PHOSPHORYLATED PROTEINS IN THE PASSAGE FROM FREE mRNP TO POLYSOMES IN MICE PLASMACYTOMA CELLS

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1. Introduction

In eukaryotic cells the messenger RNA and their precursors are invariably associated with proteins to form messenger ribonucleoprotein complexes called mRNP. In the cytoplasm, mRNP can be bound to ribosomes or can be in the non polyribosomal state, i.e., free mRNP (review [1]). Moreover kinetic studies on cytoplasmic mRNA have suggested a relationship between free mRNP and polysomes [2–6]. Free mRNP are not translated in a cell-free system [7,8] while both naked mRNA from these free particles and the polysomal mRNP can direct protein synthesis [4,6,7,9,10]. Of the mRNA-associated components, proteins or protein-associated factors may play a functional role in translational control. Among these proteins several are reversibly phosphorylated [11–15]. These observations along with the occurrence of a phosphorylation process in the initiation of protein synthesis [16] have prompted us to study the behavior of phosphorylated proteins associated with mRNA in free mRNP as well as in polysomes.

Here, we analyze kinetically the behavior of phosphorylated proteins and mRNA components in the passage from free mRNP to polysomes in mice plasmacytoma cells.

2. Materials and methods

2.1. *Isolation of free mRNP and polysomes*

Plasma cell tumours RPC_5 were grown in Balb/c mice. Each tumor-bearing mouse was injected intraperitoneally with 1 mCi [^{3}H]uridine and with 75 μg actinomycin D after 14 h; under these conditions only synthesis of rRNA was entirely blocked [17]; 30 min after actinomycin D injection the mice received 2 mCi [^{32}P]orthophosphate intraperitoneally. They were killed at different times after this injection. Tumours were homogenized in 4 vol. ice-cold, 20 mM triethanolamine–HCl buffer (pH 7.6) containing 150 mM KCl, 6 mM 2-mercaptoethanol, 4 mM magnesium acetate and 1.1 M sucrose. The homogenate was centrifuged at 20 000 × *g* for 15 min in a Spinco R 60 Ti rotor. To obtain separately free mRNP and polysomes, the post-mitochondrial supernatant was diluted to 0.25 M sucrose and centrifuged on a D_2O–sucrose gradient at 180 000 × *g* for 20 h as in [18]. The F200 fraction was obtained by centrifugation of the diluted post-mitochondrial supernatant at 200 000 × *g* for 4 h in a Spinco R 60 Ti rotor.

2.2. *Determination of radioactivity*

Radioactivity was measured as in [11] using a scintillation spectrometer. The labelled compounds were determined by differential calculation. The radioactive material was adsorbed onto Whatman filters (2 × 4 cm); the filters were immersed in cold 10% trichloroacetic acid and washed successively in cold 5% trichloroacetic acid, ethanol, ethanol–ether oxide (v/v) and finally in ether oxide. The first count of the dried papers corresponds to the ^{3}H radioactivity from long-term labelled r[^{3}H]RNA plus the ^{32}P radioactivity of ^{32}P-phosphorylated proteins plus short-term labelled m[^{32}P]RNA. In a second step, the rehydrated filters were treated with 5% trichloroacetic acid, heated 10 min at 95°C, washed as above and counted. This last count corresponds to ^{32}P-phosphorylated proteins; subtraction of the second from the first ^{32}P count corresponds to mRNA.

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2.3. *Polyacrylamide gel electrophoresis*

SDS–polyacrylamide gel electrophoresis was done in slab gels according to [19] and consisted of a 10–20% acrylamide gradient. After staining with Coomassie brilliant blue, the slab gels are treated with 5% trichloroacetic acid at 95°C for 10 min to hydrolyse the RNA, then dried and exposed to X-ray films (Kodak X-Omat).

3. Results

3.1. *Evidence of phosphorylated proteins in free mRNP and polysomes*

Cytoplasmic RNAs fractionated on a discontinuous D_2O-sucrose gradient were analysed on a CsCl gradient as shown in fig.1. The [^{3}H]uridine-labelled rRNA present in the polyribosomes bands exclusively at a density of 1.54 g/cm^2, the ^{32}P-labelled mRNA associated with ribosomes bands in the same density area (fig.1B). CsCl density gradient of free mRNP shows that the ^{32}P-labelled mRNA has a relatively broad distribution of densities, with a maximum at 1.43, whereas ribosomal particles are virtually absent (fig.1A). Since phosphorylated proteins are present in both particles, it becomes important to examine the role of phosphorylated proteins in the passage from free mRNP to polysomes. Are phosphorylated proteins from free mRNP precursors of those present in polysomes?

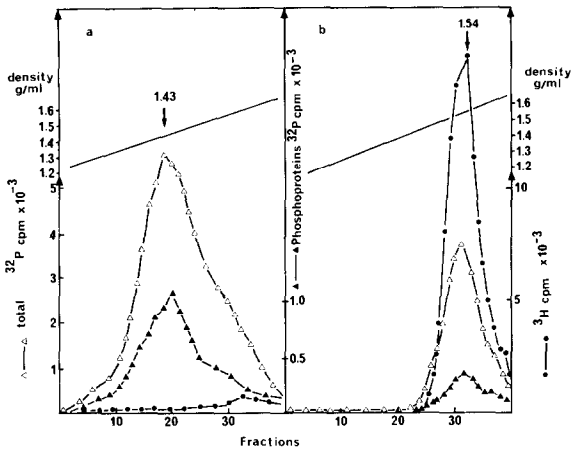

Fig.1. Isopycnic sedimentation in CsCl. Free mRNP (A) and polysomes (B) obtained as described were fixed with 1% formaldehyde, layered on top of a CsCl gradient and centrifuged during 14 h at 10°C and at 45 000 rev./min in a Spinco rotor SW 65. The radioactive material is analyzed as in section 2: (●) ^{3}H radioactivity from long-term labelled rRNA; (△) ^{32}P radioactivity from ^{32}P-labelled mRNA plus ^{32}P-phosphorylated proteins; (▲) ^{32}P radioactivity after treatment with 5% hot trichloroacetic acid; evidence of ^{32}P-phosphorylated proteins.

3.2. *Kinetic analysis*

If the above hypothesis is correct, the phosphorylated proteins might be incorporated into polysomes with the same kinetics as mRNA. To examine this point we have analyzed the rate of phosphorylation of the proteins and mRNA in both free mRNP and polysomes. To follow the movement of mRNA and phosphorylated proteins into the cytoplasm, we took advantage of the property of actinomycin D to specifically inhibit ribosomal RNA synthesis [17,20,21]. Fig.2A shows the behavior of [^{3}H]uridine-labelled

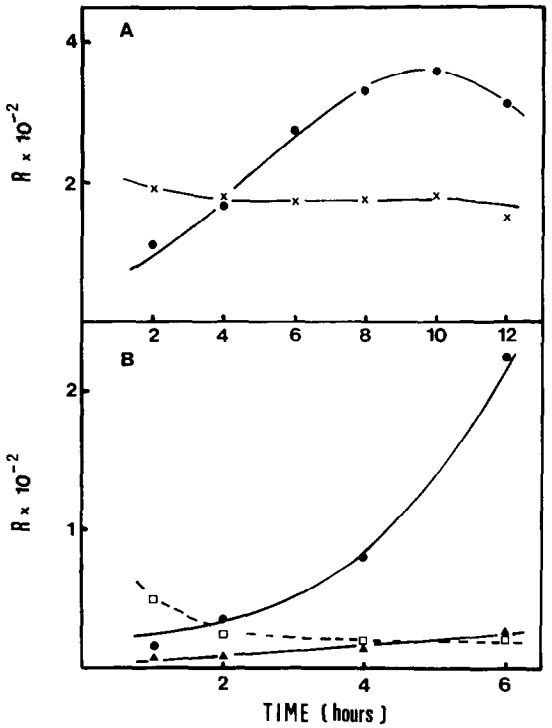

Fig.2. Evolution of labelled compounds in free mRNP and polysomes. The different labelled compounds, mRNA, rRNA and phosphorylated proteins are determined as in section 2. (A) Stability of ribosomal RNA in polysomes: (X) total polysomal protein/r[^{3}H]RNA; (●) m[^{32}P]RNA/r[^{3}H]RNA. (B) Incorporation of phosphorylated compounds: in polysomes, (●) m[^{32}P]RNA/r[^{3}H]RNA, (▲) ^{32}P-phosphorylated protein/r[^{3}H]RNA; in free mRNP, (□) ^{32}P-phosphorylated proteins/m[^{32}P]RNA. R = ratio of two different compounds. The values are av. 3 expt.

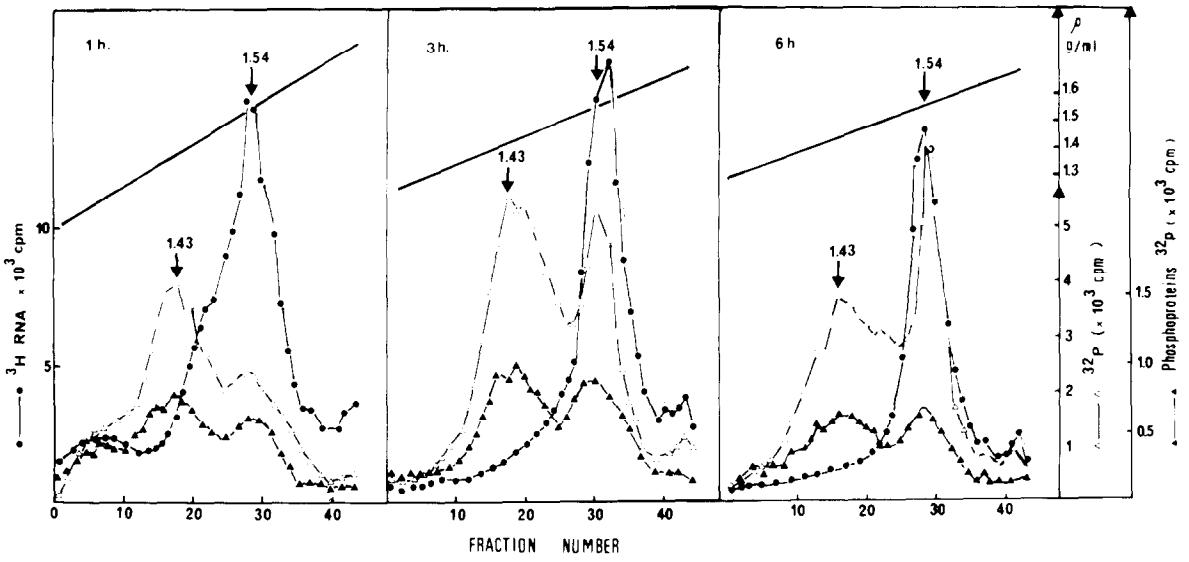

Fig.3. CsCl gradients of F200 fraction. At 1,3 and 6 h after $^{32}PO_4$ application, F200 fractions containing free mRNP and polysomes are prepared as in section 2 and analyzed on a CsCl gradient: (●) ^3H radioactivity from long-term labelled rRNA; (△) ^{32}P radioactivity from ^{32}P-labelled mRNA plus ^{32}P-phosphorylated proteins; (▲) ^{32}P radioactivity after treatment with 5% hot trichloroacetic acid; evidence of ^{32}P-phosphorylated proteins.

rRNA in polysomes, several hours after actinomycin D treatment; the ratio [^{3}H]uridine-labelled rRNA/polysomal proteins estimated as in [28] remains almost constant during the 12 h of the experiment. This indicates that:

(i) rRNA synthesis is blocked as in [17,20,21];
(ii) Ribosomes are not significantly degraded during the kinetic experiment and can be re-utilized for a reinitiation process [20,21].

Consequently, we may analyze at different times the evolution of mRNA and phosphorylated proteins from both free mRNP and polysomes by comparing them with r[^{3}H]RNA radioactivity. Thus at different times after addition of $^{32}PO_4$, the entire particulate fractions F200 are analyzed in shallow CsCl gradients (fig.3). As mentioned above, free mRNP and polysomes are characterized by their CsCl gradient density at 1.43 and 1.54, respectively. At 1 h after $^{32}PO_4$ addition, mRNA and phosphorylated proteins from free mRNP are labelled; 85% of the $^{32}PO_4$ material of density 1.43 is incorporated into mRNA: this calculation is obtained by substracting the hot trichloroacetic acid resistant ^{32}P-labelled material from the total cold trichloroacetic acid precipitable ^{32}P-labelled material occurring in the 1.43 density region (fig.3, at 1 h). At that time, few labelled mRNA appear in the

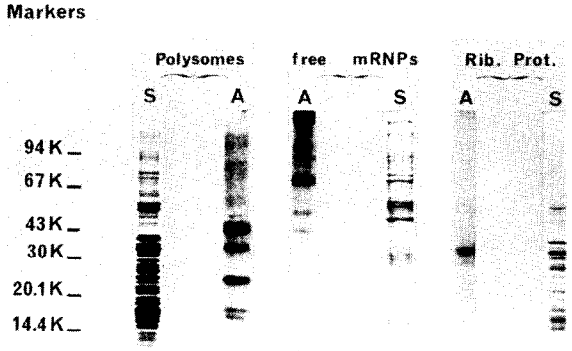

Fig.4. Analysis of phosphorylated proteins by polyacrylamide gel electrophoresis. Free mRNP and polysomes were obtained after centrifugation of a post-mitochondrial supernatant on a D_2O-sucrose gradient [15] after 3 h $^{32}PO_4$ incorporation. Phosphorylated ribosomal proteins were obtained by the extraction procedure in [29]. The proteins were analyzed by SDS gel electrophoresis on a 10–20% acrylamide gradient: (S) stained gel; (A) autoradiography. The radioactivity of free mRNP (60 μg) and polysomes (105 μg) was 14 300 cpm and 14 800 cpm, respectively. The radioactivity of phosphorylated ribosomal proteins (45 μg) was 4300 cpm. Due to the low specific activity of the phosphorylated proteins (10–15 cpm/μg protein) the gels were exposed for 10 days. ^{32}P-labelled ribosomal proteins were prepared from polysomes [28]. M_r-markers are phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400).

polysomal region (polysomal mRNA represents >70% of the total cytoplasmic mRNA [4,5]). At 3 h and 6 h, ^{32}P-label appears in the polysomes although the peak of phosphorylated proteins does not increase considerably. The ratio of mRNA and of phosphorylated proteins relative to rRNA has been measured in the polysomes. Fig.2B shows that phosphorylated proteins and mRNA are incorporated into polysomes at different rates. As the rRNA labelling is constant during 12 h, (fig.2A), the mRNA is integrated into polysomes more rapidly than phosphorylated proteins (fig.2B). This may be correlated with the fact that the ratio of phosphorylated proteins to mRNA diminishes appreciably in the free mRNP during the first hours of ^{32}P-treatment.

3.3. *Identification of phosphorylated proteins*

The preceding results show that all the phosphorylated proteins do not follow the mRNA as it is integrated into the polysomes. The protein pattern of free mRNP and polysomes has been analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Some polypeptides are common to both complexes (fig.4); however in free mRNP the labelling of high M_r species seems to be stronger than in polysomes. These latter particles show highly labelled polypeptide bands at <50 000 M_r; some of them, such as the 33 000 M_r protein are ribosomal proteins [22,23].

4. Discussion

We show here that mRNP phosphorylated proteins and mRNA are not integrated into polysomes with the same kinetics. Confirming [3–5], fig.2a,b show that mRNA is first present in free mRNP and thereafter in polysomes. Fig.3 indicates that mRNA integrates more rapidly into polysomes than phosphorylated proteins. The data suggest that phosphorylated proteins from free mRNP do not accompany the mRNA sequences into polysomes. Qualitatively and quantitatively, the phosphorylated protein patterns from both types of ribonucleoprotein complexes differ (fig.4). The major phosphorylated species present are either ribosomal subunits [22,23] or phosphorylated initiation factors [16,24,25]; some of the latter may exist in the free mRNP pool [26]. In the transition free mRNP⇆polysomes [2,5,27], either an exclusion and/or a dephosphorylation mechanism of some of the mRNA-associated phosphoproteins may favor the transition from free mRNP to polysomes.

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References

[1] Preobrazhensky, A. A. and Spirin, A. S. (1978) Prog. Nucleic Acids Res. Mol. Biol. 21, 2–38.
[2] Spohr, G., Kayibanda, B. and Scherrer, K. (1972) Eur. J. Biochem. 31, 194–208.
[3] Spohr, G., Granboulan, N., Morel, C. and Scherrer, K. (1970) Eur. J. Biochem. 17, 296–318.
[4] Zähringer, J., Baliga, B. S. and Munro, M. N. (1976) Proc. Natl. Acad. Sci. USA 73, 857–881.
[5] Mc Leod, M. (1976) Biochemistry 14, 4011–4018.
[6] Gross, K. W., Jacobs-Lorena, M., Baglioni, C. and Gross, P. R. (1973) Proc. Natl. Acad. Sci. USA 70, 2614–2618.
[7] Civelli, O., Vincent, A., Buri, J. F. and Scherrer, K. (1976) FEBS Lett. 72, 71–76.
[8] Liautard, J. P. and Egly, J. M. (1980) Nucleic Acids Res. 8, 1793–1803.
[9] Nudel, U., Lebleu, B., Zehavi-Willner, T. and Revel, M. (1973) Eur. J. Biochem. 33, 314–322.
[10] Ernst, V. and Arnstein, H. R. V. (1975) Biochim. Biophys. Acta 378, 251–259.
[11] Egly, J. M., Johnson, B. C., Stricker, Ch., Mandel, P. and Kempf, J. (1972) FEBS Lett. 22, 181–184.
[12] Schmitt, M., Egly, J. M., Mandel, P., Kempf, J. and Quirin-Stricker, Ch. (1973) Biochimie 55, 653–659.
[13] Gander, E. S., Stewart, A. G., Morel, C. and Scherrer, K. (1973) Eur. J. Biochem. 38, 443–449.
[14] Auerbach, S. and Pederson, Th. (1975) Biochem. Biophys. Res. Commun. 63, 149–156.
[15] Bag, J. and Sells, B. H. (1979) J. Biol. Chem. 254, 3137–3140.
[16] Ochoa, S. and De Haro, C. (1979) Annu. Rev. Biochem. 48, 549–580.
[17] Kempf, J. and Mandel, P. (1969) Bull. Soc. Chim. Biol. 51, 1121–1129.
[18] Kempf, J., Egly, J. M., Stricker, Ch., Schmitt, M. and Mandel, P. (1972) FEBS Lett. 26, 130–134.
[19] Maizel, J. V. (1971) Methods Virol. 5, 180–247.
[20] Perry, R. P. (1963) Exp. Cell. Res. 29, 400–407.
[21] Georgiev, P., Samarina, O. P., Lerman, M. I., Smirnov, M. N. and Severtzov, A. N. (1963) Nature 200, 1291–1293.
[22] Kabat, D. (1971) Biochemistry 10, 197–203.

[23] Genot, A., Reboud, J. P., Cenatiempo, Y. and Cozzone, A. J. (1978) FEBS Lett. 86, 103–107.
[24] Barrieux, A. and Rosenfeld, M. G. (1977) J. Biol. Chem. 252, 3843–3847.
[25] Benne, R., Edman, J., Traut, R. R. and Hershey, J. W. B. (1978) Proc. Natl. Acad. Sci. USA 251, 7675–7681.
[26] Ovchinnikov, L. P., Spirin, A. S., Erni, B. and Staehelin, T. (1978) FEBS Lett. 86, 21–26.
[27] Princen, H. M. G., Van Eekelen, C. A. G., Asselbergs, F. A. M. and Van Venrooij, W. J. (1979) Mol. Biol. Rep. 5, 59–64.
[28] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randal, R. J. (1951) J. Biol. Chem. 193, 265–275.
[29] Hardy, S. J. S., Kurland, C. G., Voynow, P. and Mora, G. (1969) Biochemistry 8, 2897–2905.