Centromere: Absence of DNA Replication during Chromatid Separation in Human Fibroblasts

Abstract. *Cultures of human fibroblasts were labeled briefly with tritiated thymidine and fixed; autoradiographs were made and exposed for 3½ months. No labeling was noted over the centromere of metaphase or anaphase chromosomes. The technique was sensitive to replication at the centromere of a DNA helix only 2.5 microns long, considerably shorter than the estimated length of a replicon in humans. This suggests that chromatid separation during mitosis is not associated with delayed replication of a short segment of chromosomal DNA.*

DuPraw (*1*) has recently proposed a folded-fiber model of chromosome structure based on the chromatid as a single DNA helix. A possible corollary of this model was that the centromere may represent a "short section of unreplicated DNA which could be partly or entirely responsible for keeping the chromatids together until anaphase" (*1*). Consistent with this was the observation that by electron microscopy the centromere was composed of fibers indistinguishable from those of the chromosome proper. Although numerous studies have demonstrated the absence of a significant amount of DNA synthesis during mitosis, unless such studies are performed under the following conditions, minute segments of DNA replicating only at the time of chromatid separation could easily be missed: (i) in the absence of colchicine, (ii) with high concentration of a specific DNA label, (iii) with relatively brief periods of labeling followed by immediate fixation, (iv) with tissues allowing excellent mitotic spreads, (v) with particular attention to the centromeres of late metaphase and anaphase, and (vi) with prolonged exposure of the autoradiograms. These conditions were incorporated into my investigation of the possibility that chromatid separation may be accompanied by replication of a short segment of DNA, maybe only one replicon (*2*) in length, located at the centromere.

Dermal fibroblasts from a normal human female and an XXXXY individual were cultured on glass cover slips in Eagle's medium with 10 percent fetal calf serum, L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin and streptomycin. Both types of fibroblasts had been in culture less than 2 months and had been transferred fewer than eight times. Two days after transfer with trypsin, during the rapid phase of growth, 10 μc of tritiated thymidine (New England Nuclear, specific activity > 10 c/mmole) per milliliter was added to the cultures. After 15 minutes of incubation, cover slips were removed and placed in a prewarmed (37°C) hypotonic solution of culture medium and distilled water, 1 : 6. They were incubated for 25 minutes, and several milliliters of a mixture of glacial acetic acid and methanol (1 : 3) were added to the hypotonic solution for 1 minute. Cover slips were then placed in this mixture of glacial acetic acid and methanol for 1 hour, air dried, stained in 1.5 percent aceto-orecin, and mounted on glass slides. Slides were coated with Kodak AR 10 fine-grain stripping film, exposed for 22 and 112 days, and developed in Kodak 19b developer. Photographs were taken by phase microscopy with a Zeiss photomicroscope.

After exposure for 112 days, 100 metaphase and 50 anaphase mitoses were carefully examined; the XXXXY culture gave the best mitotic spreads. None showed a grain count greater than the background or a greater-than-random tendency to have grains over the centromere (Fig. 1). Nuclei that were synthesizing DNA at the time of labeling were very heavily labeled (Fig. 2). Grain counts from projected photographs of 37 cells labeled for only 22 days showed an average of 32.9 (S.D. 8.4) grains per cell per day. With correction for latent image fading (*3*), in 112 days this would approximate 3100 grains per cell for cells in the S period at the time of labeling. Since the S period in these human fibroblasts was 7.5 hours in duration (*4*), this would represent 15 min/7.5 hr or 3.33 percent of the total nuclear DNA. On the basis of an average nuclear DNA content of 5.6×10^{-12} g (*5*) this would represent 1.87×10^{-13} g of DNA. With the low background that was present, specific labeling of the centromere could have been readily detected if approximately six of the chromosomes in a given mitosis had a grain over the centromere. My experiment was thus sensitive to the replication at each centromere (*6*) of $6/3100 \times 1.87 \times 10^{-13} \div 46$, or 7.9×10^{-18} g of DNA. With linear density of 3.17×10^{-22} g/Å (*4*), this would represent an extended single DNA helix 2.5 μ in length.

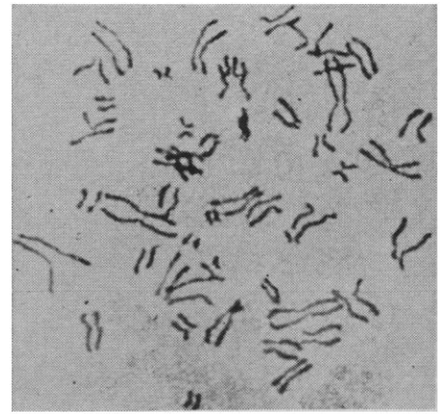

Fig. 1. Autoradiogram of an early anaphase mitosis of a human fibroblast labeled for 15 minutes with 10 μc of tritiated thymidine per milliliter and exposed for 112 days. There are no grains present at the centromeres, or elsewhere, in greater-than-background concentration.

These results suggest that if there is replication of a short segment of DNA at the centromere during anaphase separation of sister chromatids and if its base composition is comparable to that of the DNA of the whole cell, the segment must have a length of 2.5 μ or less. This is considerably shorter than the estimates of average length of a replicon in human tissues of 48 μ (*4, 7*) or longer (*1, 9*). If one assumes that a replicon is the smallest unit of DNA replication and that once a replicon begins DNA synthesis it continues to completion, this suggests that chromatid separation during mitosis is not associated with the delayed replication of a short segment of DNA. Exis-

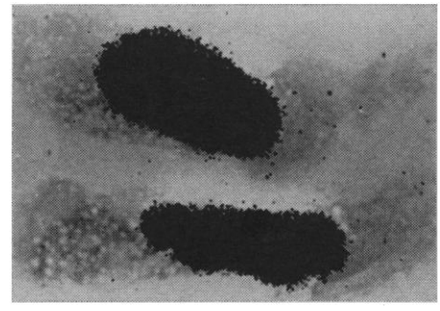

Fig. 2. Autoradiogram of two human fibroblasts labeled during the S phase with 10 μc of tritiated thymidine per milliliter for 15 minutes and exposed for 112 days. Nuclei are densely covered with grains.

tence of a replicon at the centromere with an abnormal base ratio, or one shorter than 2.5 μ, is not excluded.

DAVID E. COMINGS
Department of Medical Genetics, City of Hope Medical Center, Duarte, California

References and Notes

1. E. J. DuPraw, *Nature* **206**, 338 (1965); *ibid.* **209**, 577 (1966).
2. F. Jacobs, S. Brenner, F. Cuzin, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 329 (1963).
3. R. Baserga and K. Nemeroff, *Stain Technol.* **37**, 21 (1962).
4. D. E. Comings, *Cytogenetics*, in press.
5. C. Leuchtenberger, R. Leuchtenberger, A. M. Davis, *Amer. J. Pathol.* **30**, 65 (1954)
6. Based on the assumption that there is not a significant difference in the thickness of flattened fibroblasts and metaphase or anaphase spreads.
7. Estimation of 48 μ as the length of a replicon in humans was based on 96 minutes as the duration of replication of the heavily labeling, asynchronous and late-replicating chromatin of the inactive X chromosome (*4*) and 0.5 μ/min as the rate of replication of an extended DNA helix in HeLa cells (*8*).
8. J. Cairns, *J. Mol. Biol.* **15**, 372 (1966).
9. R. B. Painter, D. A. Jermany, R. E. Rasmussen, *ibid.* **17**, 47 (1966).
10. Supported in part by HE 3091 from PHS and general support grant, City of Hope Medical Center.

23 September 1966

Perineural Epithelium: A New Concept of its Role in the Integrity of the Peripheral Nervous System

Abstract. *A multilayered, squamous-celled epithelial cell membrane covering the individual nerve fasciculi of the entire peripheral nervous system (both voluntary and autonomic) including the sensory and motor end organs has been demonstrated in various species of animals, including man. This membrane is the direct continuation of the pia-arachnoid mater from the central nervous system. Functional significance of this membrane, especially as a diffusion barrier and as a protector of the peripheral nervous system, is briefly discussed.*

Key and Retzius (*1*) originally described what appeared to be a sheath that was composed of endothelial-like cells which surrounded nerve trunks; it has subsequently been referred to sporadically in the literature (*2*). This continuous layer of cells surrounds not only nerve trunks but all nerve fasciculi, and it underlies the connective tissue of the perineurium (Fig. 1c) (*3*). It completely surrounds the nerve fasciculi in frog, shark, whale, rat, cat, dog, guinea pig, rabbit, monkey, chimpanzee, and man.

In the frog, the membrane is two layers thick; in mammals it is from 5 to 12 layers thick, the number of layers decreasing with the decreasing size of the fasciculus. With the electron microscope, some fine collagen fibrils can be demonstrated between the layers (*4, 5*). Each epithelial cell has a basement membrane on either side (Fig. 2). Small blood vessels ramify between the layers and carry a sleeve of epithelial cells from one of these layers some distance into the nerve fasciculus (*4, 6*). In the same way, the blood vessels of the central nervous system carry a sleeve of leptomeninges.

The squamous shape of the cells and the multilayered nature of the membrane can be demonstrated (with the dissection microscope) when it is isolated and then stained to demonstrate the cellular borders, or when tangential sections are made of it. When transverse or longitudinal sections of a nerve fasciculus are cut as in standard histological preparations, the perineural epithelium appears as the lamellated structure that is described in some standard histological textbooks, but this appearance is due to the multiple layers of squamous cells. The fasciculi in sections of a nerve trunk close to the central nervous system show clear-cut and very thick lamellation because of the great number of cellular layers present, but small nerve fasciculi (terminal divisions) that are some distance from the central nervous system and are surrounded by only one or two layers of cells will not show any obvious lamellation under the microscope. Thus, the description of the perineurium as a lamellated structure cannot be applied to the perineural covering of all nerve fasciculi.

When this membrane is traced back along the nerve trunk to its point of origin in the central nervous system, it can be seen to surround the whole nerve trunk and to pass through the vertebral foramen where it becomes continuous with the pia-arachnoid membrane of the central nervous system (Fig. 1, a and b). The cells of this membrane are identical in shape and size, in nuclear structure, and in enzyme histochemistry with the cells of the pia-arachnoid mater (Fig. 1, a–c). We believe they are the same kind of cell and that this cellular sheet is embryologically derived from the same ectodermal cells as the pia-arachnoid. If the latter is regarded as a product of the ectoderm, then this membrane is an epithelium—a multilayered squamous epithelium. However, the name is not as important as its structure and relationships.

The capsule of the dorsal root ganglion is simply a reflection of the perineural epithelium around the ganglion. The epithelium also surrounds the sympathetic ganglia and the sympathetic nerve trunks and their divisions (*6*); it also surrounds the fasciculi of the vagus nerve (*7*), as well as those of other cranial nerves.

As the peripheral nerves branch, the perineural epithelium branches with them; as the branches get smaller, the number of layers of cells in the epithelium becomes less, until finally the terminal nerve fiber is surrounded by a single-layered, continuous sheet of cells (Fig. 2) which accompanies the fiber to its termination.

At the termination of the fiber, the perineural epithelium forms the capsule of the end organs. In the motor end plate it constitutes the bell mouth of Henle which envelops the end plate and attaches directly to the sarcolemma (*8*) (Fig. 3). In the Pacinian corpuscle, the various lamellae of the corpuscle are simply additional layers of cells of the epithelium which surround the naked nerve ending (*9*) (Figs. 1e, 3, and 4) as they are in the morphologically similar structure, the Herbst corpuscle of the duck. The capsule of the muscle spindle (Fig. 3) is a sculptured or molded perineural epithelium (*10*) as are the capsules of the various nerve endings in the skin (Fig. 3). In the eye, the choroid is continuous with the pia-arachnoid membrane (perineural epithelium) of the optic nerve (Fig. 1d), the cells of the two structures being morphologically and histochemically identical (*11*).

The choroid is continuous with the endothelial cells of the anterior-chamber angle meshwork and with the "endothelium" underlying the cornea, so that the retina and the contents of the eye are completely enclosed by a membrane continuous with the pia-arachnoid membrane of the optic nerve (perineural epithelium) (*11*), and the choroid is presumably derived from the same embryological origin as the pia-arachnoid and the perineural epithelium covering the nerve endings.