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Highly Purified Micro- and Macronuclei from *Tetrahymena thermophila* Isolated by Percoll Gradients[1]

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ABSTRACT. A new procedure is described that utilizes Percoll gradients for purifying micronuclei (MIC) and macronuclei (MAC) from *Tetrahymena thermophila*. Separation of MIC from MAC during certrifugation in Percoll gradients occurs as a result of their difference in size rather than density. Three kinds of tests were used to evaluate the purity of the nuclei: visualization of the nuclei by light microscopy; examination of the nuclei by electron microscopy; and Southern blots of MIC and MAC DNA probed with the 5s rRNA genes or a fragment from the MAC extrachromosomal rDNA molecule. When examined under the light microscope, the isolated MIC and MAC have much lower nuclear cross contamination levels than previous methods have reported. MIC's contaminated with less than 1 MAC in 1000 MIC and MAC's contaminated with less than 1 MIC in 500 MAC can be routinely prepared. Quantitative analyses of electron micrographs gave higher estimates of cross contamination in our purified nuclei, which may, in part, be explained by the difficulty in identifying small MIC or MAC fragments. Southern blots of MIC and MAC DNA probed with 5s rDNA confirmed the level of MAC contamination in the MIC estimated by light microscopy during purification of the nuclei. The level of nucleolar contamination in the MIC was estimated at 10% by Southern blots of MIC and MAC DNA, derived from a heterokaryon with distinctive MIC and MAC Bam HI sites, using an rDNA probe.

THE ciliate *Tetrahymena thermophila* has two kinds of nuclei in a common cytoplasm: a transcriptionally inactive diploid micronucleus (MIC) that can undergo meiosis and a transcriptionally active macronucleus (MAC) that is larger in size and has ca. 23 times more DNA than the MIC and is replaced from products of the MIC each sexual generation. Both the MIC and MAC originate from a single diploid zygotic nucleus by a series of nuclear divisions. The DNA in the G1 MAC is amplified to a value 32 times that of the G1 MIC by the end of the second cell cycle after conjugation (6). This value persists for at least 50 fissions, but by 100 fissions it drops to the level characteristic of the mature MAC (7). Chromosomes are observed in the MIC, but it is not clear if the DNA molecules seen in the MAC represent only chromosome fragments or whether whole chromosomes are also present (19, 22, 26). Indeed, fragmentation of the chromosomes in the MAC appears to accompany the generation of extrachromosomal copies of the rRNA genes and other genomic alterations associated with the tandemly repeated hexanucleotide C_4A_2 (27, 31).

Not all genes are found in the same abundance in the two nuclei. Besides amplification of the rRNA genes (29), other sequences found in the MIC appear to be underreplicated (13) or eliminated in the MAC (28).

In order to make meaningful comparisons between sequences present in MIC and MAC, cross contamination of the DNA's from the two nuclei needs to be minimized. This means obtaining each type of nucleus as free as possible from the other type of nucleus. Two procedures have been published for the purification of the nuclei. The first relies upon differential centrifugation for preparing MAC and filtration for preparing MIC (9, 10). With this procedure, purified MAC usually contained 1 MIC per 5–20 MAC, and purified MIC usually contained 1 MAC per 200–400 MIC as determined by light microscopy. The second procedure uses filtration to separate the nuclei (13). With this procedure, contamination of MIC in the MAC preparation was "negligible," and no intact MAC were observed in the MIC preparation, although small amounts of chromatin bodies and nucleoli derived from the MAC were seen under the electron microscope.

In this paper we report a procedure that makes use of Percoll gradients for separating MIC from MAC. When examined under the light microscope, the purified MAC are usually contaminated with as little as 1 MIC per 200–500 MAC and the purified MIC are contaminated with as little as 1 MAC per 1000–2000 MIC. The levels of contamination seen in the light microscope are compared to estimates made by electron microscopy and by Southern blots of MIC and MAC DNA probed with 5s rDNA or a fragment from the extrachromosomal rDNA molecule.

MATERIALS AND METHODS

Cell lines. Most of the cell lines were derived from crosses of *Tetrahymena thermophila* inbred strain D and its congenic strain D/1 (1). The principal ones used were DI_a and DI_b, which refer to samples of populations of exconjugants purified by the use of magnetic columns (3) from two recent crosses. Other cell lines included caryonides isolated from a recent cross or cloned cell lineages isolated from older crosses. For example, cell line 21a originated from a single cell isolated from the progeny of a single pair at 13 fissions following conjugation from cross 75-163. It was recloned at 13 fission intervals up to 130 fissions. It has been subcultured bimonthly, so that it has now undergone more than 1000 fissions. In addition to the D × D/1 cell lines, two strains that are heterokaryons constructed by E. Orias were obtained from P. J. Bruns: SB-530 *rdnA1/rdnA1 Chx/Chx* (rdnA$^+$ cy-s) and SB-523 *rdnA1/rndA1 Chx/Chx* (rdnA1 cy-s) (20).

Growth of cultures. For nuclear preparations, test-tube cultures were grown in 1% proteose-peptone (w/v) for two days at 30°C. Flasks containing PP210 medium (2% proteose-peptone (w/v), 10 μM $FeCl_3$) were inoculated with test-tube cultures, grown for 24 h in a shaker water bath at 30°C, and the cell concentration determined. Five-liter diphtheria toxin bottles containing 3 liters of PP210, 3 ml of antifoam B (Baker) and penicillin-streptomycin (250 μg/ml) were inoculated with an aliquot of cells sufficient to give a final concentration of 2–3 × 10^5 cells/ml after growth at 30°C with gentle aeration in a large water bath.

Cell counts. Cells were fixed in an equal volume of a 1:7 dilution of 38% formaldehyde in 10 mM Tris buffer (2). Well mixed samples of fixed cells were spread in the counting cham-

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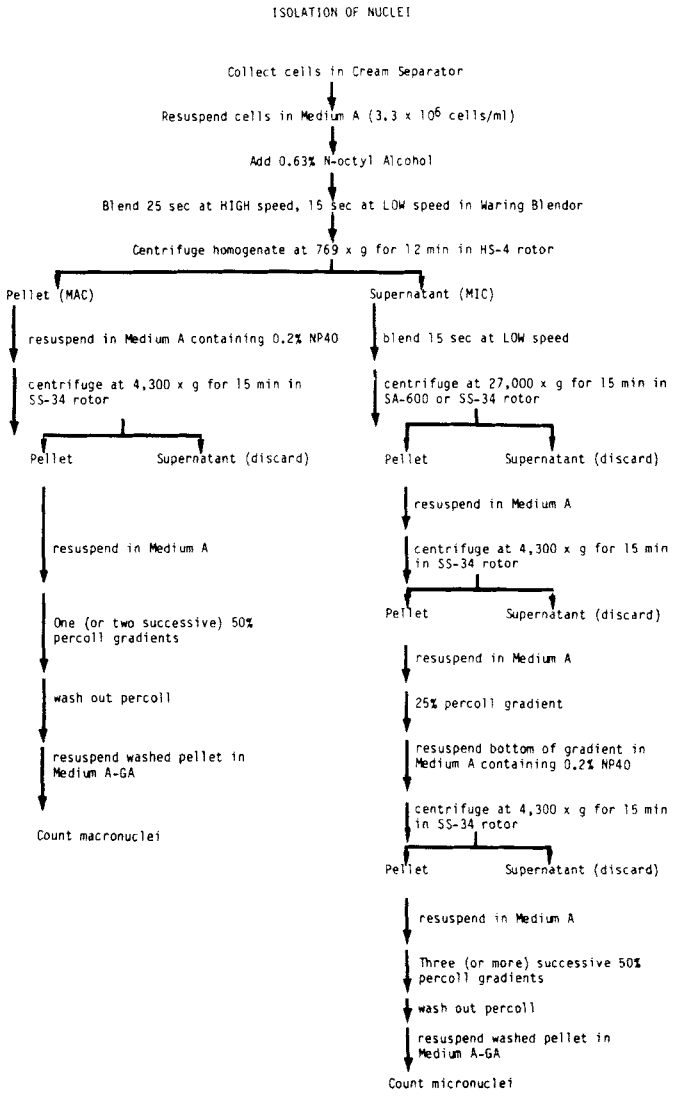


Fig. 1. Flow diagram of the steps in the nuclear isolation procedure.

ber of a hemacytometer and counted at 60× under the light microscope.

Isolation of nuclei. A flow diagram of the steps used in the isolation of MIC and MAC is shown in Fig. 1. The first part of this procedure is a modification of the Gorovsky et al. (10) method. The cells, collected by centrifugation in a cream separator, were resuspended in Medium A (0.1 M sucrose, 4% gum arabic (w/v), 0.1% spermidine-HCl (w/v), 2 mM $MgCl_2$) containing 0.1% sodium azide (w/v) and 100 μM phenylmethylsulfonyl fluoride, adjusted to pH 6.75, at a concentration of 3.3×10^6 cells/ml. To the cell suspension in Medium A was added 0.63% (v/v) n-octyl alcohol. The cells were broken by blending for 25 sec at high speed, 15 sec at low speed, in a Waring Blendor. After removing a sample for counting nuclei, the homogenate was centrifuged at 769 *g* for 12 min in an HS-4 rotor in a Sorvall RC-5B refrigerated centrifuge. The pellet containing the MAC was resuspended in Medium A to which 0.2% NP40 (Nonidet P40; Shell Chemicals) was added, and a sample was taken for counting nuclei. After centrifugation at 4300 *g* for 15 min in the SS-34 rotor, the MAC were resuspended in a small volume of Medium A, layered onto a 50% Percoll gradient, and centrifuged at 1500 *g* for 8½ min in an HB-4 rotor. The MAC band near the bottom of the tube was removed from the gradient and washed in Medium A by centrifugation at 4300 *g* for 15 min in the SS-34 rotor. The MAC were then resuspended in a small volume of Medium A, a sample taken for counting, and they were layered onto a second 50% Percoll gradient and centrifuged. The MAC band was removed, washed in Medium A, and resuspended in Medium A minus gum arabic, a sample counted, and the nuclei were lysed for extracting DNA. If a single Percoll gradient gave sufficient MAC purity, the second Percoll gradient was omitted.

The supernatant from the 769 *g* centrifugation containing the impure MIC was blended for 15 sec at low speed, centrifuged at 27,000 *g* for 15 min in the SA-600 or SS-34 rotor, resuspended in Medium A, a sample taken for counting nuclei, and the suspended nuclei were centrifuged at 4300 *g* for 15 min in the SS-34 rotor. The pellet that contained MIC heavily contaminated with MAC was resuspended in a small volume of Medium A and layered over a 25% Percoll gradient. After centrifugation at 1500 *g* for 18 min in the HB-4 rotor, the nuclei pelleted but the "scum" layer, which develops with addition of the octanol and blending, and which contains membranes and other trapped cellular components, remained suspended. The bottom of the gradient containing the nuclei was then resuspended in Medium A to which 0.2% NP40 was added, and centrifuged at 4300 *g* for 15 min in the SS-34 rotor. The pellet was resuspended in a small volume of Medium A, a sample was taken for counting, then the nuclei were layered onto a 50% Percoll gradient and centrifuged at 1500 *g* for 8½ min in the HB-4 rotor. The MIC band near the top of the gradient was removed from the gradient and washed in Medium A by centrifugation at 4300 *g* for 15 min in the SS-34 rotor. The MIC were then resuspended in a small volume of Medium A, a sample was taken for counting, and the resuspended nuclei were layered onto a second 50% Percoll gradient and centrifuged. The MIC band was removed, washed in a small volume of Medium A, a sample counted, and the resuspended nuclei were layered onto a third 50% Percoll gradient and centrifuged. If this were the final Percoll step, the MIC band was resuspended in Medium A minus gum arabic, a sample was counted, and the nuclei were lysed for extracting DNA.

Percoll gradients (25%). The gradients were made in two 15 ml siliconized Corex centrifuge tubes, each containing 3 ml of Percoll (Pharmacia), 6 ml of 2× Medium A, and 3 ml of sterile distilled water. The gradients were formed by centrifuging the tubes at 27,000 *g* for 15 min in the SS-34 rotor with the brake off. Nuclei suspended in 3 ml of Medium A were layered on top of the gradient and centrifuged in the HB-4 rotor at 1500 *g* for 18 min with the brake off. The tubes were removed only after the rotor came to a complete stop. The top of the gradient (from the top of the tube to the bottom of the smear on top) was discarded. The bottom of the gradient (the remainder of the tube contents) that contained the nuclei was then removed, placed in a 40 ml centrifuge tube, and the tube was filled with Medium A containing 0.2% NP40. The tube was inverted to mix the contents and centrifuged at 4300 *g* for 15 min in the SS-34 rotor. The pellet was resuspended in 2.5 ml Medium A, and the nuclei were counted. More than 97% of the nuclei were usually recovered in the bottom portion of the gradient.

Percoll gradients (50%). The gradients were made in 15 ml siliconized Corex centrifuge tubes and contained 6 ml of Percoll and 6 ml of 2× Medium A. Usually two tubes were used for each Percoll step in processing the MAC and one tube for each Percoll step in processing the MIC. The gradients were formed by centrifuging the tubes at 27,000 *g* for 15 min in the SS-34 rotor with the brake off. We found that the Percoll gradients could be stored for a couple of days at 4°C unless a white band

containing clumped silica formed near the top of the tube. Nuclei suspended in 3 ml of Medium A were layered on top of the gradient and centrifuged in the HB-4 rotor at 1500 *g* for 8½ min with the brake off. The centrifugation was timed with a stop watch, starting as soon as the rotor reached maximal speed. Centrifugation was stopped by turning off the timer, and the rotor was allowed to come to a complete stop before removing the centrifuge tubes. The midpoint between the top (MIC) and bottom (MAC) bands in the tube was determined. If the MIC were being processed, the MIC were removed by collecting the top of the gradient down to the midpoint, and the bottom portion of the gradient was discarded. If the MAC were being processed, the top portion of the gradient was discarded down to within one-half inch of the MAC band, and the remainder of the tube was collected. Percoll was rinsed from the nuclei by placing what was collected in a 40 ml centrifuge tube and filling the tube with Medium A. After mixing the contents by inverting the tube, the tube was centrifuged at 4300 *g* for 15 min in the SS-34 rotor. We found that it was important that a "band" from only one tube be placed in a 40 ml tube; otherwise the pellet would not be tight and nuclei might be lost. If another Percoll gradient followed, the nuclei were resuspended in 3 ml of Medium A, a sample was counted, and the nuclei were layered on the gradient. After the last gradient, the nuclei were resuspended in 1.5 ml of Medium A minus gum arabic and lysed.

Nuclear counts. Quantitative determinations of the total numbers of MIC and MAC in samples of known volume were made at each step of the nuclear isolation procedure. Fifty μl of a nuclear suspension was diluted with 50 μl of 0.4% methyl green in 6.0% (v/v) acetic acid containing 2×10^{-3} M $CaCl_2$ (16). If higher dilutions of nuclei were needed, Medium A was used as the diluent. A sample of the stained nuclei was spread in the counting chamber of a hemacytometer and counted at 600× under the light microscope.

Electron microscopy. Samples of whole cells, and highly purified MIC and MAC isolated by Percoll gradient centrifugation, were embedded and examined by electron microscopy. Isolated MIC and MAC at 2×10^7 nuclei/ml were fixed at 0°C for 1 h in Medium A containing fresh 1% (v/v) glutaraldehyde. Nuclei and debris were completely pelleted by centrifugation at 800 *g* for 10 min. The pellet was added to a small amount of fixed chicken erythrocytes used as a colored marker and then mixed into a small droplet of a 1.5% aqueous solution of agarose (w/v; Sigma Type VII) at 40°C. After the droplet cooled and solidified, it was cut into small pieces for dehydration. Several such pieces were embedded to completely assure representative sampling of nuclei and debris during subsequent microscopy. The agar fragments were dehydrated through 100% methanol, then 100% propylene oxide, and several changes of EPON 815 with hardener and accelerator. Some samples were also fixed without spermidine. Whole cells were fixed in culture medium containing 1% (v/v) glutaraldehyde and subsequently dehydrated by the same procedure used for nuclei.

Silver sections were cut using a diamond knife and subsequently stained with 2% (w/v) aqueous uranyl acetate for 5 min and 0.3% (w/v) lead citrate for 10 min. The sections were examined in a JEOL JEM 100B operated at 100 kV.

Analysis of electron micrographs. Estimates of the microscopic purity of the MIC and MAC preparations were performed by quantitative stereology of the electron micrographs of thin sections. The relative volumes occupied by intact and fragmented MIC and MAC in the sections were estimated by the point counting method, using a square grid (25). Randomly selected prints of 7000× specimen magnification were used with a square grid with a 0.5 cm spacing (corresponding to 0.7 μm on the section). Since the number of grid intersections overlapping nuclei is proportional to the volume of those nuclei in the embedded section, the relative volumes of MAC and MIC are given by the equation (25):

$$\frac{V_{mac}}{V_{mic}} = \frac{P_{mac}}{P_{mic}}, \qquad \text{Equation 1}$$

where V_{mac}/V_{mic} = the relative volumes of the two types of nuclei, and P_{mac}/P_{mic} = the relative numbers of grid intersections.

The volumes of intact MAC and MIC were estimated by analyzing serial sections. Taking sections of uniform thickness, t, the estimated volume of a nucleus is simply:

$$\bar{V} = \sum_{i=1}^{n} A_i t, \qquad \text{Equation 2}$$

where A_i = the area of the nucleus in the i^{th} section, and the summation extends over the n consecutive sections in which the particular nucleus appears.

For this analysis, the sections were cut very thick (t estimated to be 1000 Å). Although the estimated volume of a MAC or MIC is linearly dependent on our estimate of t, the ratio of volumes, $\bar{V}_{mac}/\bar{V}_{mic}$ is independent of t.

The number of nuclei, N, in our sections would be equal to the estimated volume of nuclei in the embedded sample, V, divided by the estimated volume per nucleus, $\bar{V}$. Since we were only concerned with the relative numbers of MAC and MIC, we used the following equation employing only relative quantities:

$$\frac{N_{mac}}{N_{mic}} = \frac{V_{mac}}{V_{mic}} \cdot \frac{\bar{V}_{mic}}{\bar{V}_{mac}}. \qquad \text{Equation 3}$$

In order to make further use of our data we used the ratio of DNA in a single MAC to that in a single MIC, $\bar{D}_{mac}/\bar{D}_{mic}$, to estimate the relative amounts of MAC and MIC DNA in the embedded samples, D_{mac}/D_{mic}, using the equation:

$$\frac{D_{mac}}{D_{mic}} = \frac{N_{mac}}{N_{mic}} \cdot \frac{\bar{D}_{mic}}{\bar{D}_{mac}}. \qquad \text{Equation 4}$$

DNA purification. MAC and MIC DNA was isolated by CsCl gradient centrifugation. The nuclei in 1.5 ml Medium A minus gum arabic were diluted with 1.5 ml TES (30 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.0), transferred to 50 Ti centrifuge tubes, and lysed with 60 μl Sarkosyl. After lysis, 25 μl of 10 mg/ml ethidium bromide and 6 ml of saturated CsCl in TES were added to the tube, the refractive index adjusted to 1.3920, and the tube was centrifuged at 40,000 rpm for a minimum of 40 h at 20°C in an L8-70 Beckman ultracentrifuge. The DNA band was removed and extracted with water-saturated n-butanol to remove the ethidium bromide. Two volumes of sterile distilled water were added to the aqueous phase. The DNA was precipitated with 6 volumes of cold absolute ethanol overnight at −20°C and centrifuged at 3000 *g* for 10 min in the SS-34 rotor at −10°C. The DNA pellet was drained, dried, dissolved in TEN buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl) at a concentration of 1 mg/ml and stored over chloroform at 4°C. Before use the DNA was checked for shearing by testing a sample by electrophoresis in an agarose gel.

Hybridization probes. Plasmid pRP7, obtained from R. E. Pearlman (York University), contains the 1050 bp Hind III fragment of rDNA cloned into the Hind III site of pBR322 and transformed into *Escherichia coli* strain JF 1161 (8). Plasmid pDP5, obtained from D. Pedersen (University of Rochester), contains a 280 bp Bam HI fragment of a MIC 5s rRNA gene plus AT-rich spacer cloned into the Bam HI site of pBR322 (Pedersen, personal communication). DNA from this plasmid was used to transform *E. coli* strain HB101. Plasmid DNA was isolated by a modification of the SDS/high salt cleared lysate

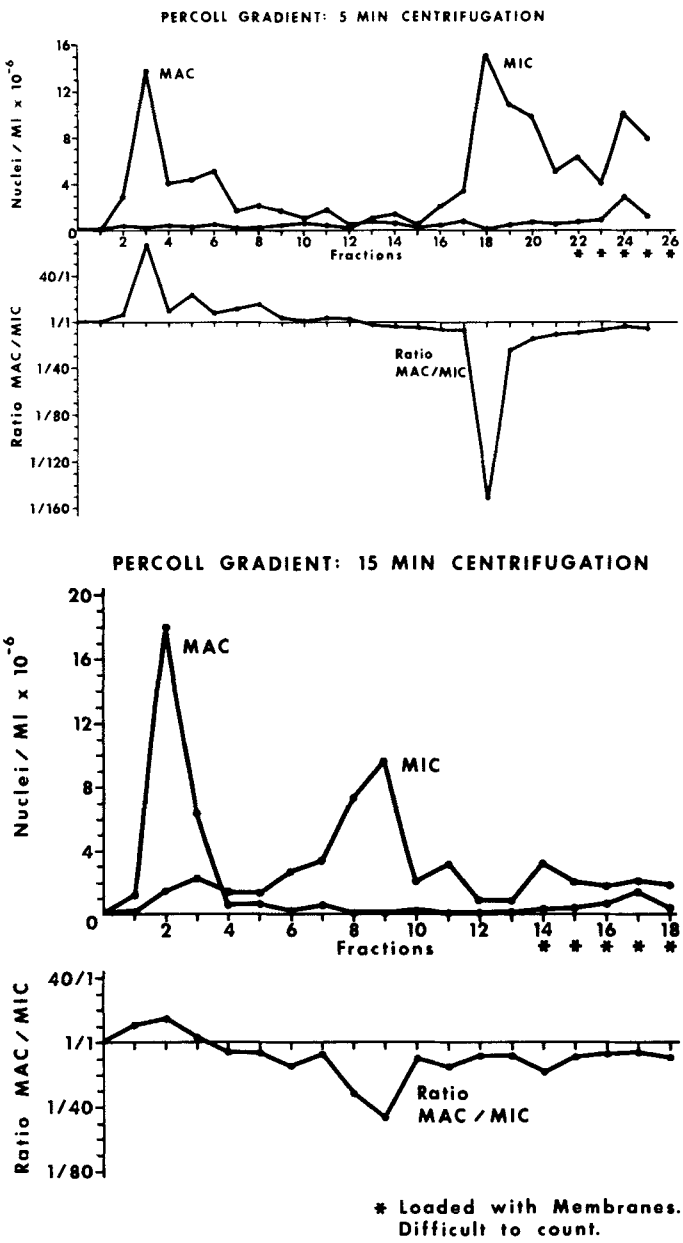


Fig. 2. Distribution of MIC and MAC in 50% Percoll gradients (a) after centrifugation for 5 min; (b) after centrifugation for 15 min. The centrifuge used was a table-top Sorvall. The centrifugation times are not equivalent to those used for the RC-5B Sorvall centrifuge. They are shorter since the braking time is longer for the table-top centrifuge.

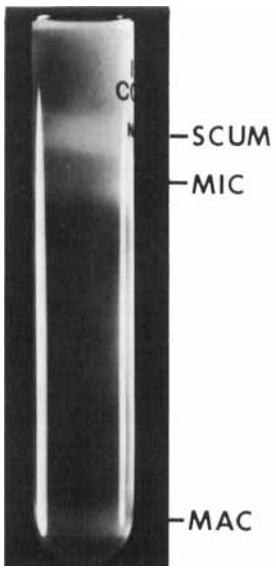


Fig. 3. Photograph of centrifuge tube after centrifugation of nuclei in the first 50% Percoll gradient without a 25% Percoll gradient. A mixture of MIC and MAC was layered onto a 50% Percoll gradient and centrifuged for 8½ min at 1500 *g* with the brake off.

procedure (11) followed by CsCl/ethidium bromide gradient centrifugation in a vertical rotor, and the DNA was labeled in vitro with α^{32}P to $\geq 4 \times 10^7$ cpm/μg by a modified nick translation procedure (17, 23).

Restriction endonuclease digestion, gel electrophoresis, and Southern blots. Digestion was carried out by the method of Davis et al. (4) using a 10-fold excess of restriction endonucleases purchased from Bethesda Research Laboratories, Inc. Agarose slab gel electrophoresis was carried out in a horizontal apparatus with a Tris-borate buffer containing ethidium bromide (12, 18, 21), using Hind III digested phage λ DNA in each gel as size markers. After electrophoresis, the DNA in the gels was visualized with a UV transilluminator. DNA was transferred from the gels to nitrocellulose filters by the method of Southern (24) with modifications. The filters were prehybridized for 3–5 h at 65°C in 10× Denhardt's solution (5), 3× SSC (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, and 25 μg/ml of denatured carrier DNA. Denatured probe (2×10^7 cpm) was added to the hybridization mixture, and hybridization was continued for 40 h at 65°C. The filters were washed six times in 2× SSC containing 1% SDS for 20 min, then once in 0.2× SSC containing 1% SDS for 30 min and once in 2× SSC for 10 min, all at 65°C. The filters were dried and exposed to Kodak XAR-5 film at −80°C with an intensifying screen.

Densitometry. Densitometer tracings of individual lanes of autoradiograms were made using an automatic recording microdensitometer (Joyce Loebl, Model Mk.111C).

RESULTS

Percoll gradients separate MIC from MAC. We were encouraged to use Percoll gradients for separating the two types of nuclei by Langmore (MS in preparation), who has worked out a chicken erythrocyte nuclear isolation procedure using Percoll gradients. The original procedure we used was a modification of this method.

The nuclei were isolated using the series of differential centrifugation steps that form the core of the Gorovsky et al. (10) procedure. The ratio of MAC to MIC was 1:1. The nuclei were resuspended in Medium A and layered onto several preformed self-generated 50% Percoll gradients. The gradients were centrifuged for various time periods using a table-top Sorvall centrifuge, the bottom of the tube was pierced, and 5–6 drops were collected in each of the fractions. Counts of the nuclei were then made for each fraction.

The results from two of the gradients are shown in Fig. 2. Separation of the nuclei was observed in both gradients, but with longer centrifugation times the MIC band moved closer to the MAC band. Thus, higher nuclear purity could be achieved with shorter centrifugation times since there was less overlap of nuclei. We also observed that the fraction near the top of these gradients was contaminated with "scum" that included trapped

TABLE I. *Purity and yields of macronuclei using Percoll gradients.*

Sample preps[a]	Total no. cells/6 liters	% MAC recovered after washing low *g* pellet[b]	No. 50% Percolls	Yield after 50% Percolls: % cells	Yield after 50% Percolls: % washed MAC	Final ratio MAC/MIC[c]
1.	5×10^8	56	1	36	64	75/1
2.	1.6×10^9	28	1	28	100	145/1
3.	9.1×10^8	61	1	58	96	877/1
4.	9.8×10^8	39	1	28	72	136/1
5.	5.9×10^8	30	1	25	83	290/1
6.	1.0×10^9	20	1	14	70	126/1
Mean	9.3×10^8	39	1	32	88	275/1
7.	2.1×10^9	45	2	23	51	646/1
8.	1.6×10^9	51	2	27	53	266/1
9.	1.4×10^9	92	2	46	50	296/1
10.	1.5×10^9	39	2	13	33	407/1
11.	2.9×10^9	25	2	16	64	231/1
12.	1.6×10^9	20	2	11	55	1485/1
Mean	1.85×10^9	45	2	23	51	555/1

[a] 1–4: DI_6; 5, 6: DI_a; 7, 10: cell clones (D × D/1); 8, 9: caryonides (D × D/1); 11, 12: heterokaryons SB-530 and SB-523.

[b] These MAC were washed once in Medium A before resuspending them in Medium A with 0.2% NP40.

[c] Nuclear purity was determined by light microscopy.

membranes and nuclei (Fig. 3). Since this region was contiguous to the MIC band, it appeared to be responsible for the MAC that contaminated the MIC band. Subsequent to these experiments, we found we could get rid of the scum region by centrifuging the nuclei through a 25% Percoll gradient.

Using Percoll gradients for the separation of the nuclei, we developed the procedure for nuclear isolation outlined in Fig. 1. Specific points about MAC and MIC purification follow.

MAC purification. If only MAC are to be purified, the 769 *g* centrifugation time can be increased for a higher yield of nuclei. With the 12 min centrifugation time, recovery of MAC after washing averaged 39–45% (Table I). Addition of NP40 to the nuclear suspension in Medium A before the first Percoll gradient aided in the separation of the nuclei and in the recovery of MAC after the gradients. An average of 88% of the washed MAC were recovered after one Percoll gradient, 51% after two Percoll gradients. However, the average purity of the MAC was doubled: from 275/1 to 555/1 for the MAC/MIC ratio. Even after a single Percoll high MAC purity may occasionally be achieved, as seen with Prep #3, where the MAC/MIC ratio was 877/1. But, with two Percolls, ratios as high as 1485/1 may be achieved, as seen with Prep #12.

MIC purification. A number of parameters in the procedure affected the final purity and the number of successive 50% Percoll gradients required to achieve purity (Fig. 4). Originally we resuspended the cells at a concentration of 10^6 cells/ml of Medium A, and the 27,000 *g* pellet, resuspended in Medium A, was layered onto a 50% Percoll gradient without using a 25% Percoll gradient or NP40. Under these conditions (A) it took five successive 50% Percolls to reach a purity of 1 MAC/416 MIC. Addition of a 25% Percoll gradient before the 50% Percolls (B) improved the yield of nuclei and the final purity was doubled. Adding NP40 to the nuclei after the 25% Percoll gradient (C) resulted in increased purity (1 MAC/1134 MIC); moreover, this level of purity was achieved in three successive 50% Percolls. NP40 appears to aid in the separation of the MIC from the MAC if added *after* the 25% Percoll. If added before the 25% Percoll, almost half the MIC stayed at the top of the gradient

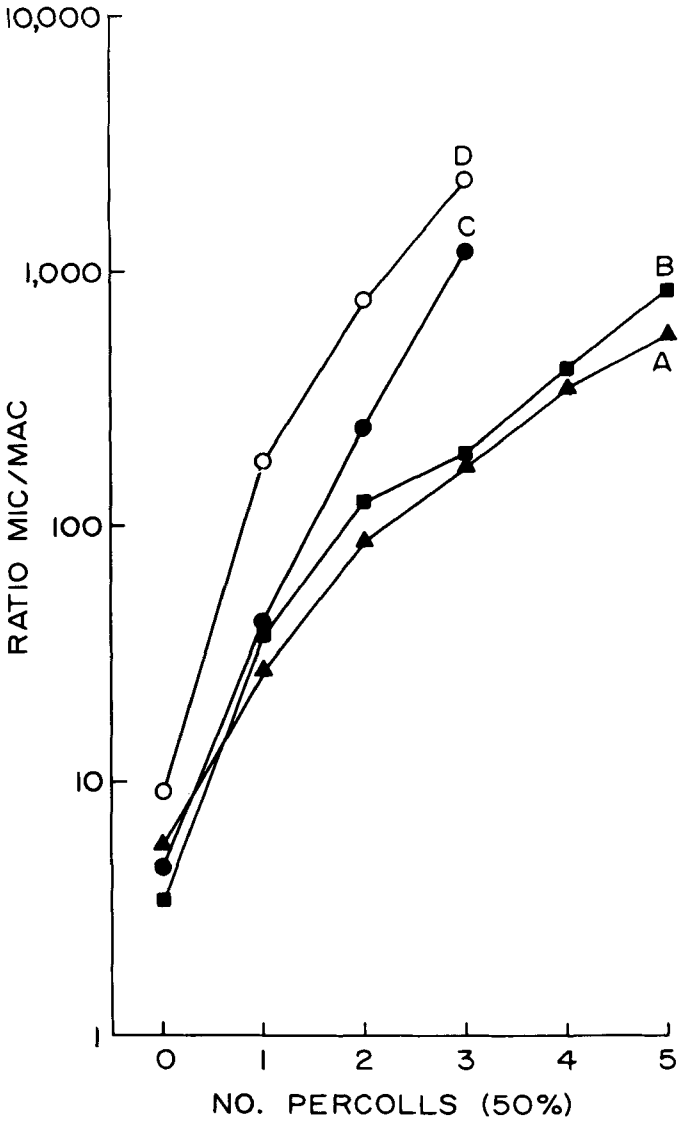
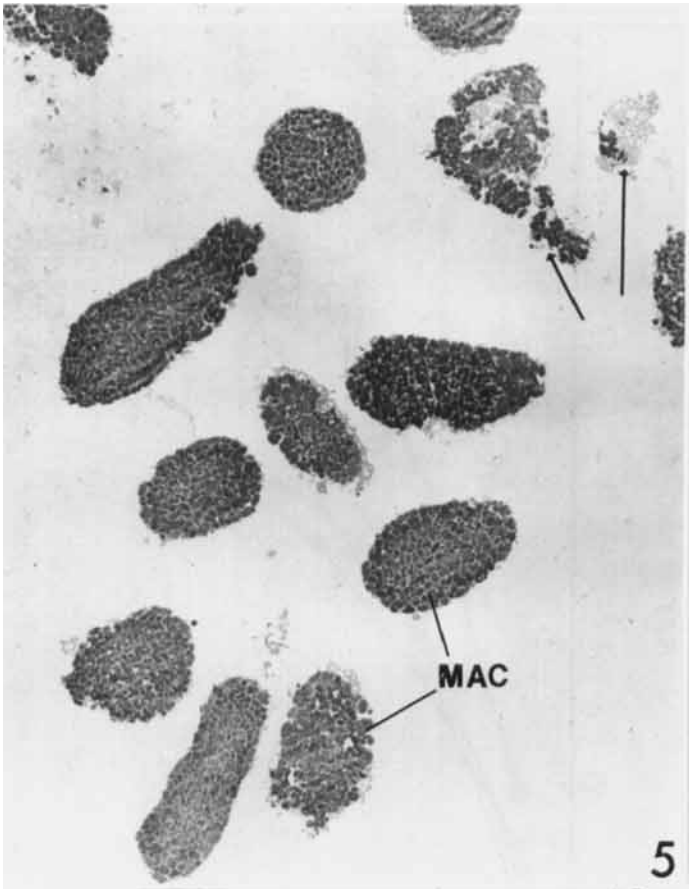


Fig. 4. Purification of MIC as a function of successive 50% Percoll gradients under four conditions: A = without 25% Percoll gradient; B = with 25% Percoll gradient; C = NP40 added to pellet from 25% Percoll gradient; D = same as C except volume of Medium A reduced to ⅓ ml per 10^6 cells.

and all of the scum sedimented with the MAC and the rest of the MIC. Even higher levels of purity were achieved (1 MAC/2198 MIC) when we reduced the starting volume of Medium A to ⅓ ml per 10^6 cells (D). We do not understand why this worked, but there are obvious practical advantages using the smaller volume.

The recovery of MIC pelleted by the 27,000 *g* centrifugation under conditions A–D averaged 79% (Table II). Without the 25% Percoll gradient, 63% of these MIC were recovered after the first 50% Percoll and 31% after five 50% Percolls. With the 25% Percoll gradient (B–D) 77% of the MIC were recovered after the first 50% Percoll and 45% after the last 50% Percoll. In all of these preparations the cell concentration was below 3.3×10^5 cells/ml. When cells in late log were used (E, F), it was much more difficult to purify the MIC's. Fewer MAC's were recovered in the low *g* centrifugation so that the initial MAC contamination in the MIC's was higher, resulting in more 50%


MAC
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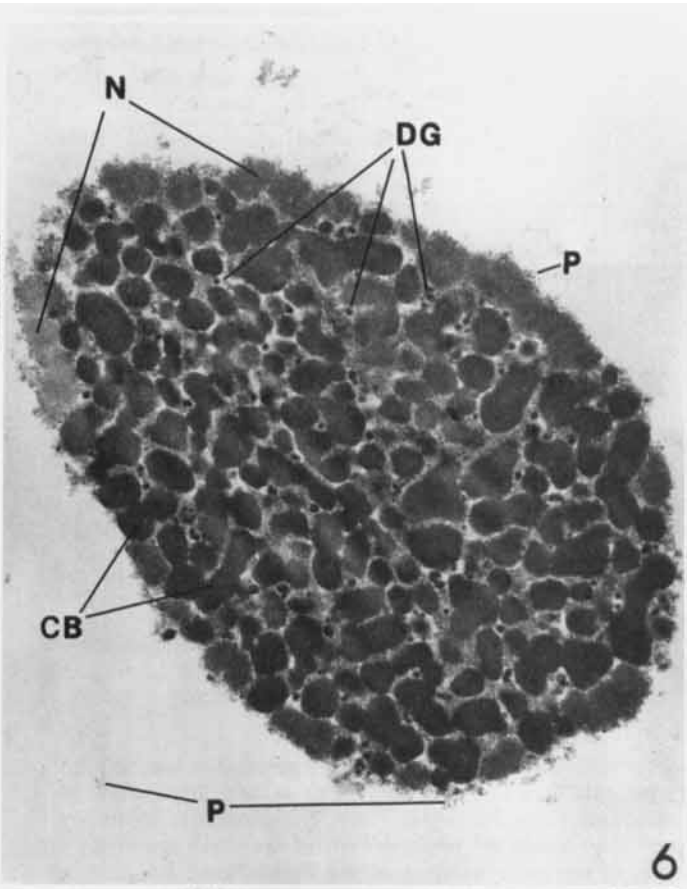
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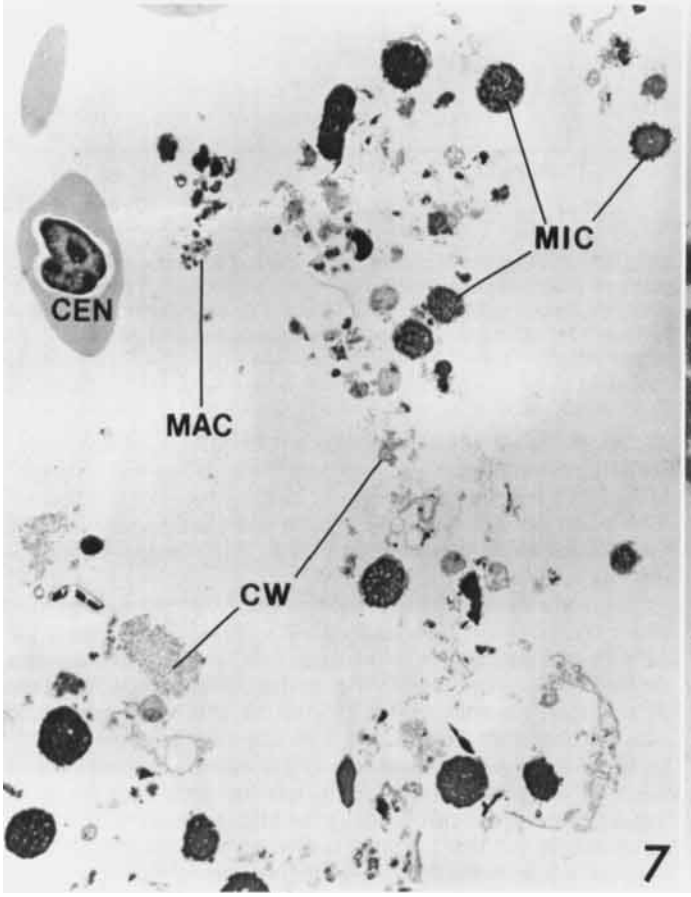
CEN
MAC
MIC
CW
7

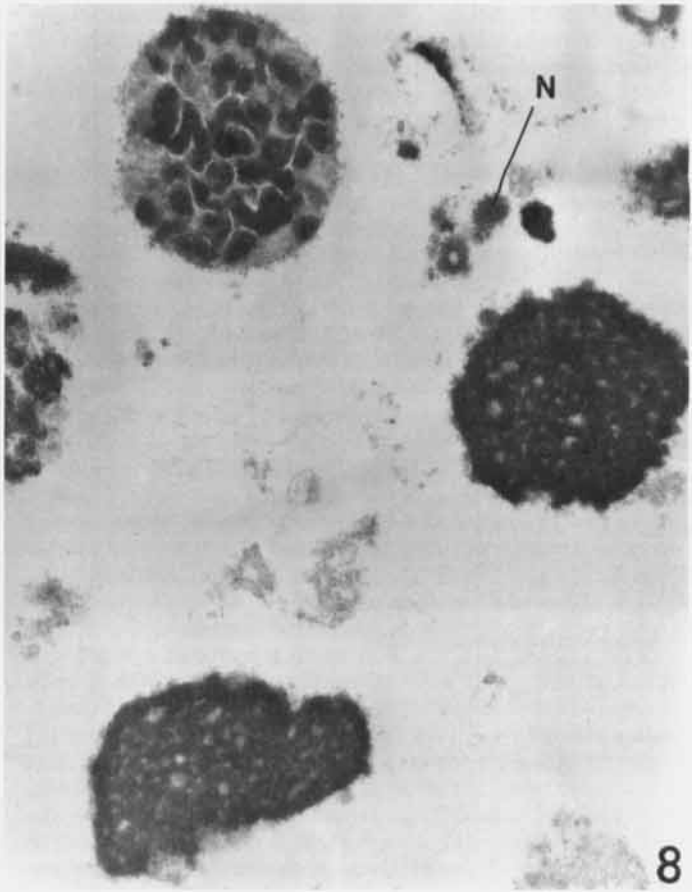
N
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TABLE II. *Purity and yields of micronuclei using Percoll gradients.*

Exptl. condition[a]	No. preps	Average no. cells/ 6 liters	MIC in 27,000 *g* pellet: % recovery	MIC in 27,000 *g* pellet: Ratio MAC/MIC	1st 50% Percoll: % of MIC in 27,000 *g* pellet	1st 50% Percoll: Ratio MAC/MIC	No. 50% Percolls	Yield after 50% Percoll: % of cells	Yield after 50% Percoll: % of MIC in 27,000 *g* pellet	Final ratio MAC/MIC[b]
A	4	7.8×10^8	106	1/3.8	63	1/27	5	33	31	1/416
B	2	9.6×10^8	75	1/3.6	80	1/38	5	36	47	1/803
C	3	1.0×10^9	73	1/4.6	73	1/41	3	29	40	1/1134
D	5	1.7×10^9	63	1/9	78	1/175	3	30	48	1/2198
E	2	3.3×10^9	52	1/1	63	1/6.3	5–6	13.5	26	1/572
F	2	3.1×10^9	37	1/1	62	1/6.8	6	3.5	9.4	1/938

[a] A = Without 25% Percoll gradient.
B = With 25% Percoll gradient.
C = NP40 added to pellet from 25% Percoll gradient.
D = Same as C except volume of Medium A reduced to ⅓ ml per 10^6 cells.
E = Cell concentration greater than 3.3×10^5/ml, Condition C.
F = Cell concentration greater than 3.3×10^5/ml, Condition D.
[b] Nuclear purity was determined by light microscopy.

Percolls being required to achieve MIC purity. This led to a lower yield and purity of the MIC's in the final sample.

Purity of nuclei. Three kinds of tests were used to evaluate the purity of the nuclei: visualization of the nuclei by light microscopy; examination of the nuclei by electron microscopy; and Southern blots of MIC and MAC DNA probed with the 5s rRNA genes or a fragment from the MAC extrachromosomal rDNA molecule.

Light microscopy was routinely used to monitor purification of the nuclei. Contamination of MAC by MIC or MIC by intact MAC or large MAC fragments could be easily observed. These observations allowed us to calculate nuclear ratios, examples of which are given in Tables I and II. Small fragments of MAC, or nucleoli, would, however, be missed at the level of magnification used.

Electron microscopy of fixed and embedded pellets of purified MAC and MIC gave information about the morphology of the nuclei and about the purity of the preparations. Several different individual preparations were examined by electron microscopy, with the same qualitative results indicating a high degree of purification of MAC or MIC. Purified MAC and MIC from a single preparation were used for all quantitative analyses, as well as for all electron micrographs shown in this paper. This preparation was carried out under the experimental conditions shown in the flow diagram (Fig. 1). MAC were taken after a single Percoll centrifugation and determined by optical microscopy to have a MAC/MIC ratio of 145/1. MIC were taken through the procedure listed as condition D in Table II, and determined by optical microscopy to have a MAC/MIC ratio of 1/1260.

Purified MAC were relatively free from contamination by MIC or other cellular organelles. Figure 5 shows a typical thin section. About 95% of the MAC are intact; the remaining MAC have been partially disrupted perhaps as a result of the homogenization. Figure 6 shows a typical intact MAC, with chromatin bodies, nucleoli, and densely stained granules of 50–150 nm diameter that are characteristic of intact and fragmented MAC. Nuclear envelopes were usually absent, presumably due to the action of NP40 during the isolation.

Purified MIC were much less free of contamination than purified MAC. Figure 7 shows a typical field in a MIC section. The major contaminants were cell wall components (including clusters of basal bodies) and fragments of MAC.

Two distinct MIC structures were found. The majority (70%) of the MIC had highly condensed chromatin, comparable in appearance to the chromatin bodies of chicken erythrocytes present in the same sections (Fig. 7). The remaining MIC (30%) had "decondensed" chromatin similar in appearance to the chromatin bodies in the MAC. Figure 8 shows two condensed and one "decondensed" MIC. Decondensed MIC can be distinguished from MAC and MAC fragments on the basis of the complete absence of 50–150 nm dense granules in all MIC and the fact that the lightly stained areas in the MIC do not resemble nucleoli.

Stereology of 12 randomly selected micrographs of purified MAC was performed. Grid intersections with MAC and MIC were determined to be 3884 and 5, respectively. Thus, by equation 1, the volume ratio (V_{mac}/V_{mic}) of MAC to MIC was 777. In order to relate this volume ratio to the ratio of MAC to MIC determined by optical microscopy we needed to determine the average volumes of intact MAC and MIC, respectively. Applying the method outlined in Materials and Methods we determined $\bar{V}_{mac}$ to be 68 μ^3 and $\bar{V}_{mic}$ to be 2.3 μ^3. Using Equation 3, the estimated ratio of intact MAC to intact MIC (N_{mac}/N_{mic}) equals 27. Equation 4 was used to give an estimate of the relative amounts of MAC and MIC DNA in the purified MAC sample. Assuming that each MAC contains ca. 20× as much DNA as each MIC, our purified MAC sample was calculated by Equation

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Fig. 5. Purified macronuclei (MAC). Fragmented MAC shown by arrows. 4300×.

Fig. 6. Macronucleus at higher magnification, with typical appearance of chromatin bodies (CB), nucleoli (N), and dense granules (DG). The nuclear envelope is absent. Small dark spots surrounding the nucleus are not ribosomes, but residual Percoll particles (P). These spots are seen also in unstained sections. 22,500×.

Fig. 7. Purified micronuclei (MIC) with a chicken erythrocyte nucleus (CEN) in a chicken erythrocyte added before embedding, for comparison. Fragments of cell wall material (CW) and macronuclei (MAC) are also seen. 4300×.

Fig. 8. Two condensed and one decondensed micronuclei. Fragments of material, possibly of nucleolar origin (N) are also shown. 22,500×.

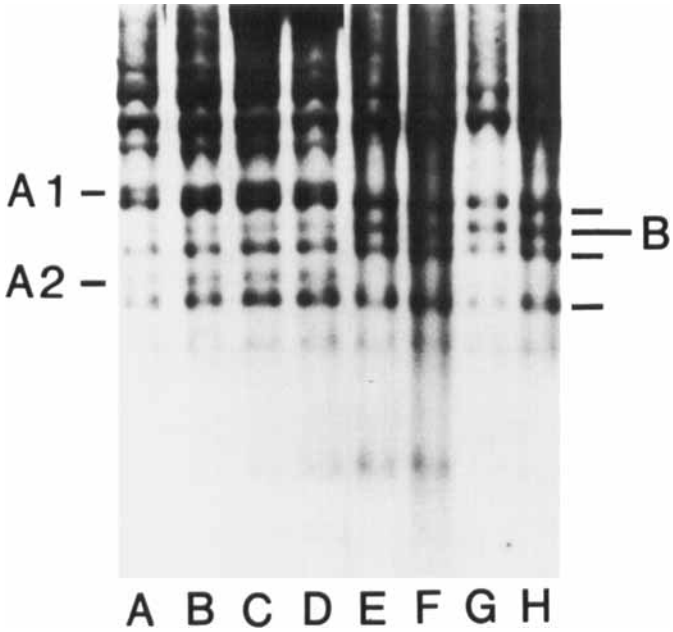

Fig. 9. An autoradiograph of Southern blots of DNA from samples of nuclei taken during purification of cell clone 21a and probed with α^{32}P labeled pDP5. Each DNA was digested with Eco RI, electrophoresed in an 0.8% gel (5 μg/lane) and blotted to nitrocellulose. Lane E represents a sample of whole cell DNA (1 MAC/1 MIC). Lanes D, C, B, and A represent samples after successive 50% Percoll gradients of the MIC. The MAC to MIC ratios for these samples are as follows: D = 1 MAC/40 MIC, C = 1 MAC/240 MIC, B = 1 MAC/424 MIC and A = 1 MAC/512 MIC. Lane F represents a sample of the 769 *g* pellet (nuclear ratio = 32 MAC/1 MIC). Lane G represents a sample after the first MAC Percoll gradient (88 MAC/1 MIC) and lane H represents a sample after the second MAC Percoll gradient (202 MAC/1 MIC). The bands labeled A1 and A2 are MIC specific bands. The band labeled B is a MAC specific band. The three bands marked simply with a line are constant bands present in both MIC and MAC and vary only with the amount of DNA loaded in each well.

4 to have a ratio of MAC/MIC DNA of 540/1. This ratio should be compared to the ratio expected on the basis of light microscopy (i.e., 2900/1).

Stereology of 21 randomly selected micrographs of the purified MIC sample was also carried out; 1582 grid intersections with MIC and 77 grid intersections with MAC fragments were recorded. MAC fragments were distinguished from MIC or MIC fragments by the presence of the densely stained granules. Ambiguous fragments were scored as MAC fragments. Thus, in our purified MIC, V_{mac}/V_{mic} equalled 0.05. Using Equation 3 and the $\bar{V}_{mac}/\bar{V}_{mic}$ determined before, N_{mac}/N_{mic} was calculated to be 0.0017 (i.e., 1/588). Using Equation 4, the ratio of MAC to MIC DNA was calculated to be 0.034 (i.e., 1/29). With light microscopy the ratio expected would be 0.016 (i.e., 1/64).

One problem with the quantitative analyses of the electron micrographs needs to be pointed out: that is, the difficulty in identifying small fragments as MIC or MAC. In the MAC sample it is possible that small MAC fragments may have been counted as MIC, which would lead to overestimating the number of MIC. In the MIC sample ambiguous fragments were scored as MAC fragments, which undoubtedly inflated our MAC calculation.

The third kind of test examined the DNA extracted from purified MIC's and MAC's for cross contamination of nuclei. Southern blots of MIC and MAC DNA were tested against two different probes.

The first probe was 5s rDNA. One well documented difference between MIC and MAC DNA is the different patterns of 5s rRNA gene clusters (15). The 5s rRNA genes are present in both nuclei as clusters of tandemly repeated genes with AT-rich spacer regions. The restriction enzyme, Eco RI, does not cleave within these repeats. When DNA is cut with Eco RI, electrophoresed, blotted, and probed with a 5s rDNA probe, the banding patterns represent the clusters of 5s rRNA genes as well as some of the flanking regions. A difference in these banding patterns was observed between MIC and MAC DNA. We used this difference to test the cross contamination of our nuclei.

MIC and MAC were purified from cell line 21a, and DNA samples were taken at several steps in the purification. These samples were cut with Eco RI, electrophoresed, blotted onto nitrocellulose filters, and probed with pDP5 that contains 5s rDNA and its spacer region. The results are shown in Fig. 9. Lane A contains purified MIC DNA. Lane H contains purified MAC DNA. Lines are drawn beside the constant bands that do not vary throughout purification except with the amount of DNA loaded into each well. Two bands marked A1 and A2 appear to be MIC specific since they increase in intensity as the MIC are purified. The band marked B appears to be MAC specific, since it increases in intensity as the MAC are purified.

Densitometry supports the continuous purification of the nuclei. MIC band A1 is barely detectable in whole cell DNA (lane E). As the MIC are purified, this band increases in intensity while band B decreases in intensity. Each lane was traced with the microdensitometer and the area under each band was measured. A1 and B were standardized against the constant bands in each lane and then compared to each other. In whole cell DNA, A1 is 1/9 as intense as B. In the DNA from purified MIC A1 is 46 times as intense as B. This gives an increase in nuclear ratio from 1 MIC/1 MAC (whole cell DNA) to 414 MIC/1 MAC (purest MIC). The ratio as calculated by light microscopy was 512 MIC/1 MAC. Therefore, the 5s rDNA data and the light microscopy data give comparable low cross contamination estimates. The degree of MIC contamination in the MAC cannot be calculated because the MIC bands are not detectable in MAC DNA blots.

Further inspection of these banding patterns shows that there are additional differences between MIC and MAC patterns, more than previously reported. This phenomenon is currently under investigation in our laboratory.

The second probe contained a fragment derived from the rDNA molecule and was used to measure the amount of MAC contamination in the MIC. The rDNA molecules with the 18s and 26s genes exist as extrachromosomal palindromes in the MAC and as integrated single copy sequences in the MIC (14, 30). The extrachromosomal palindromes are present at 10,000 copies per MAC.

The contamination levels were measured using strain SB-530, a heterokaryon, in which the MIC rDNA sequences contain an extra Bam HI restriction enzyme site not present in the MAC rDNA sequences (20). DNA from purified MIC (2% contamination with MAC by light microscopy) was cut with Bam HI, blotted onto nitrocellulose filters, and probed with pRP7, which contains the sequences of interest. The intensity of the rDNA MAC bands in the MIC DNA was compared to the intensity of rDNA MAC bands in known amounts of MAC DNA. By this criterion the MIC were contaminated with about 10% of the rDNA (data not shown). The difference in contamination levels between light microscopy and rDNA blots can be explained if some of the nucleoli released from the MAC during purification copurified with the MIC. The phenomenon was observed by Iwamura et al. (13). The fact that the estimates from 5s rDNA blots and light microscopy gave similar low levels

of MAC contamination argues that the rDNA blots measure nucleolar contamination but do not reflect MAC chromosomal DNA contamination.

DISCUSSION

Percoll gradients have been used for the purification of viruses, cells, nuclei, and other cellular components (see references listed in "Percoll: methodology and applications," supplied by Pharmacia). Often the density of the particular element has played a predominant role in achieving its separation. In the case of the two nuclei in *T. thermophila,* the basis for their separation appears to be their difference in size rather than in density. Varying the time of centrifugation with the same percentage of Percoll showed that longer centrifugation times resulted in the MIC band moving down the gradient toward the MAC band until both types of nuclei were found at the bottom of the tube. Lowering the concentration of Percoll caused both types of nuclei to be pelleted. Increasing the concentration of Percoll to 75% resulted in the spreading of both types of nuclei throughout the gradient. Further work was done to see if altering the concentration of Percoll, buffer system, or the conditions of centrifugation would effect separation of the MIC from the MAC on the basis of density. Steeper gradients were formed using 60% Percoll made up in Medium A minus gum arabic and centrifuging for 30 min at 63,000 *g*. Nuclei from the 27,000 *g* pellet were layered on top and centrifuged for 10 min at 1000 *g*. Under these conditions the MIC and MAC banded together near the bottom of the tube. Increasing the time or *g* forces did not change the position of this band. Increasing the concentration of Percoll, or changing the buffer, did not resolve the nuclei into two bands. We conclude that the densities of the two nuclei must be too similar to allow separation on the basis of density.

We did attempt step gradients instead of the continuous gradient. However, we found that Percoll contains a small amount of uncoated silica that damages nuclei. Centrifugation to form the continuous gradient pelleted the uncoated material. Under these conditions damage to the nuclei did not occur since all the nuclei could be recovered from the gradient. We also found that a fixed angle rotor cannot be used for centrifuging nuclei in a Percoll gradient since the MAC pelleted to the side of the tube in the area of the MIC band.

We also found that the 769 *g* centrifugation step was necessary both for MAC recovery as well as efficient MIC purification. If this step was skipped and the homogenate was first centrifuged at 27,000 *g,* MAC appeared to be destroyed and the scum was pelleted with the MIC, making subsequent MIC purification slow and the nuclei difficult to count.

MIC can be recovered from MAC Percolls and vice versa. However, the extent of contamination with the other type of nucleus is high. Pooling these nuclei with nuclei already partially purified slowed down purification; therefore, we found it more efficient to discard the impure nuclei.

We observed that the MAC's that contaminated MIC pellets were smaller than average in size. However, we found that MAC fragments were observed much less frequently than they were after filtration using the Gorovsky et al. (10) procedure.

Visualization of the nuclei by light microscopy indicated that our nuclei are highly purified. Greater cross contamination was suggested by the quantitative analyses of electron micrographs. However, the problem in identifying small MIC or MAC fragments may have contributed to the discrepancy in estimates. We were conservative in what we included in our estimates of contamination when analyzing micrographs. Thus, contamination is probably underestimated by light microscopy and overestimated by electron microscopy. Most workers use light microscopy to monitor nuclear purity. Hence, the level of nuclear cross contamination is probably generally underestimated.

On the other hand, Southern blots of MIC and MAC DNA using a 5s rDNA probe confirmed the level of MAC contamination in the MIC estimated by light microscopy during purification of the nuclei. This agreement in the estimates of contamination reflects the fact that in this case MAC chromosomal DNA was being measured. A higher level of rDNA contamination was observed using Southern blots of MIC and MAC DNA, derived from a heterokaryon with distinctive MIC and MAC Bam HI sites, using the rDNA probe. This observation could be explained by contamination of MIC by nucleoli, which contain the MAC extrachromosomal rDNA molecules.

The Percoll procedure permits purification of MIC and MAC that have much lower levels of nuclear cross contamination, estimated by light microscopy, than previously reported. MIC's contaminated with less than 1 MAC in 1000 MIC can be routinely prepared. MAC's contaminated with less than 1 MIC in 500 MAC can be easily prepared. For the MAC's this represents a 25- to 50-fold increase in purity over the earlier methods. This level of MAC purity was essential for our demonstrating[2] that certain repetitive MIC sequences are partially eliminated and rearranged in newly formed MAC and that subsets of these sequences persist in old MAC in different cell lineages.

Note added in proof: Recently, we have found that doubling the time of centrifugation at 769 *g* removes most of the MAC without sedimenting the MIC. This allows MIC to be purified to MAC/MIC ratios of ca. 1/5000 with one or two 50% Percoll gradients.

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Cyclic Adenosine 3′,5′-Monophosphate Binding Protein in *Tetrahymena*: Properties and Subcellular Distribution[1]

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ABSTRACT. Cyclic AMP binding activity was determined in the ciliate *Tetrahymena pyriformis* NT-1 strain. The fractions having the binding activity were eluted in a single peak coincident with a protein kinase activity. Although metal ions were not essential for activity, the binding was slightly activated by Mg^{2+} or Ca^{2+}. The binding activity was sensitive to temperature, ionic strength, and pH of the reaction mixture and was decreased by treatment of the cytosol protein with trypsin or by heating at 100°C. The binding was specific for cyclic AMP, with an estimated apparent Kd of 40 nM. When the cyclic AMP binding activity in subcellular fractions was measured, an increase in the activity of ciliary, mitochondrial, and microsomal fractions was observed during the transition from the exponential to the stationary phase of cell growth, whereas no significant change occurred in the binding activity of the whole cell homogenate. These results suggest that the redistribution of cyclic AMP binding proteins may be implicated in the regulation of cyclic AMP concentration in the cell.

SPECIES of *Tetrahymena* serve as excellent cell models for studying a number of biological processes, such as membrane structure and function (28), cell motility (23), and cell division (36). Furthermore, *Tetrahymena* contains cyclic AMP, cyclic GMP, their related enzymes (3, 10, 24, 31) and calmodulin (5, 6, 12, 19, 20, 25, 26, 35). Experimental manipulation as well as growth-related changes of cyclic nucleotide levels in the cell have implicated cyclic AMP and cyclic GMP in the control of cell division (7, 14).[2]

Previous studies from our laboratory demonstrated that the formation and breakdown of cyclic AMP are not dependent on the growth stage of *Tetrahymena* cells (8, 9). However, an immunological approach suggested that changes in compartmentalization of the intracellular cyclic AMP may occur during growth and that such phenomena might involve cyclic AMP-mediated processes in the cell (14). We could not, however, determine precisely the cyclic AMP binding site and its corresponding binding affinity in the cell by this immunofluorescence technique.

In the present study, therefore, properties and subcellular distribution of cyclic AMP binding proteins have been investigated in order to elucidate some of the mechanisms by which cyclic AMP participates in cellular control. It is conceivable that cyclic AMP binds to intracellular proteins and that the resultant cyclic AMP-protein complexes are universally involved in the actions of cyclic AMP (13, 16, 17, 22, 32). To our knowledge, this is the first report on the cyclic AMP binding activity in *Tetrahymena*.

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[2] Abbreviations used in this paper: EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N′-tetraacetic acid; cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate.