Reports

Nuclear Ghosts: A Nonmembranous Structural Component of Mammalian Cell Nuclei

Abstract. *Electron micrographs of positively stained preparations of nonmembranous ghosts prepared from HeLa cell nuclei have revealed the presence of an array consisting of rodlike and annular structures interconnected by strands sensitive to deoxyribonuclease. This array is believed to be responsible for the spherical shape of nuclei that are free of membrane. In addition, a configurational change in this array may be associated with the cyclic dissolution and reformation of the nuclear envelope that accompanies mitosis in mammalian cells.*

Interphase nuclei of mammalian cells retain their characteristic spherical shape despite treatment with detergents, which remove detectable membrane and most or all of the membrane lipid (*1–4*). This observation leads to the conclusion that neither inner nor outer nuclear membrane is responsible for the spherical shape of interphase nuclei. Candidates for the structural component that determines nuclear shape have been isolated and variously called the pore complex-lamina (*2*), the nuclear protein matrix (*3*), and the (nonmembranous) nuclear ghost (*4*). Although some of the steps used in the isolation of these three structures are similar (for example, high concentrations of cations), the procedure used to isolate nuclear ghosts (*4*) does not include a deoxyribonuclease treatment, whereas this enzyme was used in the isolation of the pore complex lamina (*2*) and the nuclear protein matrix (*3*). Nevertheless, we have shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis that the three structures mentioned above, when isolated from HeLa cells in our laboratory, have in common at least three major protein bands, which have approximate molecular weights of 67,000, 60,000, and 56,000 (*5*). From this observation, we have concluded that these three nuclear structures are derived, at least in part, from the same component of the original nucleus (*5*).

The nuclear ghosts, isolated from interphase HeLa cells as described (*4*), appear as flat disks when viewed by scanning electron microscopy (*5*). When viewed by transmission electron microscopy, each ghost appears to consist of an array of annular and rodlike structures interconnected by thin strands (Figs. 1 and 2). The intricate nature of this array is apparent in an intact HeLa cell nuclear ghost (Fig. 1). We have not yet been able to determine whether this array is derived solely from material at the nuclear periphery, such as might underlie or partially constitute the inner nuclear membrane, or whether it also extends through the nucleus (*6*), as has been suggested for the nuclear protein matrix (*3*). The diameter of the isolated membrane-free ghost is about 30 μm, which is about four times that of an isolated HeLa cell nucleus (*5*). This increase in diameter may result from the relaxation of the nuclear surface (and possibly internal components as well) brought about by the loss of large amounts of both membrane and nonmembranous nuclear material (DNA, RNA, protein, and lipid) during the isolation procedure (*4*). Although the basis of this proposed relaxation is unknown, the virtual absence of histones in the isolated ghosts (*5*) suggests that the expanded structure may result from unpackaged DNA (*7*).

More detailed information regarding the component parts of this nuclear ghost and their arrangement has been obtained from chemical studies (*4, 5*) and from ultrastructural studies (*8*). The nuclear ghosts consist of 72 percent protein, 14 percent DNA, 10 percent phospholipid, and 4 percent RNA (*4*). The protein consists of six major bands: the three mentioned above, plus proteins with molecular weights of 57,500, 51,000, and 46,000 (*5*). The ultrastructural features of the isolated ghosts as viewed by transmission electron microscopy (Fig. 2) are similar to structures observed in preparations that control for the possibility of artifacts induced by the isolation procedure (*8*). The annular structures (arrows, Fig. 2) have outside and inside diameters of 90 and 43 nm, respectively. In addition, the annular structures are present at a minimum density of about 3.4 per square micrometer, when corrected for the difference in the surface area between isolated ghosts and intact nuclei (*9*). Since these dimensions are very similar to those of HeLa cell nuclear pores (*4*), and since the density of annuli is similar to that of pores on HeLa

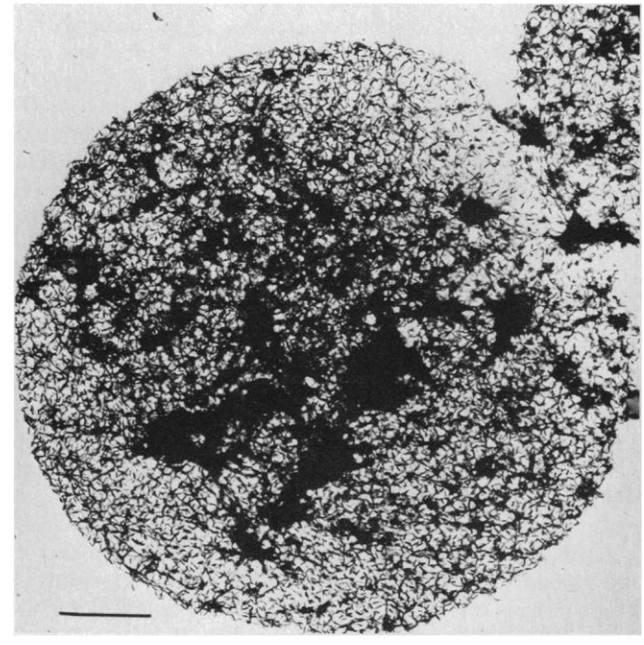
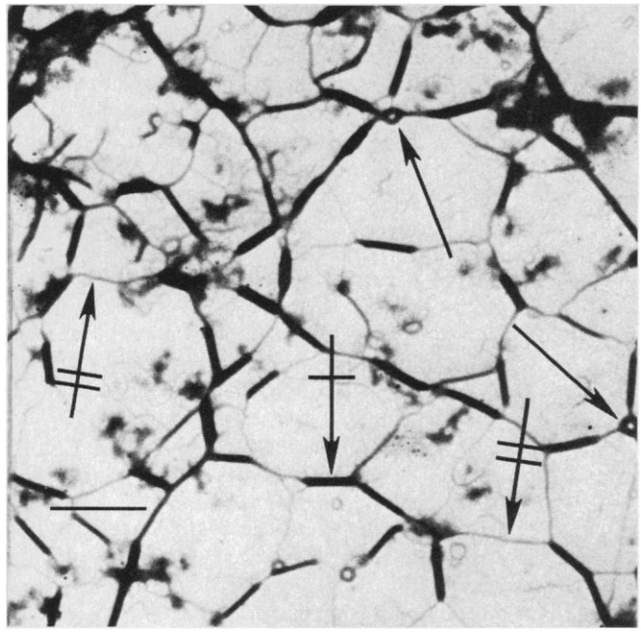


Fig. 1. Intact HeLa cell nuclear ghost as viewed by transmission electron microscopy. Ghosts were attached in whole mount fashion to a poly-DL-lysine layer on electron microscope grids coated with Formvar and carbon. The mounted ghosts were stained with 1 percent uranyl acetate and air-dried. Scale bar, 4 μm.



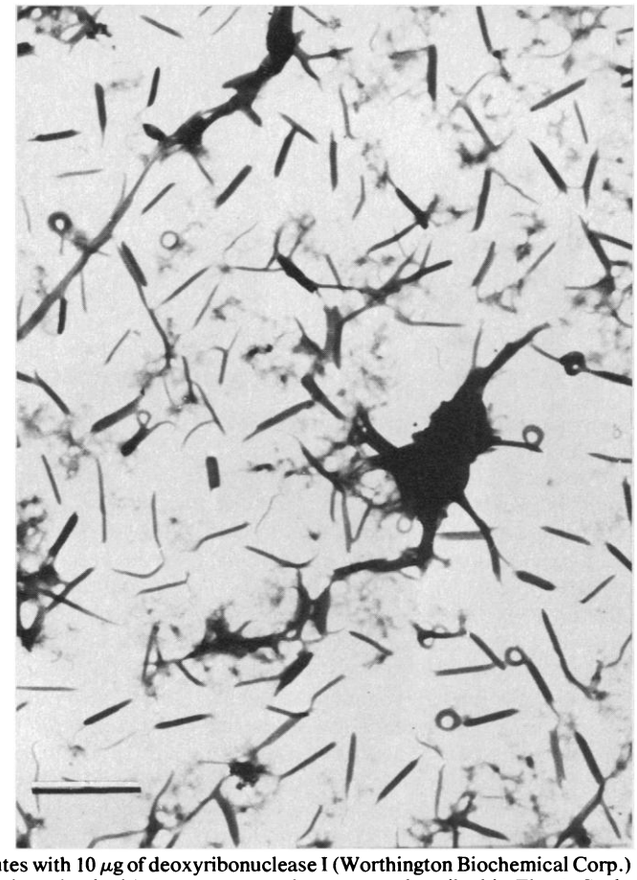


Fig. 2 (left). A transmission electron micrograph of nonmembranous HeLa cell nuclear ghosts (methods as described in Fig. 1). Arrows indicate annuli, which have an outside diameter of about 900 nm. Single-barred arrows indicate rodlike structures that are approximately 260 nm in length and 50 nm in diameter. Double-barred arrows indicate the thin strands that are sensitive to deoxyribonuclease (see Fig. 3). Scale bar, 500 nm. Fig. 3 (right). A transmission electron micrograph of nonmembranous HeLa cell nuclear ghosts treated with deoxyribonuclease I. Ghosts, previously attached with polylysine to electron microscope grids coated with carbon and Formvar, were treated for 10 minutes with 10 μg of deoxyribonuclease I (Worthington Biochemical Corp.) per milliliter. The deoxyribonuclease was removed and the ghosts positively stained with 1 percent uranyl acetate as described in Fig. 1. Scale bar, 500 nm.

cell nuclei [7 to 13 per square micrometer (*9*)], the annular structures might be thought of as nuclear pores. However, our failure to observe the eightfold symmetry characteristic of nuclear pores (*4*, *10*, *11*) prevents us from making a firm identification.

In addition to the annular structures, the array contains rodlike structures (single-barred arrows, Fig. 2) that are about 260 nm long and 50 nm in diameter. To our knowledge, similar structures in nuclei have not been reported previously. The nonrandom arrangement of these rodlike structures (*8*) in the nuclear array appears to indicate the existence of a heretofore unrecognized structure in the nucleus (*12*). The thin strands (double-barred arrows, Fig. 2) that interconnect the annular and rodlike structures are believed to contain DNA, since deoxyribonuclease treatment of mounted ghost results in the disappearance of these strands (Fig. 3). Treatment of ghosts results in the disappearance of ately prior to attachment to the grid results in the loss of the organized arrangement of the rodlike structures (*8*). It is not known if the DNA in the array is distinct from the bulk DNA (95 percent of the total) that is removed from nuclei during the preparation of nuclear ghosts (*4*) or if the DNA extends through the rodlike structures. However, the importance of DNA to the integrity of ghosts is emphasized by the fact that ghosts disintegrate and cannot be isolated if preparations are treated with deoxyribonuclease prior to the final purification on sucrose gradients (*5*).

Since the morphological integrity of nuclei is dependent on nonmembranous structures (*1–4*) such as the nuclear ghost described here, we suggest that the cyclic dissolution and reformation of the nuclear envelope that occurs during mitosis in higher eukaryotes [for reviews, see (*10–11*)] is associated with changes in the in vivo form of the nuclear ghost (*13*). The association of nuclear pores and membrane with mitotic chromosomes has been reported [for reviews, see (*11*)]. In addition, attachment of DNA to the interphase nuclear envelope has been observed (*14*). These observations suggest that at least three components associated with nuclear envelopes (nuclear pores, membrane, and DNA) can also be associated with the mitotic apparatus. The fact that DNA is an integral part of the isolated interphase nuclear ghost and that condensation of DNA occurs at mitosis suggests that chromosomal condensation may be responsible for the apparent dissolution of the nuclear envelope at mitosis. As mitosis is completed and chromosomes are decondensed, the interphase nuclear ghost would reform and the dispersed nuclear membranes would reassemble on this framework. The specific trigger (*15*) that might stimulate such a reversible change is unknown. Histones have been found to be modified very early in mitosis (*16*), and such modifications are believed to affect the interactions between histones and DNA [for review, see (*17*)]. Whatever the signal for chromosomal condensation, the DNA that maintains the integrity of the ghosts is likely to be incorporated into compact chromosomes during mitosis. This packaging would result in a dramatic distortion of a nuclear framework represented by the ghosts. This distortion, caused by incorporation of the framework DNA and associated structures into compact chromosomes, may be the cause of the nuclear envelope dissolution that occurs during mitosis in cells from higher eukaryotes.

JOHN M. KELLER, DONALD E. RILEY
Department of Biochemistry,
University of Washington, Seattle 98195

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6. Analysis of thin sections of ghosts embedded by conventional methods has revealed the presence of structures with dimensions consistent with those of the annuli, rods, and connecting strands described in the text. However, the spherical integrity of the ghosts is lost during these procedures.
7. At least part of this relaxation (expansion) could result from the release of proteins from nucleosomes, which has been shown to result in a five- to sevenfold increase in the contour length of DNA [J. D. Griffith, *Science* **187**, 1202 (1975); P. Oudet, M. Gross-Bellard, P. Chambon, *Cell* **4**, 281 (1975); C. Cremisi, P. P. Pignatti, O. Croissant, M. Yaniv, *J. Virol.* **17**, 204 (1976)].
8. The possibility that the structure of the nuclear ghost is an artifact produced by the isolation procedure is considered very unlikely for the following reasons. Similar structures are observed when ghosts are (i) critical point dried, (ii) dried on grids coated with cytochrome c, (iii) prepared in the presence of a different detergent (Triton X-100), (iv) prepared in the absence of detergent, or (v) prepared in the absence of high ionic strength (that is, the omission of 0.5*M* $MgCl_2$). In addition, nuclear ghosts isolated from synchronized HeLa cells show distinct morphological properties that are characteristic for a particular phase of the cell cycle. We believe that the changes dependent on the cell cycle would not be observable if the ghost components did not reflect inherent arrangements of nuclear structures (D. E. Riley and J. M. Keller, in preparation; J. M. Keller and D. E. Riley, in preparation).
9. The minimum density of annuli per square micrometer was determined from an enlarged copy of Fig. 1. Two of our colleagues counted the number of annuli in 20 unobscured fields, each representing 4 μm^2. The average value of these counts (1.73 and 1.6) was 0.42 annuli per square micrometer. The density of annuli on an intact nucleus before expansion was calculated with the assumptions that (i) both sides of the flattened ghost were observed and (ii) as a result of the approximate fourfold enlargement in the diameter of the nuclear ghost compared to an intact nucleus, the surface area of the ghost is about 16 times that of an intact nucleus. We believe that the final value represents a minimum density and is in good agreement with the average reported pore density on HeLa cell nuclei [H. W. Fisher and T. W. Cooper, *Exp. Cell Res.* **48**, 620 (1967); G. G. Maul, H. M. Maul, J. E. Scogna, M. W. Lieberman, G. S. Stein, B. Y.-L. Hsu, T. W. Borun, *J. Cell Biol.* **55**, 433 (1972)].
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12. Doughnut and stemlike structures have been found to form as a result of interactions between DNA and polylysine [M. Haynes, R. A. Garrett, W. B. Gratzer, *Biochemistry* **9**, 4410 (1970); D. E. Olins and A. L. Olins, *J. Mol. Biol.* **57**, 437 (1971); U. K. Laemmli, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4288 (1975)]. We believe that it is very unlikely that the structures that we observe arise by an interaction between the DNA in the ghost preparation and the polylysine on the electron microscope grid. Control experiments with purified calf thymus DNA attached to grids by our method fail to show the doughnut and stemlike structures. In addition, the morphology of ghosts is unchanged if the ghosts are attached to grids coated with cytochrome c [see (*8*)]. Finally, mild deoxyribonuclease I treatment prior to ghost attachment results in disruption of the ghosts, but annular and rodlike structures are still observed.
13. This interpretation is consistent with our preliminary observations that the specific activities of the polypeptides in the ghosts are constant through mitosis.
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18. This work was supported by NSF grant BMS 75-02467 and NIH grant CA-16902. J.M.K. is an established investigator of the American Heart Association. We thank many friends for valuable criticism.

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Interkingdom Fusion Between Human (HeLa) Cells and Tobacco Hybrid (GGLL) Protoplasts

Abstract. *The fusion of human HeLa cells with tobacco protoplasts has been accomplished with the use of polyethylene glycol. The sequence from heterocellular adherence to heterokaryon formation has been followed with light microscopy and confirmed by autoradiographs of heterokaryons containing unlabeled tobacco nuclei and tritium-labeled HeLa nuclei. The HeLa nucleus retained its integrity in the tobacco cytoplasm up to 6 days after fusion.*

Protoplasts of a genetically tumor-prone plant hybrid amphiploid, *Nicotiana glauca* Grah. (GG) × *N. langsdorffii* Weinm. (LL), were prepared by enzyme digestion of the cell wall and were fused with suspended human tumor-derived HeLa S3 cells in the presence of polyethylene glycol (PEG). The HeLa nucleus was observed inside the plant protoplast within 3 hours of incubation after treatment, thus forming a heteronucleate interkingdom somatic cell fusion product.

Since the tobacco (GGLL) tumor cells are much larger (Fig. 1) than the HeLa cells and grow rapidly under less stringent cultural conditions than normal plant cells (*1*), our strategy was to utilize the plant protoplasts as recipient carriers of the human tumor cell nuclei. Protoplasts of the hybrid GGLL were prepared from young leaves according to the method of Kao *et al.* (*2*) as modified by one of us (H.Z.L.). Abundant protoplasts were released after 4 to 5 hours in a 1:1 mixture of Kao's M3 culture medium and E1 enzyme solution. In addition to Murashige-Skoog inorganic salts (*3*), iron, micronutrients, and vitamins, the M3 culture medium contained 2,4-dichlorophenoxyacetic acid (2, 4-D), $4.52 \times 10^{-7}M$; N^6-benzyladenine, $4.44 \times 10^{-7}M$; naphthaleneacetic acid, $5.37 \times 10^{-6}M$; N-Z amine, 250 mg/liter (type AS, Sheffield Chemical); glucose, 0.38*M*; xylose, $1.66 \times 10^{-3}M$; and coconut milk, 20 ml/liter. The enzyme solution contained 2 percent cellulase, 2 percent hemicellulase Rhozyme (Rohm & Haas), and 1 percent pectinase (Sigma), all desalted. The protoplasts were washed free of enzyme and suspended in M3 medium only, and were then ready for fusion.

The HeLa S3 cells were grown in log phase in suspension culture in Eagle's (*4*) minimum essential medium (MEM) with Earle's salts (Grand Island Biological). Additions to this medium gave a final concentration of 7 percent fetal calf serum, 2 m*M* L-glutamine, and 50 units of a mixture of penicillin and streptomycin per milliliter. The cultural conditions of *p*H, osmolarity, and temperature for HeLa cells are 7.3, 283 milliosmols, and 37°C, respectively; and for tobacco protoplasts are 5.7, 500 milliosmols, and 27°C, respectively.

Preparatory to fusion, the HeLa cells were centrifuged (1000 rev/min) and were then resuspended in MEM to give a concentration of about 10^7 cells per milliliter. A comparable volume of GGLL protoplasts suspended in M3 solution was used. Five or six drops of PEG solution were cautiously added to 1 ml of the mixed cell culture in order to encourage fusion. The composition of the solution (*p*H 5.5) was: PEG 6000, 0.09*M*; glucose, 0.25*M*; $CaCl_2 \cdot 2H_2O$, 10.5 m*M*; and $KH_2PO_4 \cdot H_2O$, 0.7 m*M*.

Adherence of membranes began immediately, HeLa with HeLa, GGLL with GGLL, and HeLa with GGLL to form associations of two or more cells. Incubation in the PEG solution was continued for 40 to 50 minutes. The preparation was then eluted, first with two washings in the high *p*H–high Ca^{2+} W3 solution of Kao *et al.* (*2*) and subsequently with at least five washings in culture medium M3 until the excess $CaCl_2$ was removed. The cell mixture was then maintained in drop or centifuge-tube culture at room temperature (25° to 27°C) and low light (55 to 550 lu/m²). It was sampled and