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DEPHOSPHORYLATION OF ONE 40S RIBOSOMAL PROTEIN IN MPC 11 CELLS INDUCED BY HYPERTONIC MEDIUM

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SUMMARY

Mouse myeloma cells (MPC 11) were grown at a high cell density in the presence of [^{32}P]orthophosphate. The tonicity of the medium was raised by 100 mM NaCl in half of the suspension culture after 4 hrs of incubation. The proportions of messenger-free ribosomes and polysomes from salt-treated and control cells were quantitated after separation by sucrose gradient centrifugation and the four ribosomal preparations were analysed by two-dimensional gel electrophoresis. Two ribosomal phosphoproteins, S3 and L28 (nomenclature of Martini and Gould; ref. 1) were detected by autoradiography in all four ribosomal protein patterns. Quantitation of ^{32}P-incorporation revealed that a positive correlation exists between the degree of phosphorylation of S3, and the amount of ribosomes engaged in protein synthesis. S3 was highly phosphorylated in polysomes of control cells, only slightly phosphorylated in polysomes of salt-treated cells and almost completey dephosphorylated in messenger-free ribosomes of salt-treated cells. The $^{32}P_i$-incorporation of L28 was much less effected by the hypertonic medium.

INTRODUCTION

In recent years experimental evidence has accumulated suggesting that eukaryotic protein synthesis can be regulated at the translational level (2). Most of the studies to elucidate the mechanism of this control focussed on the early steps of peptide chain initiation: (a) the formation of the met-tRNA$_f$·GTP·eIF2-complex and the association of this complex with the 40S ribosomal subunit (3-6), (b) the binding of individual mRNAs to the 40S complex (7-9). The differences in the activity of ribosome associated factors together with the inherent physical properties of individual mRNA-species have been postulated to explain the observed qualitative and quantitative changes in cellular protein biosynthesis (2). This view may be an over-

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simplification since studies utilizing the hypertonic initiation block technique (10) suggested the involvement of ribosomes in the regulation of protein synthesis (11). Because of its simplicity this technique of elevating medium tonicity seems particularly attractive to investigate the translational control mechanism. This technique enables one to effect changes in the translational machinery in tissue culture cells in response to varying a single defined parameter namely the tonicity of the growth medium. Using this method we were interested to correlate the rate of cellular protein synthesis with the state of ribosomal protein phosphorylation in control and salt-treated cells. To this end we have isolated highly purified messenger-free ribosomes and polysomes from MPC 11 cells and analysed the protein phosphorylation pattern of these two ribosomal subpopulations after in vivo labelling with $^{32}P_i$ by two-dimensional gel electrophoresis.

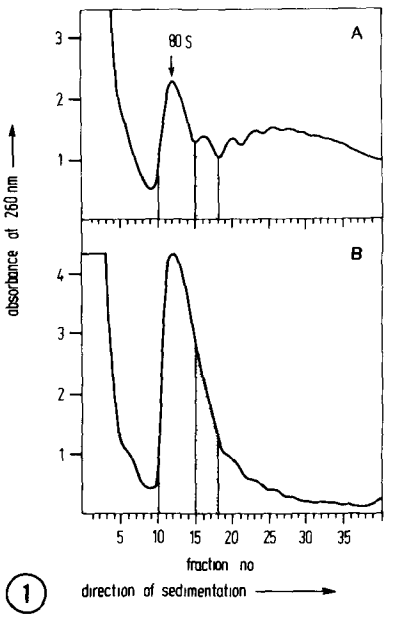
MATERIALS AND METHODS

Suspension cultures of MPC 11 cells were collected by centrifugation and suspended at 4 x 10^6 cells/ml in 700 ml medium A supplemented with 2 % (v/v) fetal calf serum. Medium A was free of phosphates and contained the following compounds: NaCl, 6400 mg/l; KCl, 400 mg/l; $MgSO_4$ x 7 H_2O, 200 mg/l; $FeCl_3$, 0.1 mg/l; $NaHCO_3$, 2250 mg/l; glucose, 2000 mg/l; and phenol red, 15 mg/l; as well as 1 mM sodium pyruvate, 4 mM glutamine, and 17 mM Hepes-KOH, pH 7.6. Amino acids and vitamins were added at twice the concentration specified by Eagle (12). Penicillin and streptomycin were used at 160 iU/ml and 100 µg/ml, respectively. The cells were labelled with 7 mCi [^{32}P] orthophosphate (carrier-free, Amersham Buchler) for 5 hrs. After one hour of incubation the medium was adjusted to 10 % fetal calf serum and incubation was continued for another 3 hrs at which time the tonicity of the medium was raised by 100 mM NaCl in half of the cell suspension, the other half served as control. Incorporation was halted one hour later by pouring the cells on isotonic ice cubes of phosphate buffered saline. The cells were collected by centrifugation at 300 xg for 5 min at 0°C, washed once with ice cold isotonic phosphate buffered saline and resuspended in hypotonic buffer (10 mM KCl, 10 mM Tris-HCl, pH 7.5, 1.5 mM Mg-acetate, and 1 mM dithiothreitol). After swelling for 10 min the cells were homogenized in a Dounce homogenizer (Kontes, Vineland, N.J.) using 6-8 strokes with the B-pestle. The homogenate was centrifuged at 700 xg for 5 min at 4°C. The postnuclear supernatant was treated with 0.1 vol of 10 % (w/v) Triton X100 and 10 % (w/v) deoxycholate and layered on a 10-40 % sucrose gradient in low salt buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM Mg-acetate, 1 mM dithiothreitol). The ribosomal particles were separated by centrifugation in the SW 27 Beckman rotor at 113,000 xg for 5 hrs at 4°C. The 80 S fraction was resuspended in high salt buffer (500 mM KCl, 50 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 1 mM dithiothreitol) and loaded on a 10-30 % sucrose gradient in high salt buffer which was centrifuged in a SW 27 Beckman rotor at 113,000 xg and 4°C for 7 hrs. The messenger-free ribosomal particles were pooled and recovered by centrifugation in the Beckman Ti 50.2 rotor at 244,000 xg and 4°C for 16 hrs. Polysomes were re-

suspended in high salt buffer and centrifuged through 1.5 ml of a 60 % (w/v) sucrose cushion in high salt buffer in a SW 65 Beckman rotor at 300,000 xg for 4 hrs at 4°C. The proteins of the ribosomal fractions were extracted as described previously (13) and analysed by the two-dimensional polyacrylamide gel electrophoresis technique of Martini and Gould (1), which combines a separation at pH 4.5 in the first dimension with a size-based separation in an SDS-gel in the second dimension. The destained gels were exposed to Kodak X-Omat films as described previously (14). Protein concentrations were determined by the method of Lowry et al. (15). ^{32}P-incorporation of the ribosomal proteins was measured by Cerenkov counting in a liquid scintillation spectrometer after cutting out the "S 3" spot.

RESULTS AND DISCUSSION

In order to achieve a large increase in messenger-free ribosomes after applying the hypertonic initiation block growth conditions were established wherein the majority of the ribosomes of MPC 11 cells were engaged in protein synthesis. A special medium was composed for this purpose which is very rich in amino acids and other nutrients but is lacking phosphate (see Materials and Methods). The cells were labelled with [^{32}P] orthophosphate in the presence of fetal calf serum (non-dialysed) as additional source of phosphate. Preliminary experiments showed that the proportion of ribosomes in polysomes was about 85-90 % of the total ribosomal population in MPC 11 cells transferred to this medium at cell densities of up to 4 x 10^6 cells/ml. This value was fairly constant over a period of 5-6 hrs. By using this medium we hoped to eliminate nutritional effects on the ^{32}P-incorporation as have been observed previously (16, 17). A high cell density was necessary in order to obtain adequate amounts of ribosomal proteins for analysis. Raising the tonicity of the growth medium by addition of 100 mM NaCl results in (a) a reduction of total protein synthesis in MPC 11 cells (10). (b) Translation of individual mRNAs is differentially inhibited as has been shown by SDS-polyacrylamide gel electrophoresis of [^{35}S]Met-labelled proteins (10). (c) Peptide chain initiation is impaired under high salt conditions leading to a massive conversion of polysomes to 80S ribosomes (10 and Fig. 1). Messenger-free ribosomal particles were purified from the 80S fraction of control (Fig. 1 A) and salt-treated cells (Fig. 1 B) by sucrose gradient centrifugation in high



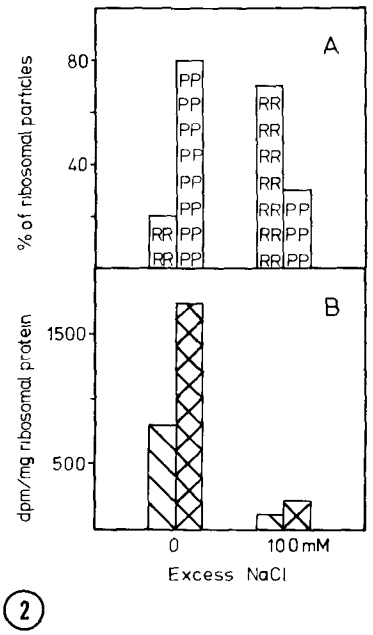


Figure 1. Ribosome profiles of control (A) and salt-treated cells (B) after sucrose gradient centrifugation in low salt buffer.
Separation was achieved on 10-40 % sucrose gradients containing low salt buffer in the SW 27 Beckman rotor at 113,000 xg for 5 hrs at 4°C. Messenger-free particles were prepared from the 80S fraction, material from the dashed area was discarded. Polysomes which were analysed after further purification were collected beginning with fraction number 18.

Figure 2. Phosphorylation of S3 of messenger-free ribosomes and polysomes from control and salt-treated cells.
(A) The proportion of messenger-free ribosomes (R) and polysomes (P) were determined from sucrose gradients in high salt buffer. Single ribosomes bound to mRNA have been included in the polysome fraction.
(B) After autoradiography the protein spot S3 was cut out of the gels, and the radioactivity was measured by Cerenkov counting in a liquid scintillation spectrometer. 700-900 µg of ribosomal protein from each of the four preparations were used for the two-dimensional gels. The dpm-values were normalized for reasons of comparison. Shaded bars and cross-hatched bars correspond to $^{32}P_i$-incorporation of S3 of messenger-free ribosomes and polysomes respectively. Results on the left side in A and B are obtained from control cells, those on the right side after raising the NaCl-concentration by 100 mM.

salt buffer. Both polysomal fractions were also subjected to a high salt treatment to remove associated proteins.

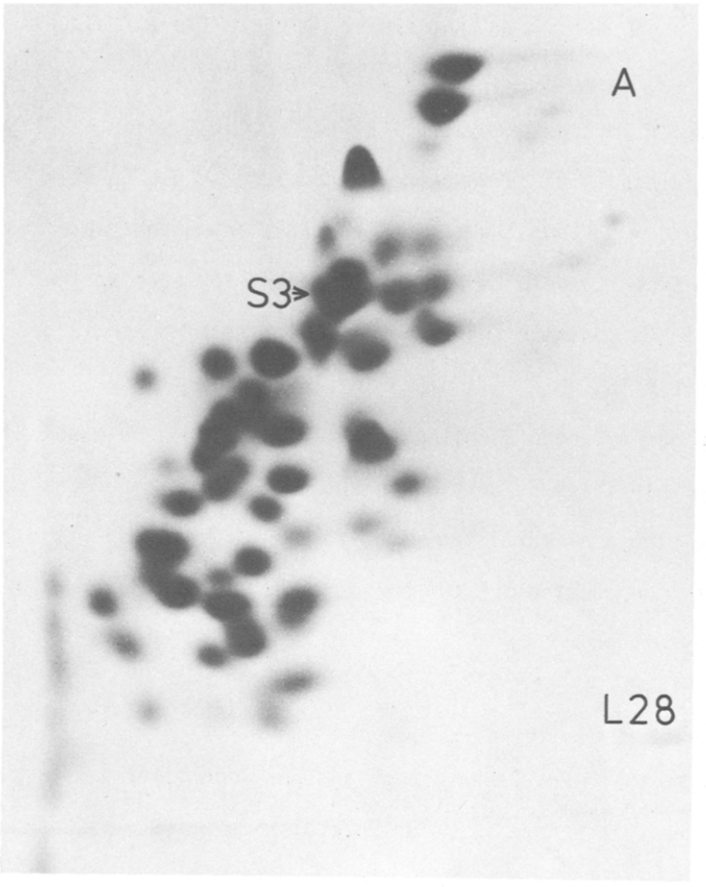
The proportion of messenger-free ribosomes and polysomes from control and salt-treated cells was calculated from gradient patterns (Fig. 2 A). In salt-treated cells the messenger-free ribosome fraction increased by over

50 % and thus is mainly derived from newly dissociated polysomes. After hypertonic shock a reduction of the ^{32}P-incorporation into the small subunit ribosomal phosphoprotein of salt-treated cells was observed (Fig. 2 B). Two-dimensional gel electrophoresis of the four ribosomal preparations with subsequent autoradiography revealed that predominantly two proteins were phosphorylated: S3 and L28 (1).

Figure 3 shows the protein pattern of polysomes from control cells (the polysomal pattern of salt-treated cells looked very similar) and the autoradiographs of both polysomal fractions. The phosphoprotein S3 was unambigously identified from a 40S subunit pattern. The extent of phosphate incorporation into S3 of polysomes was markedly reduced in salt-treated cells (Fig. 3 C) compared to control cells (Fig. 3 B). A slight increase in the ^{32}P-incorporation of L28 from 230 dpm before to 300 dpm after salt shock was detected by Cerenkov counting of the excised gel pieces. A comparison of the phosphoproteins of both messenger-free ribosome fractions showed a similar trend. The highest degree of phosphorylation was observed in S3 of polysomes from control cells, and the phosphate incorporation into S3 of messenger-free ribosomes was always lower than in polysomes (Fig. 2 B). Taken together our results imply that there exists a positive correlation between the degree of phosphorylation of S3 and the number of ribosomes engaged in protein synthesis, although phosphorylation of S3 does not seem to be a prerequisite for the involvement of the 40S subunit in protein synthesis (Fig. 2).

Whether or not the phosphorylation of S3 effects the regulation of protein synthesis remains an open question. Since S3 can be crosslinked to

Figure 3. Ribosomal protein pattern after two-dimensional gel electrophoresis.
Polysomes of control cells were purified as described in Materials and Methods and analysed with the two-dimensional gel electrophoresis technique of Martini and Gould (1). Protein pattern stained with Coomassie blue (A). Autoradiogram of polysomes from control cells (B) and from salt-treated cells (C). By comparative analyses in different two-dimensional electrophoresis systems the homology of protein "S3" (nomenclature cf. 1) of MPC 11 cells with "S6" of rat liver (nomenclature of Sherton and Gould, ref. 18) could be ascertained. In addition it is likely that protein L28 of MPC 11 cells corresponds to protein L40 of rat liver and HeLa cells (19).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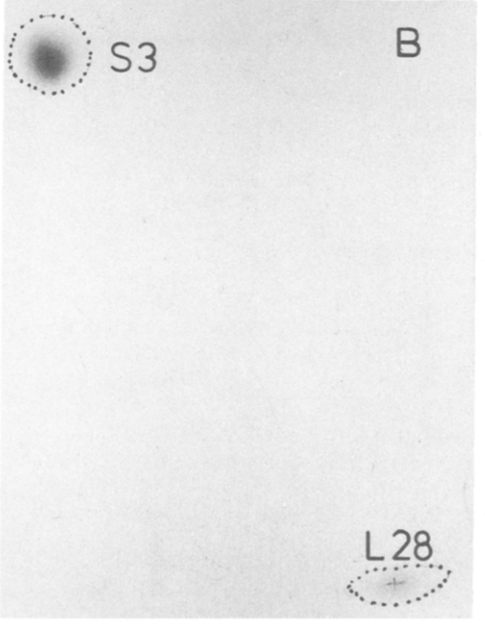
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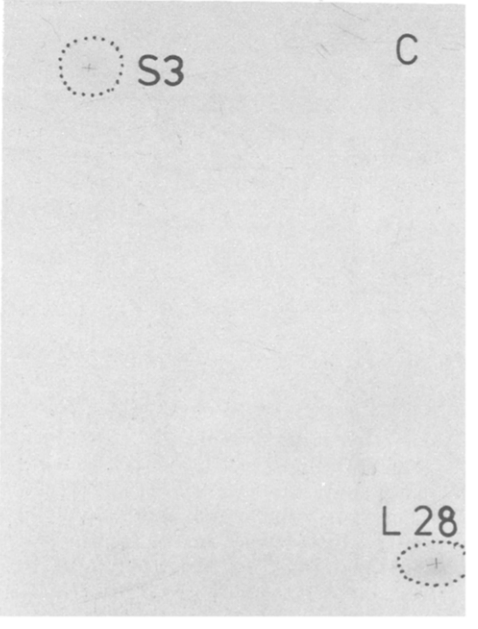
S3
C
L 28

mRNA (20) one might speculate that mRNA binding to the 40S subunit may be influenced by the level of phosphorylation of S3. Only mRNA species with high intrinsic affinities are postulated to form initiation complexes under hypertonic conditions, these messengers therefore should be able to bind efficiently to a 40S subunit which has a nearly dephosphorylated S3 protein. In this connection it would be of great interest to learn whether the cap-structure having a positive charge can interact with phosphate groups of ribosomal protein S3.

By using the hypertonic initiation block it should be possible to prepare ribosomal particles of very different, but well defined phosphorylation states. These ribosomal particles might be useful for an in vitro approach to investigate the question of the biological significance of ribosomal protein phosphorylation.

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