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A TECHNIQUE FOR THE ISOLATION OF MACRONUCLEI FROM *BLEPHARISMA JAPONICUM* (CILIATA HETEROTRICHA)

Riassunto — Una tecnica per l'isolamento dei macronuclei in Blepharisma japonicum (Ciliata Heterotricha). Nell'ambito di una ricerca volta allo studio del differenziamento sessuale di Blepharisma japonicum è parso necessario svolgere una analisi su differenziamento nucleare con particolare attenzione rivolta al macronucleo. Per tale tipo di ricerca è necessario prima di tutto disporre di una tecnica di isolamento dei macronuclei che devono conservare la loro struttura e composizione ma essere privi di contaminazioni. Sono riportate in letteratura varie metodiche di isolamento di macronuclei di ciliati, ma nessuna è risultata idonea per il Blepharisma. In questo breve lavoro descrivo una tecnica che sembra essere ottimale per l'isolamento di macronuclei di Blepharisma sia in piccole quantità per analisi citologiche che in quantità maggiori per analisi biochimiche.

Abstract — In a research planned to study sexual differentiation in *Blepharisma japonicum* at the molecular level it was decided to analyse the nuclear differentiation with special attention focused on macronucleus. For this investigation is necessary to isolate macronuclei preserving their integrity without contamination. Among the various methods reported in the literature for the isolation of macronuclei of cialiates, none have resulted as suitable for *Blepharisma*. In this short paper I explain a technique that seems optimal for macronuclear isolation both in small quantities for cytological analysis and in large quantities for biochemical analysis.

Key words — Blepharisma japonicum, macronuclei, DNA.

INTRODUCTION

Blepharisma japonicum, a freshwater species, is a large ciliate of 400-600 μ m in length with a nuclear apparatus consisting of a macronucleus 200 μ m long 20 μ m wide, plus 6 to 30 micronuclei

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approximately 2 μ m in diameter and vesicular in shape (A.C. GIESE 1973). This species has been widely used to study cell-cell interaction and gamone interaction during sexual reproduction (for a review see MIYAKE 1978-1981) and more recently for studying meiosis induction and its regulation (MIYAKE et al. 1979) in our and other laboratories. The growth techniques of the species are well standardized so that massive cultures can be easily obtained for molecular analysis.

In a research planned to study sexual differentiation at the molecular level, it seemed necessary to analyse the nuclear differentiation ensuing at each sexual process, with special attention focused on the macronucleus presiding over the vegetative life of ciliates.

The molecular structure of macronucleus is known so far only in a few ciliates: *Tetrahymena* (BORCHESENIUS et al. 1978), *Paramecium* (Mc TAVISH and SOMMERVILLE 1980, STEINBRUCK et al. 1981), and some hypotrichous species (SWANTON et al. 1980).

Such an investigation requires that macronuclear isolation be made with extreme caution, isolating the macronucleus without contaminants, yet preserving its integrity.

Several isolation techniques are reported in the literature (GOROWSKY et al. 1975, CUMMINGS 1977), however special steps for each species must be followed to obtain good, repeatable results.

In this short paper I explain a technique that seems optimal for the massive isolation of macronuclei from vegetative cells of *Blepharisma*.

MATERIALS AND METHODS

The albino clone A-5/3 of *B. japonicum* was used. The clone was grown in bacterized letture juice and phosphate buffer at pH 6.8, following the culture procedure of MIYAKE et al. (1979a). Cells were taken at different cell cycle stages, in log, stationary, and starving conditions, washed with and suspended in sterile SMB (MIYAKE and BEYER 1973) and buffered as above, for 24 h before using. They were then centrifuged at 100 g in pear-shaped ampules and the supernatant was sucked out. The cell pellet underwent the macronuclear preparation and purification procedure at 4°C according to the following protocol using the solutions reported below:

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a) Tris HCl 20 mM pH 7.4; MgCl₂ 2 mM; Cacl₂ 3 mM

b) Triton X-100 0.9%; cytric acid 10% pH 1.5

c) Saccharose 2 M; 20% glycerol; CaCl₂ 3 mM; MgCl₂ 2 mM; Spermine 100 μ ml.

1) The packed cells were incubated with an equal volume of solution A for 5'.

2) an equal volume of solution B was added and the cells were broken by gently pipetting them up to their complete lysis.

3) the homogenate was resuspended in solution A and washed several times through centrifugation at 50 g for 3' each; in this way, most cellular components were removed.

5) a further purification of macronuclei was obtained by suspending them in solution A to which an equal volume of solution C was added. This mixture was then stratified over solution C in the 1:20 ratio. Centrifugation at 3000 g for 5' then followed.

6) the supernatant was sucked out and saccharose was washed away with distilled water.

To analyse cytologically the macronuclei the following steps were undertaken:

7) the nuclei suspended in the minimum volume possible of solution A were centrifuged at 3000 rpm in centrifuge Sorval, rotor 9RA, for 40' over an appropriate slide.

The slide used was as follows: two slides were joined together with paraffin, in the upper slide a hole was made in which the nuclei in solution A were placed. This well was then covered with a coverslip.

8) The slides, kept in horizontal position, are dipped in a fixation mixture of methanol-40% formalin-acetic acid in a 85:10:5(v:v) ratio. After gently removing the coverslip, the nuclei were left in the fixative for 30'.

9) the fixative is then removed with a blotting paper while distilled water is slowly added with a micropipette in one corner of the well.

10) after the washing operation above, the nuclei were adhered to the slide by 2-3 quick baths in 95% ethanol and the upper slide was then detached.

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11) the macronuclei can now be stained according to the desidered techniques. To control the purification technique at each step, the macronuclei may be isolated and stained with acetic-orcein.

RESULTS

The observations of this macronuclear preparation (fig. 1), under the compound microscope at the highest magnification, has ascertained the almost complete absence of micronuclear contamination (< 0.1%). This fact demonstrates the successfulness of of the isolation technique of macronuclei which can be obtained purely enough for molecular comparison of the two types of nuclei.

Although cytoplasmic contaminants are present in a very low degree a good reduction in contamination can be made by washing the nuclei several times with solution A and then centrifuging them in solution C. The cytoplasmic residues which persist are mainly due to membrane debris which do not alter the molecular analysis due to their small amount.

The technique here reported permits the aquisition of macronuclei which mantain their typical shape (fig. 1) in 80% of the cases. This percentage is based on scoring the number of damaged/nondamaged macronuclei per surface unit on ten slides chosen at random. Such a percentage remains unchanged regardless of the physiological conditions or the stages of the cell cycle involved. This appears at variance with what happens in other ciliates where it is quite difficult to obtain good isolation of macronuclei from starved cells (GOROWSKY et al. 1975).

During the macronuclear preparation, there seems to have been no leakage of DNA as shown by comparing the DNA content in isolated macronuclei with the macronuclei of whole cells, all Feulgen stained, through cytophotometric analysis. It appears also that no DNA degradation occurs since the DNA electrophoretic patterns from isolated and non-isolated macronuclei are alike as will be reported elsewhere (SALVINI et al. in preparation).

CONCLUSION

Among the various methods reported in the literature for the macronuclear isolation of ciliates none have resulted as suitable

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Fig. 1 - a) macronuclei of *Blepharisma japonicum* stained with Feulgen technique, x 40. b) macronuclei of *Blepharisma japonicum* stained with Feulgen technique, x 120. c) *Blepharisma japonicum* acetic-orcein stained, x 100.

for *Blepharisma* because too many macronuclei were damaged in the process and because of the insufficient purification (CUMMINGS 1977, GOROWSKY et al. 1975, WOLF 1980).

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The technique here reported makes use of Triton-100, an ionic detergent, which allows a good cell homogenization without damaging the nuclear structure.

The Triton X-100 concentration should not be lower than 0.45% otherwise the pipette squeezing described in step 2 has to be repeated too many times to break the cell membrane, resulting in the mechanical damage of macronuclei and cellular residues larger in size that are difficult to get rid of. The 0.45% concentration of the detergent is sufficiently low avoiding any appreciable leaking of nuclear material.

Although Triton X-100 does not rupture lysosome membrane, the acid pH of the detergent solution is considered indispensable for inhibiting the lysozime activity that could be released by lysosomes.

The cell incubation in solution A, as the first step in our procedure, facilitates cellular lysis but it is recommended to limit it to 5' otherwise the nuclei become too fragile for the successive steps.

The concentration of the nuclear protective agents such as Ca^{++} , Mg^{++} and spermine are similar to those reported in the literature.

In conclusion our method appears to be quite suitable for macronuclear isolation both in small quantities for cytological analysis and in large quantities for biochemical analysis, due to the fact that the isolated macronuclei maintain their morphology and composition as *in vivo* conditions and the contaminants are negligeable.

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