PHYSICAL SEPARATION OF NUCLEI FROM TWO INDEPENDENT PLASMODIA OF *PHYSARUM POLYCEPHALUM* AFTER FUSION

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INTRODUCTION

In recent years, there has been wide interest in the interaction of nuclei and cytoplasm. Studies in this area have been carried out by the fusion of cultured cells at different stages of the cell cycle, by addition of a nucleus to a cell, or by the addition of a second nucleus to a cell (4, 6). These elegant

techniques suffer from the fact that they are done with a limited number of single cells and so one is unable to follow the effect of such changes by the usual biochemical methodology.

With the myxomycete *Physarum polycephalum*, the fusion of the synchronous, multinucleated plasmodia has been carried out by a number of workers (2, 5, 9) in studies on the control of mitosis. We have worked out a technique whereby it is possible to fuse two synchronous plasmodia of *P. polycephalum* which are at different stages of the nuclear cycle, and at any suitable time after the fusion, the nuclei derived from the two original parent plasmodia can be separated. The separation technique depends upon using two genetically compatible strains of *P. polycephalum* in which the nuclei differ in DNA content by a factor of three. By the use of velocity density gradients one can separate the nuclei after isolation so that contamination of one with the other is less than 2%.

MATERIALS AND METHODS

P. polycephalum microplasmodia were grown and plasmodia prepared as previously described (3). Prelabeling of plasmodia was carried out by adding 5 μCi/ml of [^{3}H]thymidine (Tdr) 20 Ci/mm or 0.5 mCi/ml of [^{14}C]TdR 56 mCi/mm (Nuclear Dynamics, Inc., El Monte, Calif.) overnight to a shake flask of microplasmodia in log phase growth. Plasmodia were prepared the following morning from these microplasmodia. Both microplasmodia and plasmodia are routinely cultured in semidefined medium at 26°C. The compatible strains of plasmodia, viz., 2 × 5 and 8 × 9, were developed by and obtained from Dr. Finn Haugli of the University of Tromsø, Norway.

Fusion of plasmodia was carried out by carefully laying one plasmodium on top of another so that the top of the bottom plasmodium was in contact with the top surface of the other culture. In some instances, as indicated in the text, we fused cultures so that the bottom of the upper plasmodium was on top of the lower plasmodium. To accomplish this, the upper culture was first layered on to a sheet of Saran wrap (Dow Chemical Corp., Midland, Mich.), top side against the Saran wrap, and then this culture layered on the lower plasmodium and the Saran wrap removed. It was usually necessary to tease the edge of the plasmodium loose from the Saran wrap with a spatula.

Nuclei were isolated according to the procedure of Mohberg and Rusch (7). Separation of different-sized nuclei was carried out on 0.5–1.5-M linear sucrose gradients containing 0.01 M $CaCl_2$, 0.01 M Tris-HCl (pH 7.0–7.2) in Beckman 1 × 3.5-in cellulose nitrate tubes (Beckman Instruments, Inc., Fullerton, Calif.). Tubes were carefully overlaid with nuclei suspended in 0.5 ml of 0.25 M sucrose containing 0.01 M $CaCl_2$ and 0.01 M Tris-HCl (pH 7.0–7.2) and centrifuged at 550 rpm for 30 min in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) in no. 579 stainless steel centrifuge tubes placed in the HB-4 bucket rotor. The tubes fit loosely, but this does not seem to affect the gradients.

For autoradiography, the plasmodia were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 5 μm, and autoradiographs were prepared as described by Bogoroch (1). These were developed after 2 wk, stained with 0.03% azure C, and mounted in permount.

Radioactivity determinations on gradient fractions were determined by adding 10 ml of Aquasol (New England Nuclear, Boston, Mass.) to each fraction and counting in a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

To determine the usefulness of fusion of one plasmodium with another compatible plasmodium, one needs an accurate measure of the length of time required for full mixing of fused plasmodia to occur. A recent paper suggests that fusion is instantaneous (2), whereas another suggests that it takes 1.5 h (8). To measure this parameter, we prelabeled a flask of microplasmodia with [^{3}H]TdR as described in Materials and Methods and prepared another without label. Plasmodia 4.0 cm in diameter were prepared from both cultures, and fusion of the labeled and unlabeled plasmodia was carried out during early G_2 after the first mitosis. The two plasmodia were fused together either top to top or top to bottom to determine whether this affected the rate of fusion. (This was done since Sheen et al. (11) and our unpublished data clearly show structural differences between the top and bottom of a plasmodium). Starting 20 min after fusion and at 20-min intervals for 3 h, small wedge-shaped pieces about one-tenth of the plasmodium in size were cut off with a scalpel and fixed in neutral-buffered Formalin. Autoradiographs were made as described above. Plasmodia were scored for degree of mixing by examining the time-sequence of a plasmodium to determine when the radioactively labeled nuclei could be found throughout the cytoplasm. Table I presents the data from two such experiments. It is evident that mixing is complete within 2 h and that plasmodia may be fused in either manner. Plasmodia were henceforth fused top to top since this was the simplest procedure.

To determine whether plasmodial nuclei of

TABLE I

Time Required for Complete Mixing of Fused Plasmodia

Plasmodia Fused Top to Top		Plasmodia Fused Top to Bottom	
Culture No.	Time Required (min)	Culture No.	Time Required (min)
1	120	6	120
2	140	7	100
3	120	8	100
4	100	9	120
5	100	10	120

Pieces of plasmodia were fixed in formalin every 20 min after fusion for up to 3 h and autoradiograms made as described in Materials and Methods. To determine the time when the fused plasmodia had become completely homogeneous, as judged by the distribution of labeled nuclei, the autoradiograms were examined microscopically.

different size could be separated after fusion, we carried out the following experiment. We fused plasmodia in early G_2 in which the small nuclei (8 × 9) were prelabeled with [^{3}H]TdR and the large nuclei (2 × 5) were prelabeled with [^{14}C]TdR. After allowing 3 h for complete mixing of the plasmodia, nuclei were isolated according to published procedures (7). The nuclear pellet was resuspended in 0.25 M sucrose containing 0.01 M $CaCl_2$ and 0.01 M Tris-HCl (pH 7.0–7.2) and the nuclei were counted in a hemocytometer. The nuclear suspension was adjusted so that the nuclei were present at a concentration of 22.5 million nuclei in 0.5 ml. An aliquot of 0.5 ml of the nuclear suspension was subjected to sucrose gradient centrifugation as described in Materials and Methods. Visual examination showed two light-scattering bands as diagramed in Fig. 1. Microscopic examination of the nuclei removed from these bands clearly showed that the large nuclei were in the lower band and the small nuclei in the upper band. An identical tube was fractionated by collecting fractions from the bottom of the tube and determining the radioactivity. Fig. 2 shows the radioactivity profile obtained. It is clear that the purity of the nuclear fractions is greater than 98%.

Our studies confirmed that it is necessary to follow in minute detail the technique described for preparation of nuclei (7). This is particularly true of the first step which calls for the use of a large volume of nuclear isolation medium to prevent nuclei from clumping. Clumped nuclei cannot be successfully separated on a velocity gradient. Light-scattering bands can be detected with as few as 4.5 million nuclei of one size. Gradients cannot be loaded with more than 22.5 million nuclei of one type without overloading.

DISCUSSION

We present here a technique for the successful separation of nuclei from two parent plasmodia after these have been allowed to fuse together. This type of fusion is actually equivalent to cell fusion since the cytoplasm of each parent plasmodium is fused along with the nuclei. Unlike the usual cell-to-cell fusions, however, one can control the size of the pieces undergoing fusion so that instead of using pieces of equal size, one can fuse a piece that is only one-tenth the size of the other piece and still have enough nuclei to separate physically. However, unlike the more commonly performed cell-to-cell fusion which takes place very rapidly, fusion of the comparatively large mass of two plasmodia takes up to 2 h to be completed.

From their work on fusing plasmodia from different stages in the nuclear cycle (but lacking visibly identifiable characteristics), Chin and Bernstein (2) concluded that the time of mitosis was determined by events occurring just previous to 105 min before scheduled mitosis. They arrived at this conclusion because if they fused plasmodia earlier than 105 min before scheduled mitosis, they

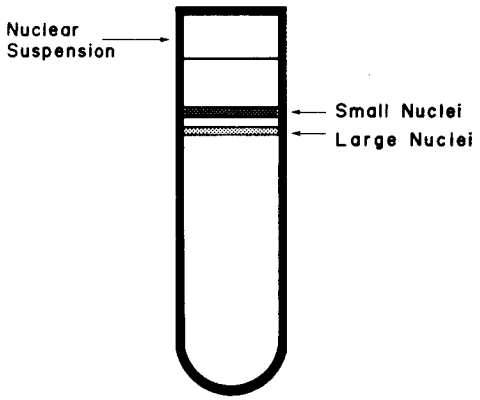

FIGURE 1. Diagrammatic representation of the separation of *Physarum polycephalum* nuclei on a 0.5–1.5-M sucrose gradient. Light-scattering bands of nuclei were clearly visible. Nuclei were isolated from plasmodia prepared by fusing a plasmodium with large nuclei to one with small nuclei. Gradients were centrifuged at 500 rpm for 30 min in the HB-4 head of a Sorvall RC2B centrifuge.

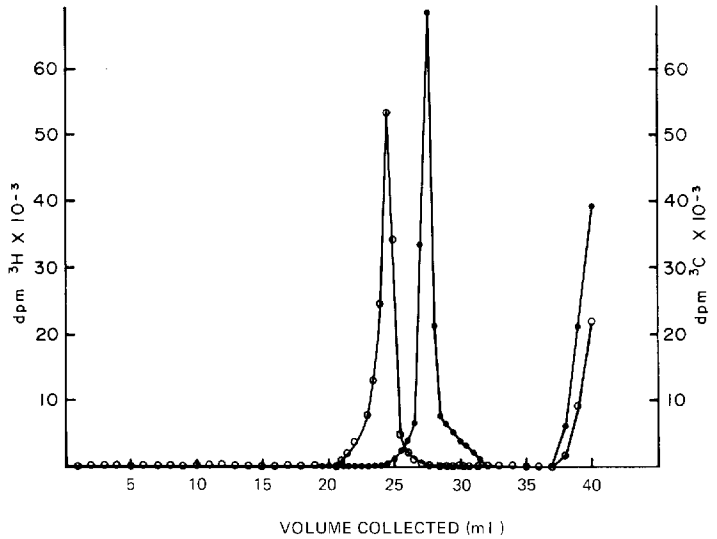

FIGURE 2 Radioactivity profile of a velocity sucrose sedimentation gradient of nuclei isolated from a plasmodium prepared by fusing a plasmodium with small nuclei (prelabeled with [^{3}H]Tdr ●—●) to a plasmodium with large nuclei (prelabeled with [^{14}C]Tdr O—O). 1-ml fractions (0.5 ml in the region where the nuclear bands could be detected by light scattering) were collected from the bottom and counted as described in the text.

observed all the nuclei in a fused plasmodium going through the process simultaneously, whereas if they fused during the last 105-min period, the nuclei still showed two separate mitosis periods. Our present studies and those of Murakami and Ohto (8) suggest that their results may well have been caused by mere lack of time for complete fusion of plasmodia.

Since under the conditions of growth used here the nuclear cycle is 8–10 h in length, this 2-h fusion time represents a sizable percentage of the nuclear cycle. This is less than satisfactory for studies on the regulation of the nuclear cycle. Therefore, in an effort to stop DNA synthesis until such fusion is complete, we investigated the use of inhibitors of DNA synthesis previously reported with this organism (10). We find that without interfering with the fusion itself, it is possible to stop DNA synthesis with 5-fluorodeoxyuridine and then start it again, once fusion is complete, by the addition of thymidine (McCormick, unpublished observations). It may be possible to use low doses of cycloheximide in a similar manner or to slow plasmodial growth without slowing fusion by growing plasmodia on dilute growth medium or by growing plasmodia at lower temperatures.

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