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PREPARATION OF METAPHASE CHROMATIN OF PHYSARUM POLYCEPHALUM WITHOUT THE LOSS OF REPRESSED RNA SYNTHESIS

Fumio Sawada*, Yasuhiko Miyauchi+, Hiroshi Tanaka+
and Shinji Matsumoto

Division of Chemistry,
National Institute of Radiological Sciences,
9-1, Anagawa-4-chome, Chiba-shi 260, Japan

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Summary --- A novel method for the preparation of intact chromatin from the slime mold Physarum polycephalum which retains the in vivo property of RNA synthesis is described. Preparations from G_2-cells were highly active, while those from metaphase-cells were inactive. The plasmodial cells were disrupted by gentle homogenization on a polyethylene sieve in a neutral isotonic sucrose medium containing Mg^{++}, deoxycholate and EGTA, a Ca^{++}-chelating agent. The nuclei were lysed in a hypotonic buffer without use of EDTA and chromatin was precipitated by centrifugation after addition of Mg^{++}.

INTRODUCTION

Physarum polycephalum is an organism advantageous for the study of biochemical events in the mitotic cycle, since nuclear division in the plasmodial cell stage takes place synchronously. The results of previous studies in vivo provide for a biphasic pattern of RNA synthesis, the minimum occurring at metaphase (1,2). Similar patterns also observed in cell-free systems of isolated

* To whom correspondence should be addressed.
+ On leave from the Faculty of Sciences, Chiba University.

Abbreviations : DOC, potassium deoxycholate; DTT, dithiothreitol; EGTA, ethyleneglycol bis(β-aminoethylether)N,N,N',N'-tetracetic acid; TCA, trichloroacetic acid.

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nuclei (3-7) and nucleoli (4-6). In studies dealing with subnuclear fractions, isolated chromatin from interphase nuclei retained a high level of endogenous RNA synthetic activity (5,8). It was reported, however, that a chromatin preparation from metaphase nuclei showed RNA synthetic activity compatible to that observed with interphase chromatin (5).

It is, therefore, indispensable, for understanding the mechanism of repression of RNA synthesis at metaphase, to develope an isolation method of intact chromatin preserving the *in vivo* characteristics. The method of preparation of chromatin from the G_2-phase and metaphase cells, which retains their *in vivo* property is described in this paper.

MATERIALS AND METHODS

Liquid culture of *Physarum polycephalum* was grown in a semi-defined medium (9). The synchronous culture was obtained by inoculating the plasmodia on filter paper at 25°C (10). The mitotic cycle were about 9 h. The surface culture at the 3rd mitosis and those about 2 h before the metaphase were used for the preparation of metaphase and G_2-phase nuclei, respectively.

The medium used for preparation of the nuclei are as follows :
STM medium = 0.25 M sucrose-10 mM Tris-HCl(pH 7.2)-10 mM $MgCl_2$;
STMG medium = STM medium containing 5 mM EGTA ;
STMGC medium = STM medium containing 5 mM EGTA and 0.01 % DOC ;
1 M sucrose medium = 1 M sucrose-10 mM Tris-HCl(pH 7.2)-10 mM $MgCl_2$-5 mM EGTA-0.01 % DOC.

The surface cultures were dipped in ice-cold STMG medium to remove the growth medium. The plasmodia of 20-30 mm diameter were scrapped from the filter paper onto a 100-mesh polyethylene sieve which covered tightly a beaker placed in an ice-box. The cells on the sieve were grinded gently by hand in STMGC medium (4 ml per one plasmodium) using the roung bottom of a polyethylene tube filled with crushed ice. The homogenate collected in the beaker was filtered through a 400-mesh nylon sieve and absorbent cotton (0.1 g per 4 ml of homogenate) loosely packed in a glass column. The filtrate was layered on the top of 1 M sucrose medium and centrifuged at 3,200 rpm for 15 min. The pellet was

resuspended in STM medium and centrifuged at 3,000 rpm for 10 min to precipitate the nuclei.

The nuclei were resuspended in 1 mM Tris-HCl (pH 7.5) containing 1 mM DTT (1 ml per about $5x10^6$ nuclei) by agitation with a Vortex mixer at the highest setting for 5 sec and allowed to stand for 10 min in ice to complete lysis of the nuclei. The nuclear lysate was supplemented with $MgCl_2$ to final concentration of 5 mM and was centrifuged at 20,000xg for 15 min. The pellet was resuspended in 1 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 1 mM DTT and was used as the chromatin preparation.

The assay system for RNA synthesis consisted of 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM DTT, 0.15 mM each of ATP, CTP and GTP, 0.005 mM [^{3}H]UTP (12.5 μCi/ml), Radiochemical Centre, Amersham) and nuclei (about 10^7 nuclei per ml) or chromatin. The reaction mixture incubated at 25°C was terminated by adding 5 % TCA containing 1 % sodium pyrophosphate. The precipitate was collected onto a Whatman GF/F filter, washed with TCA and ethanol, dried and counted for radioactivity with a liquid scintillation counter.

DNA was assayed by the fluormetric method using 3,5-diaminobenzoic acid (11).

RESULTS

At the stage of homogenization, the nuclei prepared by the present method were nearly morphologically identical with such nuclei *in vivo* as checked under a phase-contrast microscope as shown in Fig. 1. Homogenization with a Waring blender was too vigorous even at the lowest setting to retain the original morphology of metaphase nuclei. Homogenization in media containing Ca^{++} or Triton X-100, both of which had been generally used (12), brought about shrinking of the nuclei and loss of their fine structure. The combined use of $MgCl_2$ and a Ca^{++}-chelating agent (EGTA) was effective in preserving the morphological integrity of nuclei after homogenization of plasmodia. Triton X-100 was successfully replaced with low concentration of DOC.

Centrifugation of the homogenate through 1 M sucrose cushion (12) was efficient to purify the nuclei, althogh shrinking

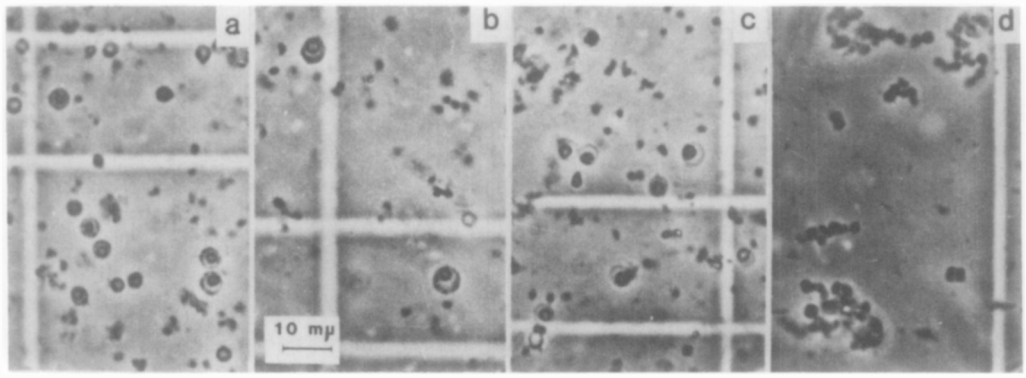

Fig. 1. Phase-contrast micrographs of nuclei in the crude homogenates from plasmodia in G_2-phase (a), prophase (b), metaphase (c) and anaphase (d).

occurred and the fine structure observed in both G_2-phase and metaphase nuclei disappeared. Recovery of the nuclei from the homogenate after the final centrifugation was about 20 % and the yields of the nuclei were about 3×10^6 per one plasmodium of 30 mm diameter.

The nuclei isolated could be lysed by simple agitation in a hypotonic and divalent cation-free buffer without use of EDTA. All of the chromatin was recovered from the nuclear lysate in the presence of Mg^{++}, but the recovery was reduced to about 80 % if Mg^{++} was not supplemented to the medium, as analyzed by DNA content. The addition of Mg^{++} was necessary to obtain precipitate of chromatin easily dispersible in the homogenization medium, otherwise the aggregates of chromatin could not be properly redispersed. DTT was essential to get active chromatin from the G_2-nuclei.

We confirmed the previous observations (3-7) that the isolated G_2-nuclei were highly active in RNA synthesis and the activity with the metaphase nuclei was strongly repressed (Fig. 2, left). As evident from Fig. 2 (right), the *in vitro* assay using our preparations of chromatin differentiated clearely between

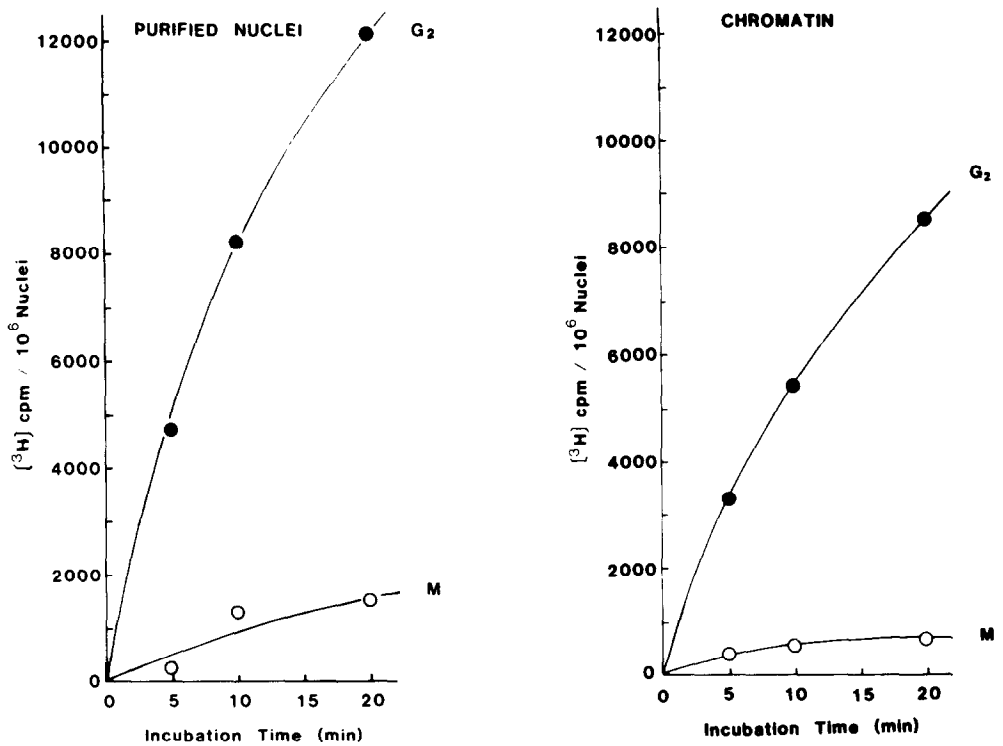

Fig. 2. Incorporation of [^{3}H]UTP into RNA of isolated nuclei (left) and chromatin (right) prepared from plasmodia in G_2-phase (—●—) and metaphase (—○—).

G_2-phase and metaphase at the subnuclear level, namely the preparation from the former was active and that from the latter was inactive just in the same manner as was observed *in vivo* and in the isolated nuclei. Since our preparations preserved *in vivo* properties, the present method seems to be preferable for studying the reguratory mechanism of RNA synthesis in the living cells.

DISCUSSION

Special attentions were given in the isolation of nuclei, especially those at metaphase, because of their extreme fragility. Our method of homogenizing the cells on a sieve was superior to other methods in order to get nuclei with intact morphology. The method was generally applicable to the plasmodia in any stages of the cell cycle including metaphase and anaphase. Triton

X-100 was omitted from the isolation media to avoid possible damages to the nuclear membranes such as inhibition of DNA synthesis (13,14) and solubilization of RNA polymerase from the nuclei (8,15).

The present method for preparing chromatin under low ionic strength conditions without the use of EDTA seems much milder compared to the conventional methods using this reagent (5,6,17). It is also simpler than the sophisticated procedure using lysolecithin (8). Bradbury et al (18) showed that nuclei prepared in low divalent ions were lysed without use of any chelating agent. Addition of Mg^{++} to the nuclear lysate before precipitating the chromatin was essential to get metaphase chromatin as observed by the same authors (18). Vigorous shearing forces such as sonication or homogenization in a motor-driven homogenizer was avoided, because they may cause some damages to chromatin structure (19,20).

The observation that our preparation of G_2-chromatin was active in RNA synthesis but metaphase chromatin was inactive suggests that it retains the reguratory machinary of RNA synthesis in the intact form. Preliminary experiments suggest that all the components essential for RNA synthesis are present in the preparation of metaphase chromatin and some experiments are in progress to characterize the putative factors by which the cell cycle-dependent reguration of RNA synthesis proceeds.

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