SYNTHESIS OF A NUCLEAR PROTEIN IN G_2-PHASE.

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Received November 13, 1969

Summary.

A specific extraction procedure for proteins present in isolated nuclei of the slime mold Physarum polycephalum is described. This extraction procedure yields more than 90% of the nuclear proteins with less than 12% polysaccharide. Gel electrophoresis of nuclear extracts from S- and G_2-phase nuclei revealed no significant differences in the densitometer tracings of the stained bands. The synthesis of at least one nuclear protein almost exclusively in G_2 phase could be demonstrated when nuclear extracts from plasmodia labeled with ^{14}C-amino acids were used for electrophoresis with subsequent fractionation of the gels. The molecular weight of this protein was determined to be about 42,000.

The relationship of the synthesis of histones to DNA synthesis is well known. Histone synthesis occurs only in the S-phase of the mitotic cycle in synchronized HeLa cells, in vivo (1) as well as in vitro (2). Since the histones are the only nuclear proteins for which such a relationship has been described thus far, we were interested in determining the time of synthesis of other nuclear proteins. The slime mold Physarum polycephalum exhibits a natural synchrony of the mitotic cycle (3) and hence seemed a suitable organism for this project.

Materials and Methods.

Physarum polycephalum was grown in submerged cultures (4). Surface plasmodia

were formed by permitting the coalescence of microplasmodia obtained from shake flasks in the logarithmic growth phase, as described previously (4,5). Mitosis in these plasmodia was determined by light microscopy of biopsies fixed in ethanol. At different times during the mitotic cycle cultures were transferred to a new petri dish containing 5 μc ^{14}C-leucine (Schwarz Bio-Research, 338 C/mole) and 5 μc ^{14}C-lysine (Schwarz BioResearch, 315 C/mole) per ml of regular growth medium. In every experiment one culture was labeled for 2 hr 30 min during the G_2-phase which preceded the third mitosis, and another culture was labeled for an equal length of time but during the S-period. Both cultures were then harvested and the nuclei isolated (6). The purified nuclei were extracted by sonication for 1 min (Branson Instr. Sonifier, Model LS 75) in 67% acetic acid containing 0.5% of either 2-aminoethanethiol hydrochloride (2-AET) or Cleland's Reagent. The insoluble residue was spun down (27,000 g, 15 min), and the supernatant was dialyzed overnight against 0.1 M sodium acetate and 0.5% 2-AET. Proteins were precipitated with acetone (final concentration, 75%) and 0.5% 2-AET, washed once in the same solution, and dissolved in the final electrophoresis buffer containing 0.1 M Tris-HCl (pH 7.5), 8 M urea, 1% SDS (sodium dodecylsulfate, w/v), and 0.5% Cleland's Reagent at 60°C.

The acetic acid insoluble residue was extracted with 0.4 N NaOH, and the protein content in both extracts was determined by a slightly modified Folin method (7), with bovine serum albumin used as a standard. The sugar content was measured after acid hydrolysis of the polysaccharides, with glucose used as a standard (8). More than 90% of the nuclear proteins were extracted by the acetic acid procedure. The final extract contained less than 12% polysaccharide. Other extraction methods which were tried yielded a much higher amount of sugar -- probably derived from the presence of intranuclear polysaccharides (6) -- which prevented the proteins from entering the gels.

The proteins were separated by electrophoresis, modified after Shapiro et al. (9), with a Canalco Electrophoresis Apparatus Model 1400. The gels used (100 mm long, 6 mm wide) contained 7.5% polyacrylamide including 0.5% bisacrylamide as a crosslinker. Gels and electrode vessels contained the same buffer as the samples, except for 0.1% SDS (instead of 1%) and with urea omitted from the upper vessel. About 250-300 μg protein with a specific activity of 80-100 cpm/μg protein (S-phase-labeled proteins showed a higher specific activity than those labeled in the G_2-phase) were loaded on each gel. Duplicate gels were run with each sample. Electrophoresis was carried out until the marker dye used (bromophenol blue) had reached the bottom of the gels (4 hr at 60 V, 7.0 mamps/gel). One of the duplicates was fixed in 50% methanol plus 10%

acetic acid for at least 2 hr, stained in 0.2% Amido Black 10 B (Harleco) in 20% methanol and 55% acetic acid for 2 hr, destained in 35% ethanol and 7.5% acetic acid for several days and transferred to 7.5% acetic acid. A Canalco microdensitometer, Model E, was used for scanning the band patterns. The other unfixed duplicate was crushed by forcing it through a handdriven gel-crushing device containing a fine wire gauze which macerated the gel. Seventy fractions per gel were thus obtained and eluted overnight in counting vials at 68°C in 0.4 ml distilled water. The radioactivity of the samples was counted in a Packard Scintillation Counter. The counting efficiency was not affected by the gel particles remaining in the vials (average counting efficiency, 60%).

Results.

The stained band profiles from the S- or G_2-phase showed no significant differences in the number and distribution of bands (Fig. 1, a and b). The three bands which migrated the furthest were identified as the main three histone bands (H, Fig. 1) by comparison with a purified histone preparation subjected to the same gel system. A considerable amount of material remained on top and in the upper region of the gel; this might be membrane proteins. The patterns also showed relatively few bands in total, probably as the result of excessive treatment with detergent during the isolation of the nuclei (6). A difference occurred in the relative amount of proteins present in the histone peaks and the main peak in the upper region of the gel (peak X, Fig. 1, a and b): the histone peaks contained more material in the S-phase pattern but the peak X contained more in the G_2-phase pattern. This is in agreement with the finding that the amount of histones doubles during the S-phase (2); thus a given amount of nuclear proteins during this stage contains a higher percentage of histones. The molecular weight of the peak X material was approximately 42,000 when compared with standard proteins in the SDS gel system (9). The radioactivity patterns, on the other hand, demonstrated completely different profiles for G_2- and S-phase labeled nuclei (Fig. 1, c and d). Cultures labeled during DNA replication contained the main radioactivity in the histone fraction, whereas cultures labeled during G_2 showed a strong

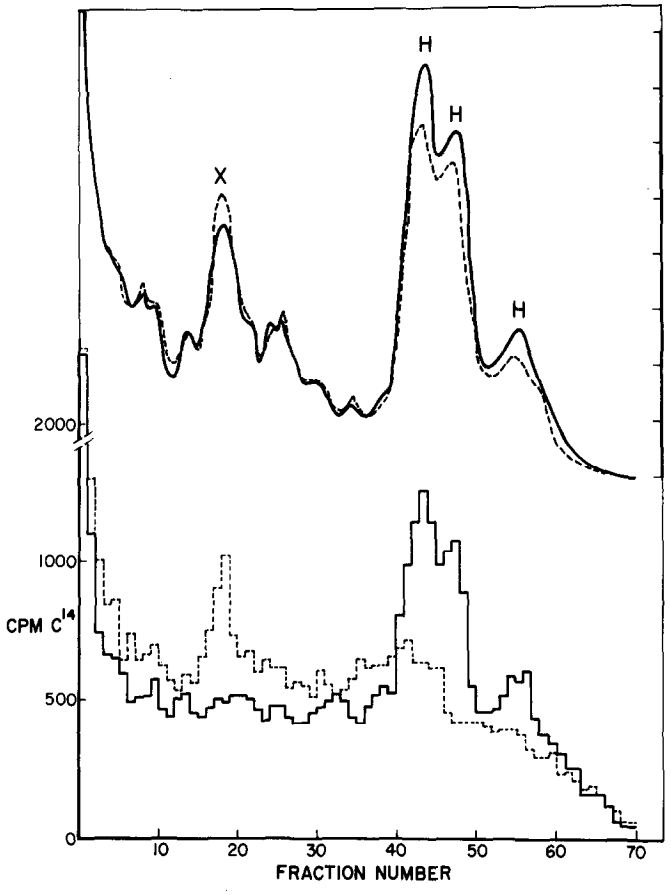

Fig. 1. Densitometer tracings of a stained gel of proteins extracted from nuclei in a) S-phase (solid line) and b) G_2-phase (dotted line), compared with the corresponding radioactivity patterns of c) S-phase (solid line) and d) G_2-phase (dotted line) labeled nuclear extracts. The top of the gels is on the left. The scale on the right represents relative units of optical density of the stained patterns.

peak of radioactivity corresponding to peak X in the densitometer tracings. The differences in radioactivity greatly exceeded the dissimilarities in the stained patterns. The conclusion that all peaks were indeed proteins was substantiated by the following facts: amino acids were incorporated into nondialyzable material, the material was soluble in acetic acid and precipitated by acetone, was stained by Amido Black, and was not removed from the gel by excessive treatment with acids. The results described

above were confirmed in seven independent experiments. We concluded, therefore, that these results demonstrate the presence of nuclear proteins synthesized during the G_2 period. The patterns indicate also that all other bands present in G_2- and S-phase nuclear extracts are synthesized at an approximately constant rate throughout the whole cycle. However, the method used does not permit us to distinguish between intra- or extra-nuclear synthesis. Previous work with HeLa cells (2) has shown that at least histones are exclusively synthesized by a cytoplasmic fraction. Studies on the identity of the protein synthesized during G_2 are currently in progress.

Acknowledgements.

We wish to thank Dr. Joyce Mohberg for providing purified histones and Dr. Harald Jockusch for technical suggestions. This work was supported in part by grants from the National Cancer Institute (CA-07175) and from the Alexander and Margaret Stewart Trust Fund.

References.

1) Spalding, J., K. Kajiwara and G. C. Mueller. Proc. Natl. Acad. Sci. U.S. 56 (1966), 1535
2) Gallwitz, D. and G. C. Mueller. Science 163 (1969), 1351
3) Guttes, E., S. Guttes and H. P. Rusch. Fed. Am. Soc. Expt. Biol. Fed. Proc. 18 (1959), 479
4) Mohberg, J. and H. P. Rusch. J. Bact. 97 (1969), 1411
5) Mittermayer, C., R. Braun and H. P. Rusch. Exptl. Cell Res. 38 (1965), 33
6) Mohberg, J. and H. P. Rusch. Manuscript in preparation
7) Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. J. Biol. Chem. 193 (1951), 265
8) Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith. Anal. Chem. 28 (1956), 350
9) Shapiro, A. L., E. Vinuela, J. V. Maizel, Jr. Biochem. Biophys. Res. Commun. 28 (1967), 815