,S. and van Obberghen-Schilling,E. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3935-3939.

Saborio,J.L., Pong,S.S. and Koch,G. (1974) *J. Mol. Biol.*, **85**, 195-211.

Scharf,K.-D. and Nover,L. (1982) *Cell*, **30**, 427-437.

Thomas,G., Siegmann,M. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3925-3956.

Thomas,G., Martin-Pérez,J., Siegmann,M. and Otto,A.M. (1982) *Cell*, **30**, 235-242.

Traugh,J.A. (1981) in Litwack,G. (ed.), *Biochemical Actions of Hormones*, Vol. **8**, Academic Press, NY, pp. 167-208.

Wettenhall,R.E.H. and Howlett,G.J. (1979) *J. Biol. Chem.*, **254**, 9317-9323.

Wettenhall,R.E.H., Cohen,P., Caudwell,B. and Holland,R. (1982) *FEBS Lett.*, **148**, 207-213.

Wool,I.G. (1979) *Annu. Rev. Biochem.*, **48**, 719-754.

*Received on 13 September 1983*