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The Isolation and Characterization of Nuclear Ghosts from Cultured HeLa Cells†

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ABSTRACT: Macromolecular complexes, which appear as ghosts when viewed by phase contrast microscopy, have been isolated from the nuclei of HeLa cells grown in culture. The preparation of these ghosts involves a detergent wash which removes the unit membranes of the nuclear envelope structure but leaves intact both the nuclear pores and the dense structure conferring nuclear margins (possibly the dense lamella). Detergent-washed nuclei are subsequently treated with 0.5 *M* $MgCl_2$ and fractionated on continuous sucrose gradients containing 0.5 *M* $MgCl_2$. The ghosts are recovered as a sharp band at an apparent sucrose density of 47–52% and consist of 72% protein, 10% phospholipid, 14% DNA, and 4% RNA. The release of the majority of intranuclear components is indicated by the large loss of nuclear DNA (95%), RNA (71%), and protein (87%) contrasted to the small loss of phospholipid (27%) during the conversion of detergent washed nuclei to isolated ghosts. Sodium dodecyl sulfate–polyacrylamide gel patterns of the ghost proteins consist of two major bands with approximate molecular weights of 20,000 and 35,000. The isolation of ghosts with a similar density and protein composition from nondetergent-washed nuclei indicates that the ghost is not an artifact induced by the detergent treatment. The absence of cytoplasmic contamination in the preparations of detergent washed nuclei and nuclear ghosts was demonstrated by chemical, enzymatic, and electron microscope studies. We suggest that the isolated ghosts represent a structural macromolecular complex which underlies and is probably attached to the inner nuclear membrane of intact nuclei. The possible additional presence of intranuclear network proteins has not been excluded.

Investigations into the biochemical behavior of the nuclear envelope during the cell cycle, with particular regard to its cyclic dissolution and re-formation in mitosis, would be greatly facilitated by the isolation of well-defined components of the nuclear envelope from cells grown in culture. Until the present, no attempt at characterization of nuclear envelope fractions prepared on a large scale from cultured cells has been reported, even though there have been a number of studies on nuclear envelopes isolated from tissue sources (e.g., Kashnig and Kasper, 1969; Zbarsky et al., 1969; Franke et al., 1970; Agutter, 1972; Berezney et al., 1972; Bornens, 1973; Monneron et al., 1972).

As a result of ultrastructural studies performed on a wide variety of systems, the nuclear envelope has been recognized as having membranous as well as nonmembranous components. These include the inner and outer nuclear membranes, the dense lamella, the heterochromatin layer, and the annuli or pore structures, which occur in varying numbers on the surfaces of nuclei (Kay and Johnston, 1973).

Although many authors have emphasized the membranous nature of their isolated nuclear envelope fractions, recent evidence suggests that the presence in these fractions of nonmembranous nuclear envelope components may need to be considered as well. For example, while our work was in progress, Aaronson and Blobel (1974a) demonstrated that the nuclear pores, which are often taken to be diagnostic for nuclear membrane, seem to be firmly embedded in a layer (dense lamella or heterochromatin layer) normally internal to both inner and outer nuclear membranes. These investigators have shown that the structural integrity of nuclei is maintained in the absence of any membrane. Furthermore, there have been reports of fibrous protein networks which are continuous with the nuclear envelope and extend throughout the nuclear volume (Smetana et al., 1963; Steele and Busch, 1966; Narayan et al., 1967; Berezney and Coffey, 1974). Despite their obvious importance in maintaining the structure of the nuclei, the likely presence of nonmembranous nuclear and nuclear envelope com-

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ponents such as the dense lamella, heterochromatin layer, and internal protein networks has not been accounted for in putative "nuclear membrane" preparations.

We have isolated a macromolecular complex from HeLa cell nuclei which occurs in the form of spherical particles with distinct peripheral margins. The maintenance of the gross morphology of nuclei, despite the loss of considerable intranuclear material, has led us to refer to the isolated macromolecular complex as nuclear ghosts.[1] Individual ghost formation from individual nuclei can be directly observed. The present report deals with the isolation and biochemical characterization of these ghosts. It is hoped that further study of these and related structures will lead to a biochemical understanding of the structure and function of nonmembranous nuclear envelope components.

Future reports will also be concerned with cell cycle dependent changes in nuclear envelope components and with the maturation of *Herpes simplex* virus, whose life cycle is intimately related to structures at the nuclear surface (Darlington and Moss, 1968; Morgan et al., 1954; Shipkey et al., 1967; Siegert and Falke, 1966).

Materials and Methods

Cells and Cell Culture. The human HeLa S_3 cell line was obtained from Dr. Paul Atkinson (Albert Einstein College of Medicine). These cells were grown in suspension culture at 37° with medium composed of Eagle's minimal medium modified for suspension culture (GIBCO) and supplemented with 3.5% fetal calf serum and 3.5% calf serum. Cells were routinely maintained in logarithmic growth by dilution with fresh medium. All cultures have been routinely found to be free of mycoplasma by Dr. George Kenny (University of Washington).

Chemical Determinations. For most of the experiments involving chemical determinations, the starting material consisted of 0.6 to 1.0 l. suspension cultures of HeLa cells. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (Calbiochem, A grade) as a standard. The determination of RNA was carried out according to the method of Munro and Fleck (1966) which involves removal of acid soluble components, digestion with KOH, and perchloric acid precipitation of DNA and protein. DNA was extracted from separate samples with hot 0.5 *N* $HClO_4$ and determined by the diphenylamine reaction (Burton, 1956). Calf-thymus DNA (Calbiochem) was used as a standard.

To determine lipid phosphorus, total lipids were extracted with chloroform–methanol (2:1, v/v) and washed with 0.88% KCl as described by Folch et al. (1956). The lipid extracts were then taken to dryness under a stream of nitrogen, and the phosphate present in the dried extracts was determined by the method of Bartlett (1959). The total amount of phospholipid present was assumed to be 25 times the amount of phosphorus (in micrograms) present in the extract. For the purpose of identifying and quantitating the various phospholipids present in the chloroform–methanol extracts, amounts of the dried lipid extracts corresponding to 10 μg of phosphorus were dissolved in 200 μl of chloroform–methanol (2:1, v/v) and spotted quantitatively onto plates of silica gel H (20 × 20 cm, 100 μm thick). The samples were chromatographed in two dimensions, employing the solvent systems and washing procedures of Eichberg et al. (1973). After drying, lipids were made visible with I_2 vapor and outlined with a pencil. The identification of each spot was verified by testing for the co-migration of excess amounts of standard phospholipids with each of the individual spots. A background determination for each visualized spot was obtained by tracing a spot of equal area onto a blank region of the same plate. The visualized spots and their corresponding controls were then scraped off the plate and the phosphate content of each of these samples was determined by the ultramicromodification of the method of Bartlett (1959), except that digestion was with 0.3 ml of 5 *N* H_2SO_4 instead of 0.3 ml of 10 *N* H_2SO_4. Silica gel was removed in all cases by centrifugation before the absorbance values were read. The recovery of lipid phosphates from the thin layer plates was 90 to 95%. Phospholipid standards used were phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylinositol, lysolecithin, lecithin, cardiolipin, and sphingomyelin (General Biochemicals, chromatographic purity).

Estimations of mature ribosomal 18S and 28S RNA in detergent-washed nuclei and total cytoplasm obtained from the supernatant of the cell homogenate were performed on material isolated by the hot phenol extraction method of Penman (1969), followed by centrifugation in sodium dodecyl sulfate–sucrose gradients. The gradients were fractionated and the absorbance at 254 nm was continuously monitored using an Instrumentation Specialties Co. continuous flow monitor.

The densities of fractions isolated from sucrose gradients were determined with a sugar refractometer. Observed values are reported as apparent sucrose densities without correction for other interfering substances (e.g., 0.5 *M* $MgCl_2$).

Radioactive Labeling. Falcon plastic tissue culture dishes (150 × 25 mm) containing 20 ml of medium were each seeded with 8.0×10^6 cells. In parallel cultures, [9,10-^{3}H]palmitate (New England Nuclear, 230 mCi/mmol) adsorbed to serum proteins was used at a final concentration of 3.0 μCi/ml, a ^{3}H-labeled amino acid mixture (15 amino acids, general label, New England Nuclear) was used at a final concentration of 2 μCi/ml, and [*Me*-^{3}H]thymidine (New England Nuclear, 20 Ci/mmol) was used at a final concentration of 0.25 μCi/ml. Incubation was for 35 to 40 hr, after which a sufficient quantity of unlabeled cells was combined with each of the labeled cultures such that the final number of cells for each analysis was 0.5 to 1.0×10^8. Labeling with [^{3}H]fucose was carried out for 16 hr in 150-ml cell cultures at an initial density of 40×10^4 cells/ml with L-[^{3}H]fucose (New England Nuclear, 4.6 Ci/mmol) at a final concentration of 1 μCi/ml.

For the assay of radioactivity in samples containing ^{3}H-labeled amino acids, [*Me*-^{3}H]thymidine, or [^{3}H]fucose, each aliquot was diluted with water to 1 ml. One drop of a 0.3% RNA (total yeast RNA, Miles) solution was added as carrier to each sample, followed by 1 ml of 15% trichloroacetic acid. Precipitation was allowed to proceed for 10 min in the cold. The precipitates were then collected on glass filter disks (Schleicher and Schuell, Inc.), washed twice with 5 ml of 5% trichloroacetic acid, once with 5 ml of a 75% ethanol solution containing 2% potassium acetate, and once with 5 ml of 70% ethanol. For all samples containing [^{3}H]palmitate the final two ethanolic washings were replaced by five washings with 5-ml quantities of cold water since ethanol was found to remove labeled lipid from the

[1] This definition of ghosts is not intended to imply the presence of membranes as have been found in other ghost preparations (e.g. red blood cell).

precipitates. The filter disks were placed in glass scintillation vials and dried overnight at 110°. Finally, 2 ml of scintillation fluid (Omnifluor, New England Nuclear) was added for counting.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Analysis of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using the discontinuous buffer system of Laemmli (1970). A periodic acid sensitive cross-linking agent, *N,N'*-diallyltartardiamide (Anker, 1970), was used mole for mole in place of methylenebisacrylamide. Solubilization of 100 to 200 μg samples of protein was accomplished in a solution composed of 2% sodium dodecyl sulfate, 0.0625 *M* Tris-HCl (pH 6.8), 10% glycerol, and 5% 2-mercaptoethanol at 100° for 2 min. Samples were heated immediately after mixing. The gels were run at a current of 1.5 mA per gel for a period of 6 to 7 hr and stained with 0.1% Coomassie Brilliant Blue in aqueous 50% Cl_3CCOOH. Molecular weight standards used were bovine serum albumin, catalase, and trypsin.

Glutamate Dehydrogenase. The activity of glutamate dehydrogenase in the various subcellular fractions was assayed by the procedure of Schachter et al. (1970). The oxidation of NADH in the reaction mixture was followed by monitoring the absorbance at 340 nm at 25°. The enzyme activities were calculated from the initial slopes, and the measured activity was found to be directly proportional to the concentration of added protein. Control incubations lacking in α-ketoglutarate failed to demonstrate any possible interfering NADH oxidase activities in the various samples.

Staining of Nuclear Ghosts. The nuclear ghosts were stained with a freshly prepared and filtered solution of 0.1% Coomassie Brilliant Blue in water. A drop of the staining solution was placed directly on top of a drop of the ghost suspension on a microscope slide. Mixing, by agitation of the ghost suspension with staining solution, tended to disrupt the fragile ghosts.

Electron Microscopy. Isolated nuclei originally were prepared for thin-sectioning by fixation in glutaraldehyde, followed by post-osmification, dehydration, and embedment in Spurr resin (Spurr, 1969). The results with this procedure indicated that the detergent-washed nuclei lacked envelope membranes. To confirm this finding, all subsequent observations were made on material treated with tannic acid during fixation. This method, developed from the modification by Tilney et al. (1973) of procedures by Futaesaku et al. (1972), entailed the following. Pelleted nuclei were fixed for 5 min at 20° in 2% glutaraldehyde in 0.05 *M* sodium phosphate (pH 6.9), containing 0.1 *M* $CaCl_2$. They were then transferred to tannic acid made up to 8% in an identical buffered glutaraldehyde solution containing the same buffer and were left in this solution for 16 hr at 20°. Pellets were subsequently rinsed in 0.1 *M* sodium acetate at pH 5.4, post-fixed for 5 min in 2% OsO_4 in the same buffer, again rinsed in this buffer, and treated for 60 min in 1% uranyl acetate. Pellets were then dehydrated in a graded series of ethanol solutions, transferred to propylene oxide, and embedded in Spurr resin. Thin sections were stained successively with uranyl acetate and lead citrate before viewing in a Phillips EM 300 electron microscope.

Results

Preparation of HeLa Cell Nuclei. Nuclei were prepared by the following modification of the method of Penman (1969). After chilling to 4°, HeLa cells (1.0×10^8 to 10.0×10^8 cells) were harvested by centrifugation at 600*g* for 4 min. The use of longer centrifugation times resulted in a subpopulation of cells that did not respond to the swelling step described below. The pelleted cells were then gently redispersed and washed in divalent cation-free phosphate buffered saline (PBS-A)[2] (Dulbecco and Vogt, 1954). Following centrifugation, the walls of the centrifuge tube were carefully dried and the pellet was dispersed in 5 ml (for every 1.0×10^8 cells) of $\frac{1}{3} \times$ RSB[2] (0.01 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.4), and 0.0015 *M* $MgCl_2$) (Penman, 1969). The cells were allowed to swell for 5 to 10 min and were then homogenized with 20 to 25 strokes in a glass Dounce-type homogenizer. This treatment was usually sufficient to rupture more than 95% of the cells with minimal damage to the nuclei as monitored by phase contrast microscopy. One volume of $\frac{1}{3} \times$ RSB was then added and the homogenate was centrifuged in an International PR-2 centrifuge at 1800 rpm for 0.5 to 1.0 min in order to pellet the nuclei. Resuspension of the nuclei was facilitated by the use of minimal centrifugation times and large centrifuge tubes (e.g., 40 ml, round bottom) in order to obtain thin pellets. However, the centrifugation time could be increased slightly for the collection of nuclei at subsequent stages of the preparation, since the nuclei became less "sticky" after each washing. Yields were generally optimized if resuspension of nuclear pellets was accomplished with Pasteur pipets that had been presoaked in $\frac{1}{3} \times$ RSB, as opposed to vortexing, which seems to damage the nuclei with respect to subsequent steps of the preparation. When nuclei were to be fractionated on the 0.5 *M* $MgCl_2$ gradients without prior detergent treatment, the nuclear pellet obtained from the cell homogenate was washed once in $\frac{1}{3} \times$ RSB and then dispersed in 0.25 *M* sucrose–TKM[2] (0.025 *M* KCl, 0.005 *M* $MgCl_2$, and 0.05 *M* Tris-HCl (pH 7.5)) (Monneron et al., 1972). For all other purposes, the nuclear pellet was washed once in $\frac{1}{3} \times$ RSB and finally redispersed in a volume of $\frac{1}{3} \times$ RSB equal to twice the volume of the original homogenate. Representative thin sections of non-detergent-treated nuclei isolated in $\frac{1}{3} \times$ RSB are shown in Figure 1. There appears to be no significant contamination by cytoplasmic organelles. The inner and outer membranes are seen to be present, though occasionally disrupted (Figure 1a), and numerous well-detailed pores exhibiting eightfold symmetry were observed in partially tangential views (Figure 1b).

The membranes of the nuclear envelope were removed with a mixed detergent solution. A 0.15-ml aliquot of a 2:1 mixture of a 10% solution of Tween 40 and a 10% solution of sodium deoxycholate was added to every milliliter of nuclear suspension (Penman, 1969). After addition of detergents, the suspension was gently swirled for 5 sec and centrifuged at 1800 rpm for 0.5 to 1 min. The resulting pellet was easily redispersed if the mild centrifugation conditions described above were employed. The total duration of the detergent treatment, including the time of centrifugation, was 4 to 5 min at 4°. The nuclei were then washed twice in $\frac{1}{3} \times$ RSB and finally suspended in 0.25 *M* sucrose-TKM. DNA determinations were used as an indication of the yield of detergent-treated nuclei from whole cells. For 10^6 cells starting material, there was a total of 9.8 and 5.9 μg of DNA in the whole cell and nuclear suspensions, respective-

[2] Abbreviations used are: RSB, buffer composed of 0.01 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.4), and 0.0015 *M* $MgCl_2$; TKM, buffer composed of 0.05 *M* Tris-HCl (pH 7.5), 0.025 *M* KCl, and 0.005 *M* $MgCl_2$; PBS-A, divalent cation-free phosphate buffered saline.

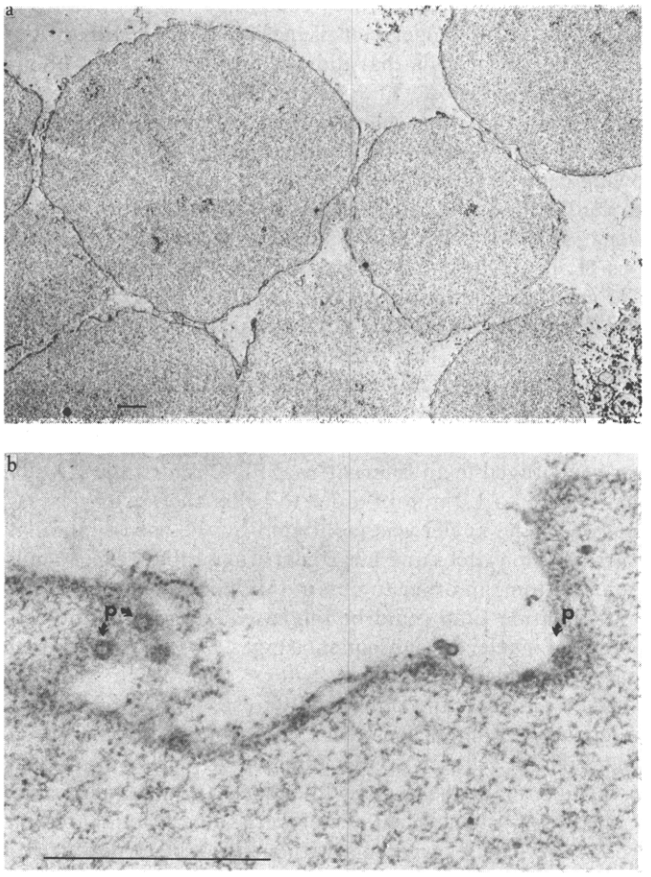

FIGURE 1: Electron microscopy of non-detergent-washed nuclei isolated in ⅓ × RSB: (a) thin sections at low magnification showing nuclei with intact inner and outer membranes; (b) partially tangential view showing detailed pore structures, p, in areas where the section grazes the surface; scale bar, 1 μm.

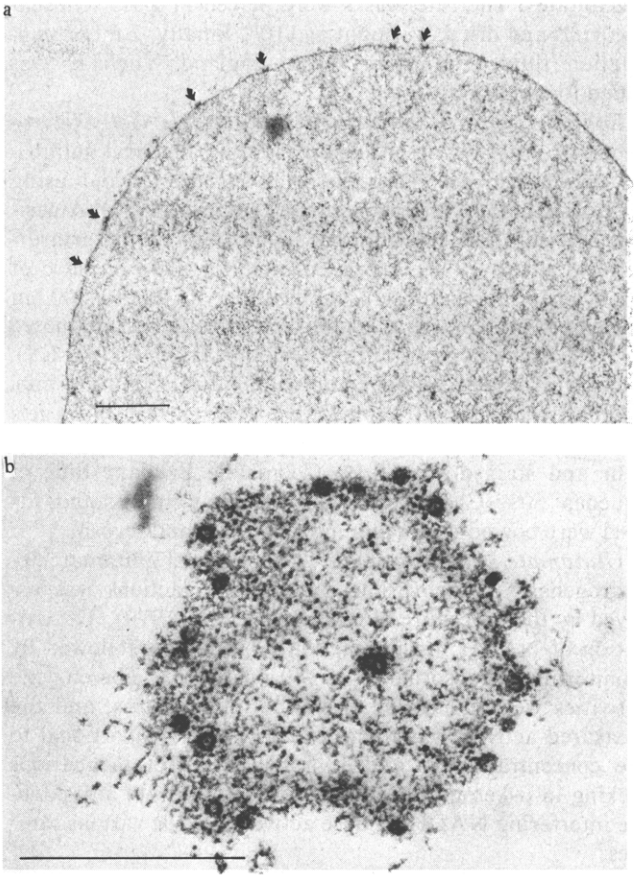

FIGURE 2: Thin sections of detergent-washed nuclei isolated in ⅓ × RSB: (a) a representative section demonstrating the absence of outer nuclear membrane and the presence of "cuff"-like nuclear pores (arrows); (b) grazing section showing frontal views of numerous detailed pore structures; scale bar, 1 μm.

ly, indicating a 60% yield of nuclei. Representative thin sections of detergent-washed nuclei are shown in Figure 2. The detergent treatment has clearly caused the release of the outer nuclear membrane as described by others (Holtzman et al., 1966), but nuclear pores are still seen to be present in cross-sectional views (arrows, Figure 2a). We further note that, although the nuclei exhibit dense borders, no distinct trilaminar inner unit membrane is visible. Numerous detailed pore structures, which exhibit eightfold symmetry in partially tangential views, are clearly seen to be embedded in the dense nuclear borders (Figures 2a and 2b).

Preparation of Nuclear Ghosts. Nuclear suspensions were first made 0.5 *M* in $MgCl_2$, a treatment which has been shown to release the chromatin of rat liver nuclei (Monneron et al., 1972). Equal volumes of nuclear suspension and 1.0 *M* $MgCl_2$ in 0.25 *M* sucrose–TKM were mixed by gentle swirling for 5 sec. Longer or more vigorous mixing did not enhance the removal of DNA from the nuclei but did decrease the yield of the ghost-like material described below. Samples (4 ml) of the 0.5 *M* $MgCl_2$ treated nuclear suspension were layered over 20 to 55% (w/w) continuous sucrose gradients (40 ml) containing 0.5 *M* $MgCl_2$–0.05 *M* Tris-HCl (pH 7.5). Usually, two such gradients were used for every 1.0×10^8 cells starting material. Centrifugation at 25,000 rpm for 10 to 15 hr at 3° in a Beckman SW-27 rotor resulted in the appearance of a sharply defined, turbid band of material at an apparent sucrose density of 47–52%.[3] Fractionation of nondetergent nuclei in the same manner resulted in a similar band appearing at the same density (see Discussion). The band of turbid material was removed with a Pasteur pipet, diluted with 5 vol of TKM buffer, and centrifuged at 4000*g* for 30 min in a Sorvall RC2-B centrifuge. Higher centrifugal forces tend to disrupt the ghost structures described below. The resulting pellet was washed twice with TKM buffer and suspended in four to five times its volume of TKM buffer.

A preliminary characterization of the fractionation of nuclei on the 0.5 *M* $MgCl_2$–sucrose gradients was based on the incorporation of various radioactive precursors. Parallel cultures were each labeled for 35 to 40 hr with either [9,10-^{3}H]palmitate, a general ^{3}H-labeled amino acid mixture, or [*Me*-^{3}H]thymidine (see Materials and Methods). Nuclei prepared from each of these cultures were treated with detergent followed by 0.5 *M* $MgCl_2$ and applied to continuous sucrose gradients containing 0.5 *M* $MgCl_2$. After centrifugation, the gradients were fractionated and the fractions analyzed for incorporation of the various radioactive precursors. The results shown in Figure 3 and Table I indicate that the band at 47–52% apparent sucrose density contains about 70% of the total residual phospholipid of detergent washed nuclei but only 6% of the total nuclear DNA. These results were verified by chemical determinations performed on isolated nuclei and the isolated 47–52% apparent sucrose density band (Table III). The data obtained by incorporation of radioactive precursors (Table

[3] The exact position of the band varies between 47 and 52%, but is usually found as a sharp band at 47%.

Table I: Incorporation of Radioactive Precursors into Various Subnuclear Fractions.

	[9,10-^{3}H] Palmitate Incorpd,[a] 10^{-4} × Radioact. (cpm)	^{3}H-Labeled Amino Acid Mixture Incorpd,[a] 10^{-3} × Radioact. (cpm)	[*Me*-^{3}H] Thymidine Incorpd,[a] 10^{-4} × Radioact. (cpm)
Non-detergent-treated nuclei	8.3 (100)[b]	2.6 (100)	1.5 (100)
Detergent-treated nuclei	1.7 (20)	2.3 (88)	1.4 (93)
Detergent supernatant	6.2 (75)	0.3 (11)	0.04 (3)
Gradient fractions 1–6 for detergent-treated nuclei[c]	0.5 (6)	1.8 (70)	1.3 (87)
Ghosts from detergent-treated nuclei	1.2 (14)	0.2 (8)	0.09 (6)
Gradient fractions 1–6 for non-detergent-treated nuclei[c]	5.0 (60)	2.2 (84)	1.2 (80)
Ghosts from non-detergent-treated nuclei	2.3 (27)	0.2 (8)	0.1 (7)

[a] Expressed as the total counts per minute per 10^6 nuclei analyzed. See Materials and Methods for labeling procedure. [b] Parentheses indicate the percentage of the values obtained from non-detergent nuclei. However, note that, in the text, reference is sometimes made instead to the percentage of the values for detergent-washed nuclei. [c] Refer to Figure 3.

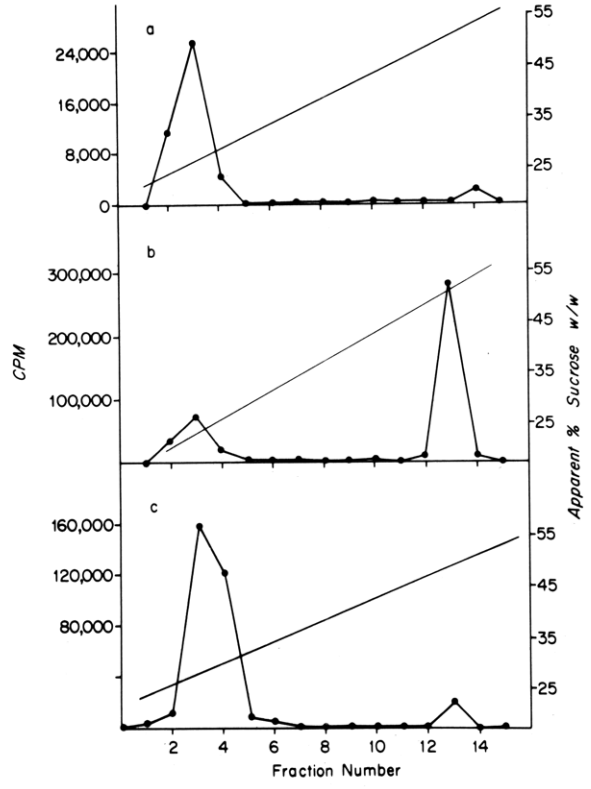

FIGURE 3: Fractionation of detergent-treated nuclei on continuous sucrose gradients containing 0.5 *M* $MgCl_2$. Each of three parallel cultures was labeled as described in Materials and Methods. Detergent-treated nuclei prepared from each culture were treated with 0.5 *M* $MgCl_2$ and fractionated on three separate 20 to 55% continuous sucrose gradients containing 0.5 *M* $MgCl_2$: (a) ^{3}H-labeled amino acid mixture; (b) [9,10-^{3}H]palmitate; (c) [*Me*-^{3}H]thymidine.

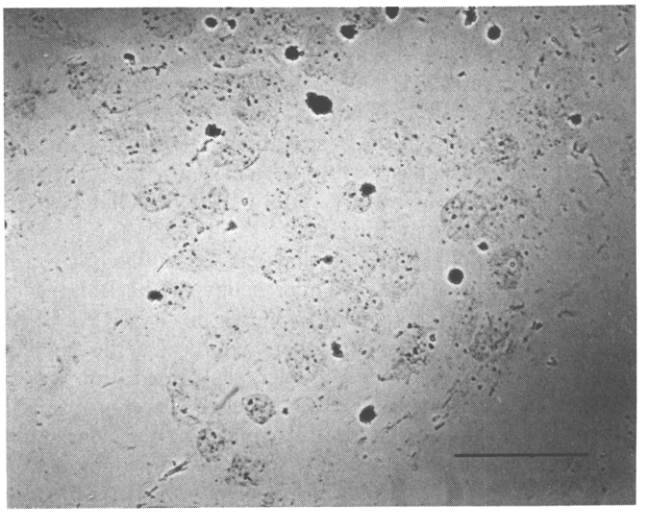

FIGURE 4: Phase-contrast microscopy of ghosts isolated by gradient centrifugation of detergent-washed nuclei which had been treated with 0.5 *M* $MgCl_2$. The dark, "non-ghost" particles represent a significant level of contamination in this preparation. Staining was as described in Materials and Methods; scale bar, 0.10 mm.

I) and the chemical data (Table III) were in good agreement for all stages of the nuclear fractionation. Essentially the same results were obtained when non-detergent-treated nuclei were fractionated on the 0.5 *M* $MgCl_2$–sucrose gradients, except that there was a greater loss of phospholipid to the gradient loading zone (Table I).

Phase Contrast Microscopy. The progress of the homogenization procedure was generally closely followed in order to ensure adequate lysis of the cells with minimal damage to the nuclei. It was found that when the nuclear suspensions were made 0.5 *M* in $MgCl_2$ the nuclei rapidly appeared to swell to about four or five times their original volume and then became transparent (probably due to a depletion of chromatin, see below) except for a very faint outline and apparently unaltered nucleoli. This process could be visualized by examining the nuclear suspension under a phase microscope while introducing a drop of 1.0 *M* $MgCl_2$ at the edge of the coverslip.

Examination of the turbid band of material isolated from the 0.5 *M* $MgCl_2$–sucrose gradients consistently revealed aggregates of ghost-like structures (Figure 4) identical with those obtained upon treating nuclei with 0.5 *M* $MgCl_2$, except that in the former case the nucleoli seem to be absent. We have calculated that the number of whole cells used as starting material that are finally represented in the ghost preparation is 45%. This value is based on the yield of detergent-treated nuclei from whole cells (60%, see above), and

Table II: Estimation of the Levels of Cytoplasmic Components in Various Subcellular Fractions.

Cell Fraction	Sp. Act. of Glutamate Dehydrogenase (μmol/min per mg of Protein)	[^{3}H] Fucose Incorpd, 10^{-4} × Radioact.[a] (cpm)	% of Total Cellular 18S Ribosomal RNA
Whole cell sonicate	9.2	1.52	
Total cytoplasm		1.42	100
Non-detergent-washed nuclei		0.11	
Detergent-washed nuclei	<0.3	<0.01	<1
Ghosts isolated from detergent-treated nuclei	<0.3	<0.01	

[a] Expressed as the total counts per minute in the various fractions per 10^6 cells analyzed. See Materials and Methods for labeling procedures.

the recovery of the residual phospholipid of detergent treated nuclei in the isolated ghosts (72%, derived from data in Table III). The ghost structures were most readily observed by staining with 0.1% Coomassie Blue (see Materials and Methods). Among the aggregates of ghosts were varying amounts of refractile bodies which are thought to be the remnants of dead cells since, when observed, they were present at all steps in the preparation. The ratio of these refractile bodies to ghosts could be kept below 1% if the starting cells were in a healthy state and were pelleted at low speeds prior to the swelling step.

The quality of the preparation was best judged by examination with phase contrast microscopy of the isolated nuclei, which appear as smooth spheres in $\frac{1}{3}$ × RSB. When more than one or two nonspherical or refractile particles were observed per hundred nuclei, the preparation was rejected. Ghosts prepared from nuclear suspensions with three to four times this level of contamination consistently contained extra protein bands when examined by sodium dodecyl sulfate–acrylamide gel electrophoresis (see below). It is suggested that the co-purification of dead cells and other debris could be a serious problem in methods attempting to isolate nuclear envelope fractions from tissue, in which the proportion of dead or damaged cells is less subject to control.

Cytoplasmic Contamination. Our examinations by phase and electron microscopy demonstrated a virtual absence of cytoplasmic organelles and debris in the preparations of detergent-treated nuclei and ghosts from detergent-treated nuclei. However, as a further means of assessing the possible extent of cytoplasmic contamination, several approaches were used. Since HeLa cell nuclei have been shown to contain a very small fraction of the total cellular 18S RNA, it has been suggested that analysis of the RNA species present in nuclear preparations can be used as an indication of purity (Penman, 1969). Sucrose gradient analysis of the RNA species present in both detergent-treated nuclei and total cytoplasm (see Materials and Methods) indicated that less than 1% of the total cellular 18S RNA was present in the preparations of detergent-treated nuclei (Table II).

The virtual absence of mitochondria was demonstrated by the absence of the mitochondrial enzyme, glutamate dehydrogenase. The specific activity of glutamate dehydrogenase in detergent-treated nuclei and their corresponding ghosts was less than 4% (the lower detection limit) of that of the whole cell sonicate (Table II). The low activity of this enzyme in nuclei and ghost preparations was not due to the effects of the brief detergent treatment, since the detergent mixture used did not appreciably affect the activity of the whole cell sonicate, even after a period of 30 hr at 4°.

Finally, contamination by plasma membrane fragments was found to be minimal as monitored by the incorporation of fucose, a specific marker for the HeLa cell plasma membrane (Atkinson and Summers, 1971). The amount of L-[^{3}H]fucose incorporated into various subcellular fractions (see Materials and Methods) after a period of 17 hr indicated that the detergent-washed nuclei and the ghosts from detergent-treated nuclei each contained less than 1% of the total L-[^{3}H]fucose incorporated into cells (Table II).

Chemical Characterization. In order to characterize the composition of the ghosts isolated from nuclei, as well as to determine the effects of the Tween 40–sodium deoxycholate detergent mixture on nuclei, chemical analyses (Materials and Methods) were performed on non-detergent-treated nuclei, detergent-treated nuclei, and the corresponding ghosts. Based on the results shown in Table III, the composition of the ghosts isolated from detergent-treated nuclei is approximately 72% protein, 10% phospholipid, 14% DNA, and 4% RNA (see also Discussion). The phospholipids present in the ghosts from detergent-treated nuclei were analyzed by two-dimensional thin-layer chromatography (Figure 5). The relative proportions of the various phospholipids are

Table III: Chemical Composition of Subnuclear Fractions.[a]

	Phospholipid	Protein	DNA	RNA
Non-detergent-treated nuclei	2.15[b] (100)[c]	23.1 (100)	9.8 (100)	1.2 (100)
Detergent-treated nuclei	0.51 (24)	19.6 (85)	9.6 (98)	0.49 (41)
Detergent supernatant	1.68 (78)	3.2 (14)		0.66 (55)
Ghosts from detergent-treated nuclei	0.37 (17)	2.6 (11)	0.5 (5)	0.14 (12)
Ghosts from non-detergent-treated nuclei	0.64 (30)	2.4 (10)	0.7 (7)	

[a] All values represent the average values obtained from two to four independent preparations. The number of significant digits shown for each value is an indication of the limits of variability. Except for the isolated ghosts, gradient fractions could not be individually analyzed since trichloroacetic acid precipitates of these fractions could not be quantitatively pelleted from sucrose–$MgCl_2$, and collection on filters was unsuitable for chemical analysis (refer to Table I). [b] These values are expressed as micrograms per 10^6 nuclei analyzed. [c] Parentheses indicate the percentage of the values for non-detergent-treated nuclei. However, note that in the text reference is sometimes made for convenience to the percentage of the values for detergent-treated nuclei.

Table IV: Phospholipid Composition of the Ghosts Isolated from Detergent-Treated Nuclei.[a]

Phosphatidylcholine	56.3
Phosphatidic acid	2.9
Phosphatidylethanolamine	6.8
Cardiolipin	3.1
Sphingomyelin	16.5
Unidentified	14.1

[a] Expressed as the percent of total phospholipid phosphorus contributed by each. Values are the average of determinations performed on two independent ghost preparations.

shown in Table IV. Spots B and C seem to represent two distinct species of sphingomyelin since the sphingomyelin standard used covered both of these spots in co-migration studies. Spot A, comprising 14.1% of the total lipid phosphate, did not correspond in migration with any of the standards tested (see Materials and Methods). The phospholipid composition of detergent-washed HeLa nuclei was indistinguishable from the composition shown for the isolated ghosts, indicating that migration on 0.5 *M* $MgCl_2$–sucrose gradients does not have the effect of selectively removing phospholipid species from the ghosts. This finding is consistent with the high recovery in the ghost preparation of the residual phospholipids of detergent-washed nuclei (Table III and Figure 3).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Patterns. The sodium dodecyl sulfate–polyacrylamide gel patterns for the ghosts isolated from detergent-treated nuclei were surprisingly simple, consisting of two major bands, which have apparent molecular weights of 20,000 and 35,000 (Figure 6). The appearance of these two major bands with an apparent absence of significant amounts of higher molecular weight species was reproducible from preparation to preparation, provided that the criteria described above for eliminating the co-purification of dead or damaged cells with ghosts were observed. The sodium dodecyl sulfate–polyacrylamide gel patterns of ghost preparations containing three to four times (still only six to eight nonghost particles per hundred ghosts) the accepted level of contamination were considerably more complex (Figure 6). Ghosts prepared from non-detergent-washed nuclei contained the two prominent low molecular weight bands as well as a number of faint, higher molecular weight bands which were probably due at least in part to ribosomal proteins (Figure 6). Preliminary results (data not shown) indicate that the lower molecular weight band observed on the sodium dodecyl sulfate–polyacrylamide gels can be extracted from the ghosts with 0.2 *N* H_2SO_4 and migrates as a single band coincident with histone F-3 on the 6.25 *M* urea–acrylamide gel system of Panyim and Chalkley (1969).

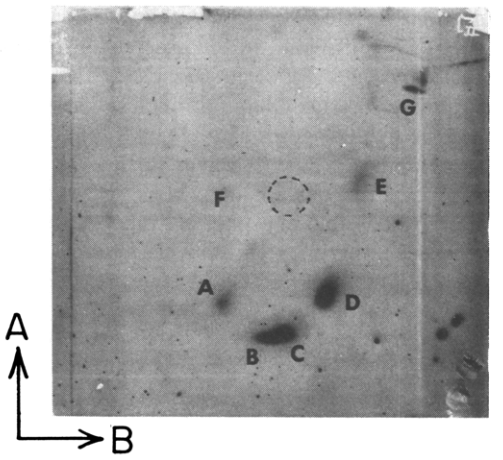

FIGURE 5: Two-dimensional thin-layer chromatogram of phospholipids extracted from the ghosts of detergent-treated nuclei. Development in direction A was with chloroform–methanol–acetic acid–water (52:20:7:3, v/v). Development in direction B was with chloroform–methanol–40% methylamine in water–water (63:35:5:5, v/v). The identities of the spots were as follows: (A) unidentified; (B and C) sphingomyelin; (D) phosphatidylcholine; (E) phosphatidylethanolamine; (F) phosphatidic acid; (G) cardiolipin.

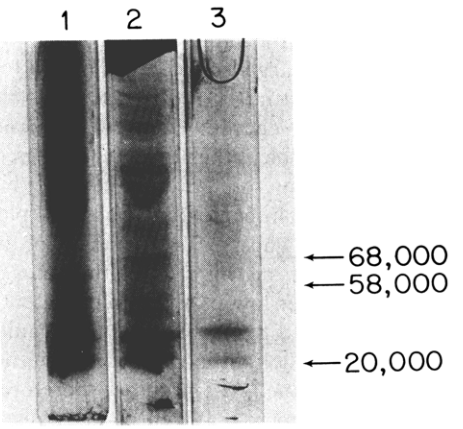

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of nuclear ghost preparations: (1) ghosts from detergent-treated nuclei contaminated by the co-purification of a small number of "dead" cells (see text); (2) nuclear ghosts prepared by fractionating non-detergent-treated nuclei on continuous sucrose–$MgCl_2$ gradients; (3) ghosts obtained by fractionating detergent-treated nuclei on continuous sucrose–$MgCl_2$ gradients. Molecular weight standards used were bovine serum albumin, 68,000; catalase, 58,000; and trypsin, 20,000.

Discussion

The biochemical basis for the dramatic dissolution and reappearance of the nuclear envelope during mitosis of higher eukaryotic cells remains undetermined. Our current approach to the study of this phenomenon is to identify specific cell cycle dependent changes in nuclear envelope components. In this paper, we have described the isolation and characterization of a macromolecular complex from HeLa cell nuclei which retains the nuclear form when viewed by phase contrast microscopy. This retention of form coupled with the loss of the bulk of the nuclear DNA (95%), RNA (71%), and protein (87%) during the preparation of these complexes has led us to refer to these complexes as "nuclear ghosts". The relationship between these ghosts and the intranuclear protein networks reported by others (Smetana et al., 1963; Steele and Busch, 1966; Narayan et al., 1967; Berezney and Coffey, 1974) is uncertain.

The ghosts that we have studied most extensively are derived from detergent-treated nuclei, although we have obtained very similar ghosts from non-detergent-treated nuclei. Our studies by electron microscopy have shown that the isolated non-detergent-treated nuclei are surrounded by both inner and outer nuclear membranes, whereas the detergent-treated nuclei are surrounded by neither, a result in agreement with the ultrastructural data of Holtzman et al. (1966). Despite this lack of organized membrane, the detergent-treated nuclei retain the integrity and appearance of nondetergent nuclei when observed by phase contrast or electron microscopy. These observations suggest that the dense marginal borders (which may represent the dense

lamella) alone or in conjunction with other nonmembranous components are sufficient, in the absence of membrane, for the physical confinement of nuclear DNA and for the maintenance of the spherical shape of nuclei. The structural integrity of the nuclear pores also seems to be independent of a limiting membrane at the nuclear surface.

These interpretations are supported by a calculation that the amount of possible phospholipid bilayer present in comparison with experimental bilayer systems (Engelman, 1969; Levine and Wilkins, 1971) is approximately 1.8 and 0.4 bilayer equivalents per non-detergent- or detergent-treated nucleus, respectively. These results, based on a value of 350 μm^2 for the average surface area of a HeLa cell nucleus (Maul et al., 1973) and our own values of phospholipid per nucleus (Table III), indicate the absence of an intact inner nuclear membrane after detergent treatment. Both our calculations for non-detergent-treated nuclei and the calculations of Gurr et al. (1963) for rat liver nuclei are consistent with the idea that most of the nuclear phopholipid resides at the nuclear surface.

As mentioned previously, when ghosts are isolated from detergent-treated nuclei, the bulk of the residual phospholipid of the detergent-treated nuclei is recovered in the isolated ghosts (Figure 3, Table III). It is interesting that the ghosts from both detergent-treated and non-detergent-treated nuclei band at the same density on the $MgCl_2$–sucrose gradients with a large loss of phospholipid to the loading zone of the gradient in the latter case. This observation suggests that there is a characteristic complement of phospholipid that is removable either by detergent or, to a large extent, by the $MgCl_2$–sucrose gradient itself. In either case, however, a residual complement of phospholipid remains tightly bound to the ghosts. The absence of observable membranes in electron micrographs of the detergent-treated nuclei and the rather low phospholipid content (10%) of the ghosts isolated from detergent-treated nuclei both suggest that the phospholipid in these isolated ghosts is not part of a unit membrane.[4] We propose, therefore, that the phospholipid of the ghosts may represent remnants of the nuclear membrane present as boundary lipid (Jost et al., 1973) attached to ghost macromolecules which were originally in close association with the inner membrane of intact nuclei.

The nuclear ghosts isolated from detergent-treated nuclei are composed primarily (72%) of protein which has been shown by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels to consist of two major components with molecular weights of about 35,000 and 20,000. Whether there are multiple polypeptide species within either of these bands is presently unknown. The fact that the two major protein species present are the same for the ghosts prepared from either non-detergent- or detergent-treated nuclei strongly suggests that the detergent mixture used in this preparation has not in itself altered the ghost composition by gross removal of inherent proteins or by causing the adsorption of extraneous ones.

It seems possible that much of the protein found in association with the isolated ghosts serves a structural role in the numerous pores present on nuclei (4000 per HeLa cell nucleus; Maul et al., 1973) as well as in the dense material in which pores are embedded. These possibilities are presently being investigated.[4,5] In addition, we are examining possible roles of the ghost proteins in the cyclic dissolution and re-formation of the nuclear envelope during the mitotic process in higher eukaryotic cells. The fact that mitosis occurs in the absence of the nuclear envelope could permit the incorporation of these same proteins in alternative loci in the mitotic apparatus. Such re-utilization might provide a mechanism preventing the mutual presence of the mitotic apparatus and nuclear envelope at the same phase of the cell cycle. In any case, the reassembly of the nuclear envelope itself at the end of mitosis may well be controlled by alterations of its constituent macromolecules. We are currently testing the hypothesis that the assembly and disassembly of this structure are accompanied by covalent modification (e.g., phosphorylation) of one or more of the ghost proteins detected in the present study.

With regard to the finding of DNA associated with the ghosts of this preparation and in reported preparations of nuclear envelope, it should be noted that a characteristic type of DNA may well prove to be bound to the nuclear envelope. Observations by electron microscopy suggest firm attachments between nuclear envelopes and chromatin fibers in a variety of species (e.g., Dupraw, 1965; Beams and Mueller, 1970; Comings and Okada, 1970). Moreover, it has been shown in one system (Agutter, 1972) that reagents or procedures which lead to extensive loss of DNA from the nuclear envelope also tend to disrupt pore and envelope integrity. We suggest that the ghosts that we isolate from cultured animal cells by the described procedure may be useful for characterizing the composition and cell cycle behavior of DNA associated with the nuclear envelope.

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Added in Proof

Our recent studies indicate that the polyacrylamide gel pattern reported for the isolated ghost proteins (Figure 6) is incorrect. We have recently taken special precautions to desalt the isolated ghosts before solubilization in the sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Under these conditions we find that the ghost polypeptides are composed of three major bands of between 65,000 and 68,000 in molecular weight, a low molecular weight band which probably corresponds to histone, and several minor bands of intermediate molecular weight. The triplet seen in the 65,000–68,000 molecular weight region is consistent with the gel patterns reported for an isolated lamina–pore complex (Aaronson and Blobel, 1975) and for isolated envelope–matrix (Berezney and Coffey, 1974). We believe that our failure to remove excess salt from the TKM washes of the isolated nuclear ghosts led to anomalous migration of the ghost polypeptides reported in the present study.

[4] Ultrastructural studies of the isolated ghosts have been unsuccessful since conventional methods of sample preparation have failed to preserve the ghost integrity. Trilaminar unit membranes were not observed in the ghost preparations, even though the methods of fixation and staining used were known to preserve membranes. Attempts to identify pores in the ghost preparations have been inconclusive, but suggestive structures have been observed. This work is still in progess.

[5] The isolation from rat liver of a proteinaceous dense lamella with associated pore complexes has been reported (Aaronson and Blobel, 1974b).

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