Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3123–3127, May 1984
Cell Biology

Different central nervous system cell types display distinct and nonrandom arrangements of satellite DNA sequences

(cerebellum/*in-situ* hybridization/centromeres/three-dimensional structure)

LAURA MANUELIDIS

Section of Neuropathology, Yale University Medical School, New Haven, CT 06510

Communicated by Sanford L. Palay, February 3, 1984

ABSTRACT **Paraformaldehyde-fixed tissue from mouse cerebellum was hybridized with biotin-labeled satellite DNA for identification of centromeres. By using avidin-peroxidase conjugates, it was possible to define the nuclear position of centromeres at the ultrastructural level. Three-dimensional analysis of well-resolved centromere arrays were aided by computer reconstruction of serial sections. Different cell types displayed distinct, nonrandom centromere locations. In Purkinje neurons, the majority of detected sequences were clustered together around the central nucleolus, whereas in granule neurons, more numerous, dispersed centromere clusters were associated with the nuclear membrane. In Purkinje cells, peroxidase-labeled regions corresponded to dense heterochromatic aggregates that capped the nucleolus; similar ultrastructural aggregates were detected in Purkinje cells of several different species. These observations suggest that in these highly differentiated cells, the nuclear position of centromeres is maintained in evolution despite species differences in centromeric DNA sequence. Such defined ordering of centromeres may be integral to specific functional capacities.**

Nuclei in different tissues exhibit distinct patterns of organization at both the light and electron microscopic (EM) levels, yet our understanding of these patterns with respect to specific DNA sequences is limited. In contrast to mitotic chromosomes, which are relatively inactive, interphase nuclei express major functions of the cell and are responsible for the direction of differentiation. The recent assignment of specific DNA sequences to individual mitotic chromosome regions makes it possible to explore the interphase nucleus with defined probes and to reexamine the concept of nonrandom interphase chromosome organization originally posed by cytologists in the early part of this century (cf. refs. 1–3). Two major technical obstacles have limited the usefulness of this approach. First, aldehyde immobilization of cell structures prior to *in situ* hybridization was not successful. Acid-methanol fixation, although useful for evaluation of mitotic chromosomes, severely distorts tissue and also may affect the precise three-dimensional placement of interphase chromosomes because histones and other proteins are extracted. Second, autoradiographic detection of hybridized sequences has limited resolution at the ultrastructural level and can obscure the cytological features of interest. Recently, significant technical improvements have been obtained with biotin-labeled DNA probes (4–8); these techniques were extended for EM study of centromere position in CNS tissue.

Tissue of adult cerebellum was chosen because neurons and the vast majority of glial cells do not replicate and, thus, will not contain variants that may be related to the cell cycle (6, 9). The architecture and cytology of the cerebellum is also well-defined (10), and this is helpful for unambiguous identification and comparison of individual cells—e.g., large and small neurons, astrocytes, and oligodendroglial cells. Mouse satellite DNA was used because it is abundant in the centromeres of all chromosomes except the Y chromosome (11, 12), and its sequence is well-characterized (13). I here assess the three-dimensional organization of centromeres in interphase nuclei of several highly differentiated CNS cells types.

MATERIALS AND METHODS

Purified mouse satellite DNA used for sequence analysis (13) was nick-translated as described (6, 8) by using biotin-UTP with a linker arm of 11 or 16, generously supplied by D. C. Ward. Incorporation of biotin-UTP into DNA was assayed by spotting 1–2 μl of Sephadex-purified fractions onto DEAE paper. The paper was blocked with horse serum and treated for 10 min with avidin–biotinylperoxidase (Vector Laboratories, Burlingame, CA) at the manufacturer's recommended concentrations. The paper was washed in phosphate-buffered saline and developed in diaminobenzidine as described (6).

Swiss mice were perfused with freshly prepared 4% paraformaldehyde in buffer A (14). Slices of cerebellum were cut perpendicular to the folia and allowed to fix for an additional 40 min, washed extensively in buffer A, and stored at 4°C. Slices of cerebellum were cut into 25- to 40-μm sections on a vibratome. Just prior to hybridization, sections were digested with Pronase (25 μg/ml) in phosphate-buffered saline containing 5 mM EDTA for 10–12 min at 22°C. Incubations with higher Pronase concentrations led to tissue disruption on the external surfaces and incomplete probe delivery to more central portions of the sections. Digestion was terminated by washing sections (3 times for 10 min each) in phosphate-buffered saline containing 5 mM EDTA, glycine (4 mg/ml), and 0.5 mM phenylmethylsulfonyl fluoride. We also tried several other methods to avoid Pronase treatment, which could distort nuclear structure. We have found that freezing sections in 20% glycerol in liquid nitrogen (3 times for 5 min each) also permits probe delivery and detection. Results were the same as those shown here with Pronase.

Sections were then equilibrated in 50% formamide/0.3 M NaCl/0.03 M Na citrate, pH 7 (buffer B) (twice for 15 min each) and then in the same solution containing the biotin-labeled DNA probe (0.5 μg/ml) and sonicated herring sperm DNA (250 μg/ml). The tissue–probe mixture was denatured for 3.5 min at 78°C and incubated at 34°C overnight. After hybridization, sections were washed extensively with buffer B, 0.3 M NaCl/0.03 M Na citrate, pH 7, and phosphate-buffered saline, and then were blocked for 15 min with 10% serum in phosphate-buffered saline at 22°C. For detection of biotin-labeled sites, sections were incubated for 2 hr at 37°C in avidin–biotinylperoxidase in phosphate-buffered saline, washed with phosphate-buffered saline, and developed for 10 min in diaminobenzidine. When using the shorter biotin-UTP with linker arm, additional steps of antibody incubation

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: EM, electron microscopy/microscopic.

were required as described (6, 8). Vibratome sections were osmified, dehydrated through ethanol, flat-embedded in Epon, and stained with lead citrate. Centromere areas in serial 1-μm sections were traced and computed by using an Apple graphics tablet (computer mode) mixed with a video display of the microscope image (15). Three-dimensional true perspective displays were reconstructed from photographs or video displays using a program developed and kindly lent by J. Russ. Reconstructions were rotated in computer movies and also examined in stereo pairs for more precise analysis.

RESULTS

Vibratome slices observed using Nomarski optical section revealed a consistent centromere pattern in Purkinje neurons; there were generally two large clusters of staining abutting the central nucleolus. These stained regions had no obvious connection with the nuclear envelope. Further resolution was obtained by examination of 1-μm Epon serial sections. Two prominent, large centromeric clusters symmetrically capped both sides of the nucleolus in most Purkinje cells; in a few cells these clusters were connected to give a C-shaped configuration, and in some cells there was a less balanced symmetry (Figs. 1 and 2). In general, the two facing central caps were oriented on a line perpendicular to the extending dendrite, although a few examples of other rotations were found. In serial section, two to four additional smaller regions of hybridization also were identified in each Purkinje cell, and these smaller clusters were adjacent to the nuclear membrane. Three-dimensional reconstruction and perspective display detailed both the relative size and the configuration of these centromeric aggregates (Fig. 2).

In contrast to Purkinje neurons, small granule neurons displayed 4 or 5 large centromeric clusters that were positioned peripherally and abutted the nuclear membrane. A single focus of satellite DNA without membrane attachment also was seen in some cells. Each peripheral cluster tended to be arrayed opposite to another cluster, giving an impression of 4-fold symmetry (Fig. 1*a*).

Astrocytes generally displayed more than six individual centromere clusters close to or on the nuclear membrane (Fig. 1) regardless of their location in specific layers (e.g., molecular or internal granule cell layers). These clusters were dispersed along the entire circumference of the nucleus; as with granule neurons, there was no collection of label at one end of the nucleus. The peripheral centromeric clusters in astrocytes tended to be somewhat smaller (and more numerous) than those of the granule neurons.

EM studies confirmed the assignments above and also gave a more precise picture of satellite DNA location with respect to the nuclear membrane and the nuclear cytology. The most striking finding in Purkinje neurons was that the large central satellite clusters corresponded to dense heterochromatic aggregates observed in nonhybridized tissue that had been treated to detect nucleolus organizers (Fig. 3*b*). These centrally placed aggregates were readily visualized abutting either side of the nucleolus in tissue stained with silver for detection of active nucleolus organizers (15). They did not contact the nuclear membrane in any section examined. Individual centromeres, estimated to be 0.2–0.3 μm wide (6, 14), could not be unambiguously distinguished within each of the large, compact satellite DNA clusters in hybridized tissue. A few examples of C-shaped clusters capping the nucleolus were comparable to those seen by light microscopy. Smaller peripheral centromeric clusters were also observed by EM in Purkinje cells, and most of these showed direct attachment to the nuclear membrane (Fig. 3).

Ultrastructurally peripheral centromere clusters in granule

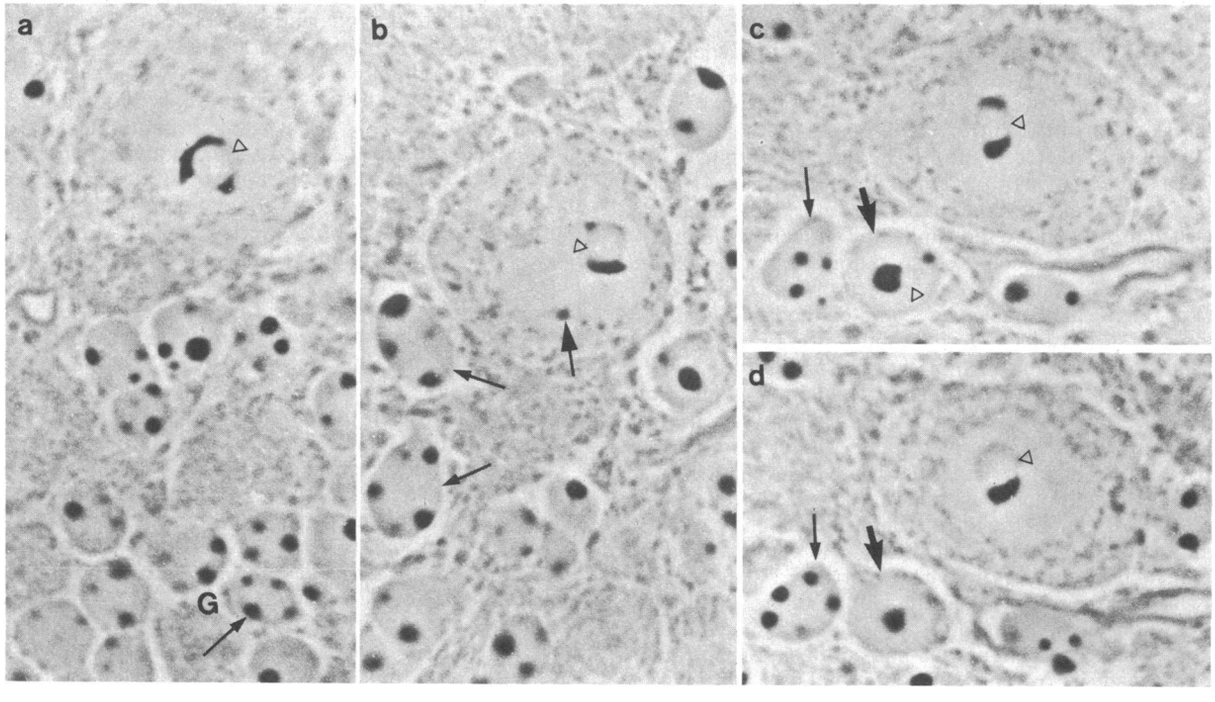

FIG. 1. Phase-contrast 1-μm Epon sections. Satellite DNA-positive regions are black. (*a*) Large C-shaped cluster around the nucleolus (△) without connection to the nuclear membrane. G is adjacent to granule cell neurons that show 4 or 5 large clusters of centromere-positive DNA abutting the nuclear membrane. The arrow shows a granule neuron with centromeres placed symmetrically opposite each other. (*b*) Nucleolus (△) capped by satellite DNA and a smaller region of satellite DNA on the nuclear membrane (large arrow). In contrast, adjacent astrocytes (arrows) have multiple regions of staining abutting or close to the nuclear membrane. (*c* and *d*) From adjacent serial sections, typical symmetrical placement of centromeres capping the nucleolus (△) of Purkinje neuron. The large arrow shows a Lugaro neuron with a large central cluster of satellite DNA adjacent to the nucleolus (△) and a smaller peripheral cluster. Five of the six satellite DNA clusters visualized in the astrocyte (small arrows) are adjacent to the nuclear membrane. (×1700.)

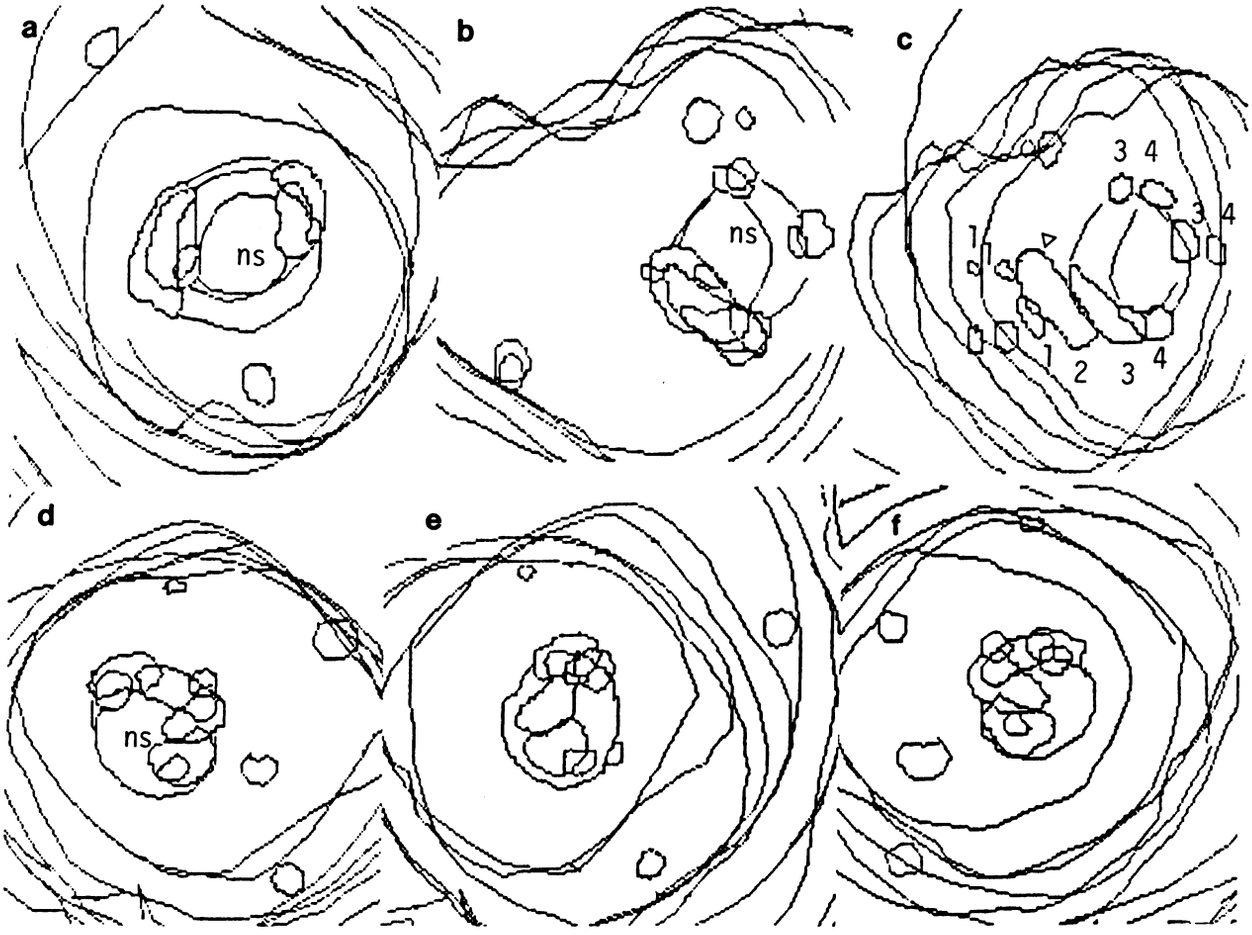

FIG. 2. Computer perspective display of 1-μm serial sections of Purkinje neurons. (*a*) Typical example of the symmetrical position of satellite DNA clusters abutting the nucleolus (ns). (*b* and *c*) Another example with less symmetry of central satellite DNA clusters, rotated at 6° and 45°. (*c*) Contiguous central satellite DNA clusters labeled by relative slice numbers 1–4. Adjacent serial sections without detectable satellite DNA and nucleolus outline (at △) were deleted to simplify the computer display. (*d*, *e*, and *f*) A cell with a C-shaped centromere cluster on the nucleolus (ns) and three smaller peripheral centromere clusters on the nuclear membrane, at rotations of 2°, 24°, and 175°, respectively.

neurons were closely associated with the nuclear membrane (Fig. 3*a*). It was expected that the dense central heterochromatin, which is characteristic of granule neurons in nucleolus organizer-stained tissue (15), would contain the bulk of satellite DNA, but this was not the case. Typically at least two small nucleoli were identified adjacent to the peripheral satellite DNA clusters in granule neurons (data not shown). Other types of cerebellar neurons had characteristic centromere locations (to be described in detail elsewhere) and tended to have the majority of centromere label closely associated with the position of the largest nucleolus, which in some cases is more peripherally placed (10). As with granule neurons, centromere clusters in astroglia were also associated with the nuclear membrane. Oligodendroglial cells which could be unambiguously identified by EM, also displayed peripheral satellite DNA clusters; these were associated with dense heterochromatin on the nuclear membrane that did not contain detectable satellite DNA.

Computer measurement of centromeric clusters indicated that ≈80% of the label detected in Purkinje cells was centrally placed around the nucleolus, and it is likely that the majority of the 40 mouse centromeres are contained in this central array. In comparison, each of the peripheral clusters identified in granule neurons or in astroglia is likely to contain 4–8 centromeres, estimated from the average size of mitotic chromosome centromeres (6) and from the number of satellite DNA clusters found in each nucleus. In contrast to Purkinje cells, peripheral clusters represented ≈80% of the detected satellite DNA in these latter two cell types.

DISCUSSION

The present results indicate that centromeres are arranged in characteristic and different three-dimensional patterns in interphase nuclei of neuroectodermal cells. Nonrandom locations of centromeres typify each differentiated cell type, even within each cell class (e.g., neurons). The present findings are compatible with a previous description of C-banded heterochromatin on acid-fixed squashes of cerebellum (16), where neurons were reported to have fewer large blocks of heterochromatin, and glial cells showed numerous smaller blocks of staining. In an autoradiographic hybridization study, nonrandom satellite distributions were suggested also for several other cell types (17). The present study shows, with superior resolution and three-dimensional preservation, the location of centromeric satellite DNA. In Purkinje cells the major focus of satellite DNA can be directly correlated with intense Feulgen-stained material around the nucleolus (10) and with ultrastructural aggregates identified by EM (15). Guinea pigs and hamsters also have similar heterochromatic blocks around nucleoli of cerebellar Purkinje cells. A similar situation is seen in large neurons of the cerebral cortex (15). The nuclear cytology of large neurons is highly conserved in evolution. However, centromeric satellite DNA sequences are markedly different in different mammalian species—for example, mouse and man (18). The cytological observations above suggest that despite marked differences in sequence, amount, and chromosomal location (acrocentric or metacentric) of satellite DNA in different species,

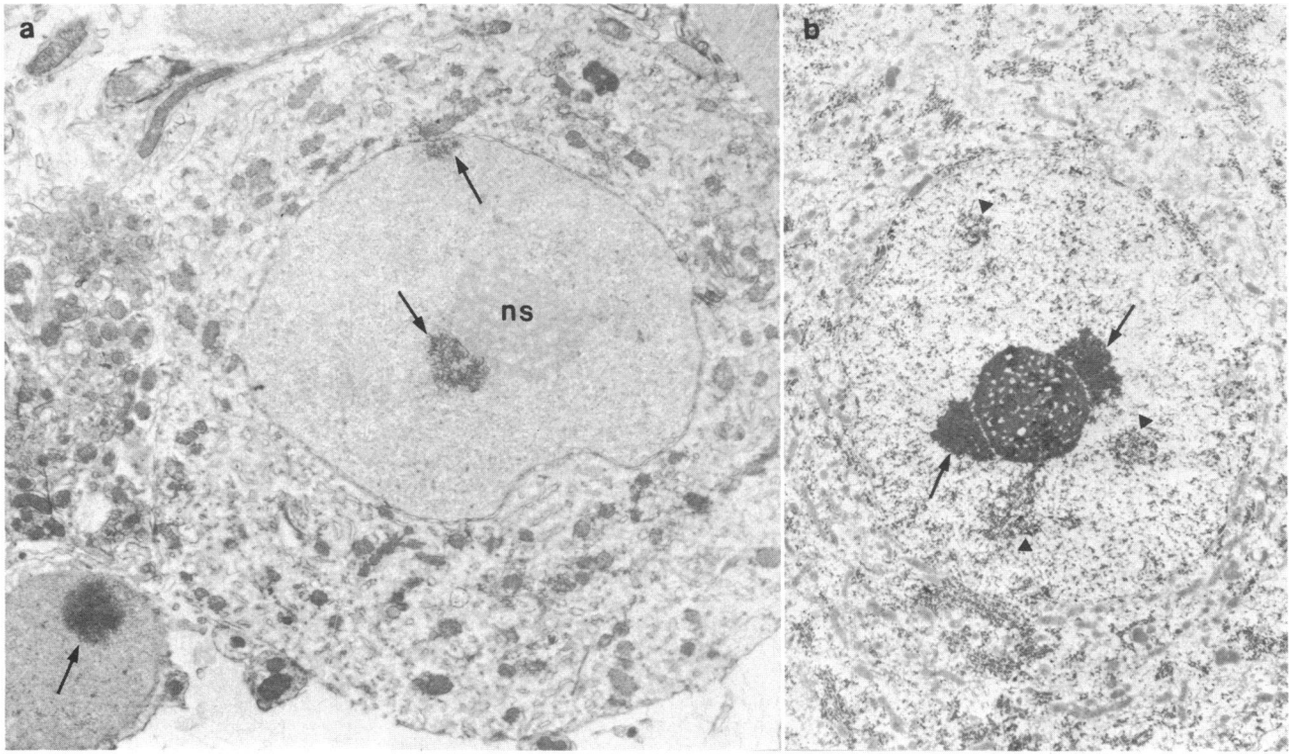

FIG. 3. (*a*) Hybridization to satellite DNA. A large cap of satellite DNA on nucleolus (ns) and a smaller cluster on the nuclear membrane (arrow) are seen in a Purkinje cell. A granule neuron displays a large centromere cluster adjacent to the nuclear membrane (arrow), counterstained with lead only to enhance peroxidase contrast. (×6500.) (*b*) Tissue stained for active nucleolus organizers with silver (dark regions in nucleolus). Note that, in this Purkinje cell, symmetrically placed heterochromatin adjacent to the nucleolus (arrows) corresponds to satellite DNA localization seen in *a*. Other dense regions in the nucleus (▲) are likely to be sites of transcriptional processing (15). (Counterstain, uranyl acetate; ×5000.)

centromeres may be positioned identically in specific cell types. Previous studies have shown that active ribosomal sequences also are positioned in a characteristic way for each neuroectodermal cell type (15), even though ribosomal sequences reside at different chromosomal locations in different species. It is possible that specific centromeric proteins are important in maintaining these defined three-dimensional locations.

Several studies of nonmammalian mitotic cells have shown that centromeres are positioned together at one side of the nucleus, with telomeres at the opposite side in a so-called "polar" orientation (3, 19, 20). It has generally been assumed that during interphase this polar mitotic arrangement is maintained (3, 19–21). No interphase cells here showed such a "polar" arrangement, although Purkinje cells showed defined aggregation of the majority of centromeres in the center of the nucleus. Since chromosomes are integral units, the chromosomal arms must extend radially from this central aggregate in Purkinje neurons. In contrast, there was no evidence of a major single collection of centromeres in granule neurons or astroglia, and centromere clusters were dispersed along the nuclear membrane. Again chromosome arms must radially extend from each of these clusters. In this context, it would be of interest to know where telomeric sequences reside. As in plant cells, mouse cells *in vitro* during metaphase and anaphase show dramatic clustering of centromere sequences at one pole of the cell, with the telomeres oriented at the opposite side, but during G_1, the centromeres are repositioned along the nuclear membrane, without a single polar focus (unpublished observations). Thus, the relative position of centromeres in interphase cannot be simply or directly extrapolated from their metaphase order. It is likely that during G_1, and probably with differentiation, there are dynamic changes in centromere location that reflect the functional commitment of a given cell. Indeed, preliminary evidence here indicates that during postmitotic differentiation, centromere clusters in Purkinje neurons may be repositioned.

The order of chromosomes in different interphase cells can have functional consequences (21, 22), and it is possible that, in addition to centromeric sequences, repeated noncentromeric DNAs may participate in this order (22). In the present study, there was apparent symmetry of centromere clusters both in Purkinje cells and in granule cells. Specific individual centromeres were not defined. It would be of interest to determine if homologous centromeres (and their radiating chromosome arms) are represented symmetrically in each mirror image array, for example as seen on each side of the nucleolus in Purkinje cells. Further experiments using the present detection techniques with cloned probes specific for a single identified centromere or chromosome segment should allow us to further evaluate such problems of symmetry and three-dimensional order in different interphase cells.

I thank D. Ward for critical evaluation of this manuscript, and David Plaskon for technical assistance. This work was supported by National Institutes of Health Grant CA 15044.

1. Wilson, E. B. (1925) *The Cell in Development and Heredity* (Macmillan, New York), 3rd Ed.
2. Comings, D. E. (1968) *Am. J. Hum. Genet.* **20,** 440–460.
3. Ashley, T. & Pocock, N. (1981) *Genetica (The Hague)* **55,** 161–169.
4. Langer, P. R., Waldrop, A. A. & Ward, D. C. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6633–6637.
5. Hutchinson, N., Langer-Safer, P. R., Ward, D. C. & Hamkalo, B. A. (1982) *J. Cell Biol.* **95,** 609–618.
6. Manuelidis, L., Langer-Safer, P. R. & Ward, D. C. (1982) *J. Cell Biol.* **95,** 619–625.

7. Singer, R. H. & Ward, D. C. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7331–7335.
8. Brigatti, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C., Hsiung, G. D. & Ward, D. C. (1983) *Virology* **126,** 32–50.
9. Kurnit, D. M. & Maio, J. J. (1973) *Chromosoma* **42,** 23–36.
10. Palay, S. L. & Chan-Palay, V. (1974) *Cerebellar Cortex* (Springer, New York).
11. Gall, J. G. & Pardue, M. L. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 378–383.
12. John, H., Birnsteil, M. L. & Jones, K. W. (1969) *Nature (London)* **223,** 582–587.
13. Manuelidis, L. (1981) *FEBS Lett.* **129,** 25–28.
14. Sedat, J. & Manuelidis, L. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42,** 331–350.
15. Manuelidis, L. (1984) *J. Neuropathol. Exp. Neurol.*, in press.
16. Hsu, T. C., Cooper, J., Mace, M. L. & Brinkley, B. R. (1971) *Chromosoma* **34,** 73–87.
17. Rae, P. M. & Franke, W. W. (1972) *Chromosoma* **39,** 443–456.
18. Wu, J. C. & Manuelidis, L. (1980) *J. Mol. Biol.* **142,** 363–386.
19. Fussel, C. P. (1975) *Chromosoma* **50,** 201–210.
20. Avivi, L. & Feldman, M. (1980) *Hum. Genet.* **55,** 281–295.
21. Bennett, M. D. (1982) in *Genome Evolution*, eds. Dover, G. A. & Flavell, R. B. (Academic, London), pp. 240–261.
22. Manuelidis, L. (1982) in *Genome Evolution*, eds. Dover, G. A. & Flavell, R. B. (Academic, London), pp. 263–285.