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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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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

fixed at periods ranging from 1 hour to 6 days. The samplings were stained with carbol fuchsin (5), a nuclear stain which effectively differentiates HeLa nuclei from tobacco nuclei (Fig. 1, A and B), and were examined as liquid mounts.

In samples taken after 1 hour of incubation, following removal of the PEG solution, one or more HeLa cells were observed firmly attached to, but exterior to, GGLL protoplasts (Fig. 1A). Similar instances were found for up to 4 days. After 3 hours and up to 6 days, careful focus revealed a number of examples of one HeLa nucleus inside the tobacco protoplast (Fig. 1B). The frequency of heterofusions, in cultures incubated for 18 hours after PEG treatment, was about 0.2 percent, as determined by the number of GGLL protoplasts observed to contain a HeLa cell nucleus. At this time many examples were seen of continuity between the outer membranes of HeLa cell and protoplast (Fig. 1C) concomitant with dissolution of the intervening membranes. Suspensions prepared with four to ten times more of the smaller HeLa cells, relative to GGLL protoplasts, yielded the most heterokaryons. In 4 to 6 days a number of tobacco cells were found with two GGLL nuclei and some with two GGLL nuclei plus one HeLa nucleus (Fig. 1D). The presence of the three nuclei may have been due to fusions involving more than one protoplast with a HeLa cell or, more likely, because of the relative scarcity of trinucleate cells in early samplings, to a GGLL nuclear division after fusion.

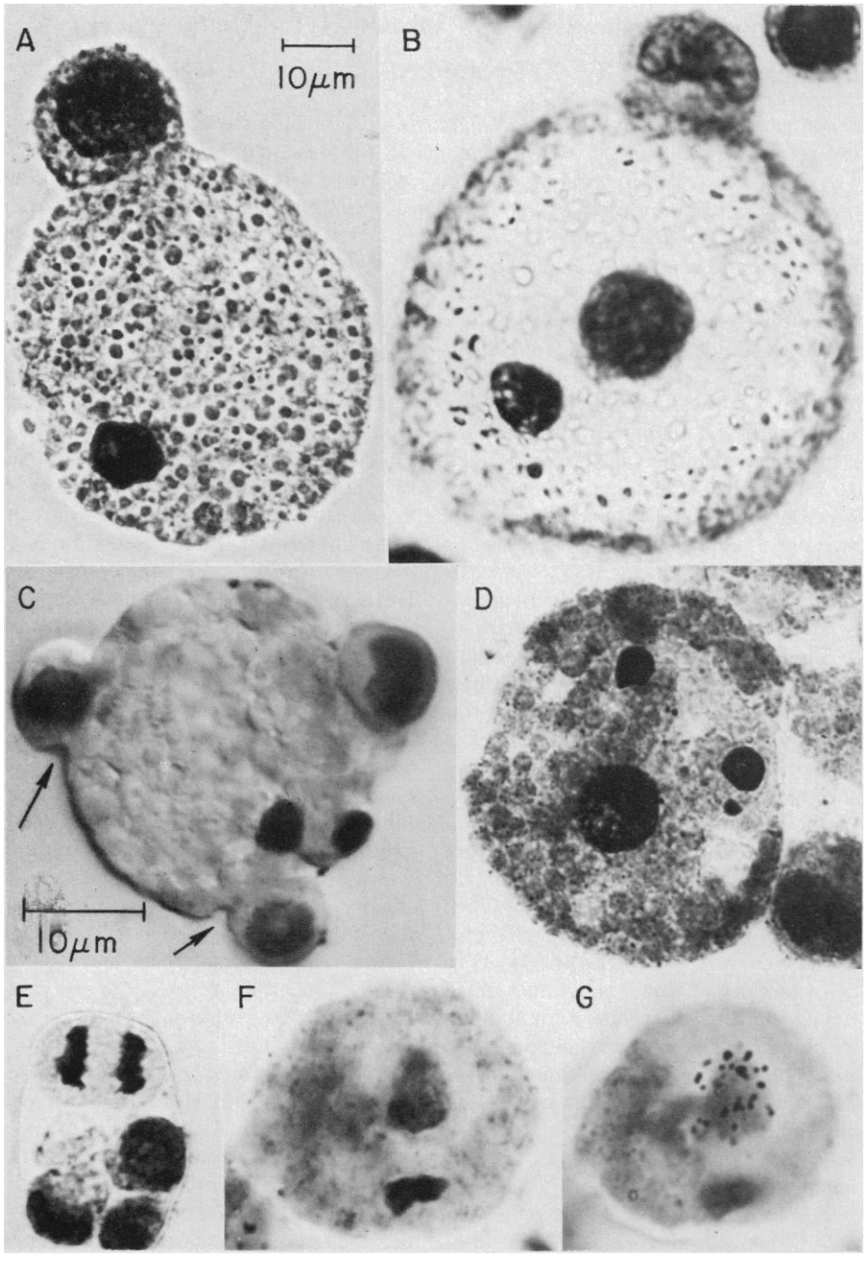

Fig. 1. Cultured human-derived HeLa cells and tobacco hybrid (GGLL) protoplasts. (A) HeLa cell adhered to surface of GGLL protoplast 1 hour after treatment with PEG. (B) Heterokaryon with HeLa nucleus (the larger) inside tobacco protoplast, 3 hours after fusion. (C) Multiple fusion of two separate HeLa nuclei (arrows) with tobacco protoplast, showing continuity of membranes 18 hours after fusion. (D) Tobacco cell with two GGLL nuclei (the smaller, darker) and one HeLa nucleus, 6 days after fusion. (E) Division figure of HeLa nucleus after 6 days in M3 medium. (F) and (G) Autoradiograph 18 hours after fusion showing heterokaryon with GGLL nucleus unlabeled (F), and silver grains over the tritium-labeled HeLa nucleus (G).[(A), (B), (D), and (E): × 800; (C), (F), and (G): × 1600]

In the liquid mounts the cover glass was spaced from the slide with Bio-Gel P-6 (Bio-Rad) so that the cells remained spherical. Under these conditions, where no intervening membranes were seen at a series of focal planes, there is little likelihood that the HeLa nuclei in question could have been merely superimposed, rather than contained within, the tobacco protoplasts. Furthermore, the carbol fuchsin stain delineated only whole HeLa cells free in the liquid, whereas within the protoplasts only HeLa nuclei were observed (Fig. 1, B and D).

Cytological evidence for heterofusion was confirmed by labeling the HeLa nucleus with tritiated thymidine. To an exponentially growing HeLa cell culture 0.2 μc of [^{3}H]thymidine per milliliter (specific activity, 6 c/mmole) was added and the cells were incubated for 24 hours to allow for incorporation of the radioactive label. The cells were then washed four times in MEM and suspended in M3 culture medium. Eighteen hours after fusion of the labeled HeLa cells with GGLL protoplasts the preparation was fixed in a solution of acetic acid, ethanol, and sorbitol, hydrolyzed for 9 minutes at 60°C, and stained with Feulgen. The stained cell samples were air dried onto slides, dipped in nuclear track emulsion (one part distilled H_2O and one part NTB_2), and exposed for 2 days. This process caused some shrinkage (Fig. 1, compare F and G with A and B). Autoradiographs were obtained (Fig. 1, F and G) that clearly showed within the tobacco protoplast an extra nucleus that was labeled and thus was identified as of HeLa cell origin.

It may not be unrealistic to expect viable products from interkingdom fusion in view of the following considerations. (i) Polyethylene-induced fusion of hen erythrocytes with yeast protoplasts was reported recently (6). (ii) Widely disparate forms may already comprise an integral part of the normal eukaryote cell. According to the symbiotic theory of the origin of eukaryotes (7), similarities between certain organelles and prokary-

otes suggest that mitochondria and chloroplasts arose, and reside in eukaryote cells, as symbionts with origins related to those of present-day bacteria and blue-green algae, respectively. (iii) We have observed what appears to be a normal HeLa nuclear division stage (Fig. 1E) after 6 days of growth in M3, a medium that was devised specifically for plant protoplast culture. The HeLa cells apparently can tolerate a range in cultural conditions, and a cell line is now under selection for growth in a mixed medium also adapted for protoplast maintenance.

If the fused HeLa-GGLL cells remain viable, a number of new avenues of research are opened. With the use of various immunological tools (for example, fluorescence antibody labeling and immune precipitation) it should be possible to answer questions about the continued synthesis of both tobacco cell and HeLa cell products, as well as the intracellular distribution of those products having specific intracellular locations. If nuclear and cell divisions occur in the fused cells and are accompanied by loss of human chromosomes, as in man-mouse somatic hybrids, it may be possible to extend the number of human genes located by association of particular chromosomes with biochemical products more likely to be uncharacteristic of the widely disparate plant host cell, provided that the necessary regulatory components are present.

A number of differences have been observed between normal and tumorous or transformed cells of both plant and human tissues. These include membrane differences (*8*), differences in rate of uptake and transport of certain solutes (*9*), and changes in the organization of membrane-associated components (*10*). Since fusion is essentially a membrane phenomenon, the altered membrane condition of the tumorous cells used in our experiments may have influenced, and possibly aided, in the fusion process.

Lastly, we call attention to the fact that the *N. glauca* × *N. langsdorffii* tumor cells are totipotent. Complete mature hybrid tobacco plants can be routinely differentiated from single GGLL tumor protoplasts. The consequences of introducing a human tumor cell, which has not been shown to be capable of differentiation, into a plant cell that retains this capability remains to be explored.

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Thyrotropin Releasing Hormone: Development of Inactivation System During Maturation of the Rat

Abstract. *Whereas thyrotropin releasing hormone is rapidly and extensively degraded by plasma of adult rats, no appreciable loss of biological or immunological activity is caused by plasma from rats 4 or 16 days old. The plasma of neonatal rats does not appear to contain an inhibitor of thyrotropin releasing hormone peptidase or a peptidase with altered substrate affinity. The development of an active peptidase in rat plasma suggests a physiological role for inactivation of thyrotropin releasing hormone.*

Hypothalamic releasing hormones act directly on the brain in addition to their well-established endocrine functions of releasing pituitary hormones. For example, thyrotropin releasing hormone (TRH) elicits behavioral changes—including mood-elevating effects in some depressed women (*1*), neurochemical responses in brain tissue (*2*), electrophysiological alterations in neurons (*3*), and neuropharmacological effects in counteracting phenobarbital toxicity (*4*)—in addition to releasing thyrotropin (TSH) and prolactin from the pituitary (*5*). The hypothesis that hypothalamic releasing hormones have neurotropic functions has been strengthened by the finding that TRH is present in extrahypothalamic regions of the brain (*6*).

In order to understand the extent and duration of the neurotropic and endocrine activities of hypothalamic releasing hormones and the manner in which these activities are regulated, the process of inactivation must be considered. Although active TRH degradation systems have been reported in adult plasma (*7, 8*) and brain tissue (*9*), the role of degradation in controlling TRH activity has not been clearly elucidated. However, the rapid degradation of TRH by plasma may account for the brevity of the antidepressant effects of TRH in women and for some of the controversy surrounding these effects (*1, 10*).

In an effort to explore the possible role of degradation in the regulation of TRH activity, we investigated the degradation of TRH by rats during the postnatal period, a time in which thyroid hormone is vital for the normal development of the central nervous system (*11*). Here we present evidence that the plasma of neonatal rats does not actively degrade TRH whereas that of adult rats rapidly inactivates TRH.

The biological and immunological activity of TRH was measured following incubations of TRH with plasma from rats 4 days, 16 days, and about 1 year old. Immunoreactive TRH was measured by a radioimmunoassay for TRH similar to that of Bassiri and Utiger (*12*). (Modifications of the assay are given in the legend to Fig. 1.) The sensitivity of our assay is 20 pg of TRH in a 300-μl assay system. Some likely TRH degradation products such as the free acid form of TRH (pglu-his-proOH), pglu-his, proNH$_2$, pglu (*13*), histidine, and proline do not cross-react with our TRH antibody.

The plasma of adult rats rapidly destroys the immunological activity of TRH (Fig. 1). The half-life of TRH (about 30 minutes) is in general agreement with that reported by others (*14*) if differences in plasma and TRH concentrations are considered. The striking feature of Fig. 1 is the inability of the plasma of 16-day-old rats to degrade TRH. Even after a 3-