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Genetics

Heterochromatic features of an 11-megabase transgene in brain cells

(chromosome ultrastructure/*in situ* hybridization/transgenic DNA/heterochromatin/three-dimensional reconstruction)

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ABSTRACT **Transgenic mice provide a remarkable experimental setting for the study of nuclear architecture. The three-dimensional localization and fine structure of a foreign DNA within the mouse genome can be conveniently followed by using high-resolution *in situ* hybridization. Foreign DNAs designed with specific characteristics, such as base bias, sequence motif(s), and size can stably integrate into finite positions on host chromosomes. Thus the relative importance of each of these characteristics in determining the three-dimensional nuclear position and the detailed morphology of the transgene can be evaluated in different cell types. The aim of this study was to evaluate a transgene with sequence characteristics that might contribute to the *de novo* formation of heterochromatin in interphase nuclei. The structure of a phenotypically silent 11-megabase transgene, containing tandem repeats of β-globin–pBR sequences integrated into the peritelomeric region of both mouse chromosome 3 homologs, was determined in adult brain cells. Neurons that are largely euchromatic were especially informative in three-dimensional studies of transgene position. The two transgenic loci behaved much like centromeric or paracentromeric A+T-rich satellite DNAs of comparable length from a single chromosome; one or both transgene domains localized together with centromeric satellite DNA on the nucleolus. This is an unusual nuclear position for a telomeric or chromosome arm region that does not contain a substantial amount of constitutively heterochromatic satellite DNA. G+C richness did not prevent these regions from assembling as dense heterochromatic bodies of ≈1 μm^3 in volume. Ultrastructurally, transgenic domains were often intimately connected with constitutive heterochromatin and were highly condensed. Labeled supercoils, formed by a discrete ≈250-nm-wide fiber, were observed in oblique thin sections through the center of the domain. The structural data were consistent with negligible transcriptional activity detected for this locus, as well as the predicted conformation of constitutive heterochromatin. Interestingly, in transgenic but not control mice, a substantial number of large neurons, including ≈30% of cerebellar Purkinje cells, showed excessive invaginations of the nuclear membrane.**

The introduction of foreign DNA into mouse embryos has recently been exploited to study selected sequence features and functions of specific genes. Expression of these transgenes, however, can additionally depend on the chromosomal integration site, which is usually random. In order to override position-dependent effects, foreign DNAs have been designed to include cis-acting promoter, enhancer, and nearby regulatory DNA sequences to drive transcription. Nonetheless, transgenes containing all these control regions are often expressed at a level that is one order of magnitude or less than that of the endogenous mouse gene. In the case of the β-globin gene, only a β-globin minilocus construct of 38 kilobases that contained more distant control regions was fully active (1). In nonerythroid tissues, the inactive β-globin minilocus chromatin was postulated to be inaccessible to trans-activating factors (1).

Inactive or "inaccessible" chromatin might be relatively condensed or morphologically heterochromatic in the nucleus. Little is known about the obligatory sequence features needed to signal the formation of heterochromatin in the nucleus. These prerequisites can be addressed in transgenic experiments. Because adjacent chromosome domains might override or influence the expression and structure of a small transgene, it was initially advantageous to study a very large transgenic insert that should resist such influences. Founder mice bearing a huge [≈11 megabase (Mb)] insert of tandemly repeated pBR322–β-globin sequences were selected (2) and back-crossed (3) to develop the homozygous mice studied here. An intervening dispersed genomic repeat within the β-globin gene had been removed, and only 7 kilobases of the β-globin gene was represented. Despite the integration of ≈1000 copies of this construct, Northern blots of total RNA had undetectable levels of β-globin or plasmid; this lack of expression was consistent with the fragmentary nature of the β-globin insert as well as the transcriptionally repressive features of companion pBR322 sequences (2).

This transgenic model was also chosen for study because the transgene is localized on the arms of chromosome 3, at a considerable distance from the centromere. In the mouse, large blocks of constitutive (C-band) heterochromatin are found exclusively in and around centromeres and are not present in chromosome arms or telomeric regions. In most mammalian species, C-bands contain tandem repeats of relatively A+T-rich satellite DNA, which can be as long as 9 Mb in length (4), and are known to be transcriptionally silent in almost all cells. Because neurons are highly differentiated, postmitotic, and show cell type-specific nuclear positions for satellite DNAs (5–7), it was advantageous to study brain tissue. In euchromatic large neurons, centromeres from many chromosomes typically aggregate together to form tightly packed heterochromatin that abuts the nucleolus (5–7). These large heterochromatic bodies exclude LINE sequences (6), which are represented in all chromosome arm regions including telomeric regions of most chromosomes; similarly, G+C-rich Alu sequences, which can be enriched in some telomeric regions, are excluded from C-band DNA (4, 8). The current study shows that (*i*) the modified telomeric domain could be collected together with centromeres on the nucleolus and (*ii*) the ultrastructural features of this transgenic region were compatible with the predicted conformation of a constitutively inactive domain in interphase (9). These findings raise several salient questions with respect to underlying mechanisms of chromosome condensation and decondensation during differentiation.

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Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

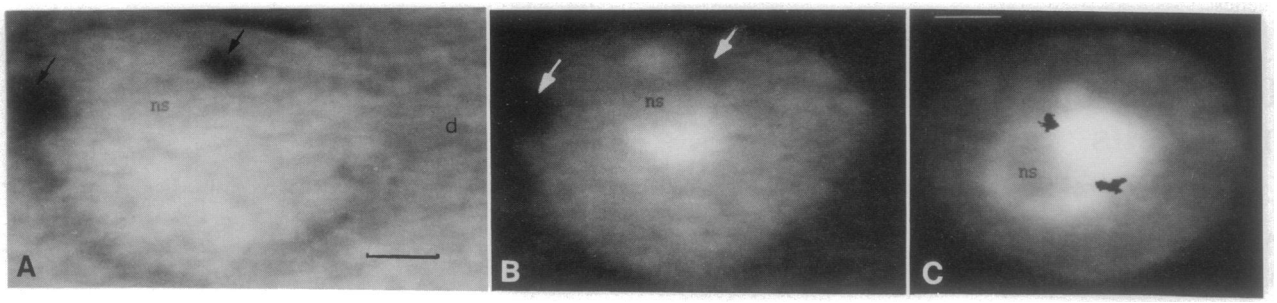

FIG. 1. (*A*) Nomarski image of spinal cord motor neuron with two transgenic hybridization signals detected with alkaline phosphatase (arrows). One signal is on the nuclear membrane and the other is on the nucleolus (ns). d, Dendrite. (*B*) Same cell using mixed Nomarski and epifluorescence light. Brightly staining centromeres with satellite DNA, detected with DAPI, collect on the nucleolus and are adjacent to the perinucleolar transgene (arrow). (*C*) DAPI-stained neuron with two transgenic signals (black) digitally superimposed from a Nomarski image at the same *z* plane. Both transgenic domains closely adhere to the very bright perinucleolar centromeres. (Bars = 2 μm.)

MATERIALS AND METHODS

Adult transgenic C57b mice (3) were perfused with 4% paraformaldehyde in buffer; ≈30-μm-thick vibratome sections were collected, permeabilized by freeze–thaw cycles, and hybridized as described (5) by using biotinylated probe DNA (0.5–1 μg/ml) with an average size of 200–300 bases for enhanced probe penetration (10). Structural artifacts may be associated with less fastidious fixation and processing methods (3). The transgenic plasmid–β-globin probe (pMBd2; ref. 5) and the transgenic mice were a generous gift of C. Lo (2, 3). *In situ* hybridizations with mouse satellite DNA probe gave patterns comparable (5) to those obtained with 4′,6-diamidino-2-phenylindole (DAPI) fluorescence and were thus not extensively pursued in the present studies. However, double-labeling experiments (11) using both digoxigenin-labeled mouse satellite DNA and biotinylated transgenic DNA (detected with fluorescein isothiocyanate and tetramethylrhodamine B isothiocyanate, respectively) confirmed the tight positional association of the transgene with satellite DNA in the nucleus. Methods for peroxidase, alkaline phosphatase, and fluorescent detection of probes have all been described in detail (5, 6, 11). Nontransgenic C57b mice have been examined extensively in previous studies (12).

To avoid photobleaching, a 0.5 neutral density filter was inserted in front of the mercury light source; a videoscope with high gain and short lag (13) was used for imaging. Analog (nonaveraged) *z*-plane images were rapidly collected on a TQ-3031F Panasonic optical laser disk recorder. These serial 0.33-μm optical sections were subsequently digitized and three-dimensionally reconstructed as described (6). In double-labeling studies, our epifluorescent mirror sets gave a slight image shift at higher magnifications. This shift was largely corrected by using a single 90% transmission mirror at 45° and inserting appropriate excitation/barrier filters to distinguish each fluorochrome. A simpler method could be used for accurate superimposition of fluorescent and alkaline phosphatase signals. One could simultaneously view epifluorescent (satellite) and transmitted Nomarski (transgenic dense alkaline phosphatase) signals using light from both sources. More than 500 cells from different brain regions were assessed by optical three-dimensional sectioning, and >100 transgene domains were assessed ultrastructurally, with and without silver intensification of peroxidase detectors (5, 7, 8).

RESULTS

Previous *in situ* hybridization studies with probes specific for a single chromosome have shown that homologous chromosomes are spatially separated from each other in the nucleus (reviewed in ref. 14). Since the β-globin–pBR transgene was present on both chromosome 3 homologs, two hybridization signals would therefore be expected in each nucleus. Almost all cells showed two separate hybridization signals. Fig. 1*A* shows these hybridization signals detected with alkaline phosphatase in a spinal cord motor neuron using Nomarski transmitted light. One dense signal abuts the nucleolus, and the other is on the nuclear membrane. Both signals are relatively compact and discrete. In Fig. 1*B*, epifluorescent illumination is added at this same *z* plane. Intense DAPI fluorescent bright signals are superimposed, and although the density of both transgene signals is reduced, they are still apparent. The intense DAPI fluorescence around the nucleolus is a consequence of preferential dye binding to megabase-long stretches of A+T-rich satellite DNA, possibly visually enhanced by the high degree of satellite DNA condensation (5). Notably, one of the transgenic signals appears to be closely associated with the perinucleolar centromeric heterochromatin. In other large neurons from spinal cord, cerebral cortex, and cerebellum, both transgenic signals were associated with C-band heterochromatin on the nucleolus. Fig. 1*C* shows an example of both transgene signals in this position. Here, dense alkaline phosphatase signals, discriminated by thresholding, were superimposed on an epifluorescent image at the same *z* plane. In all

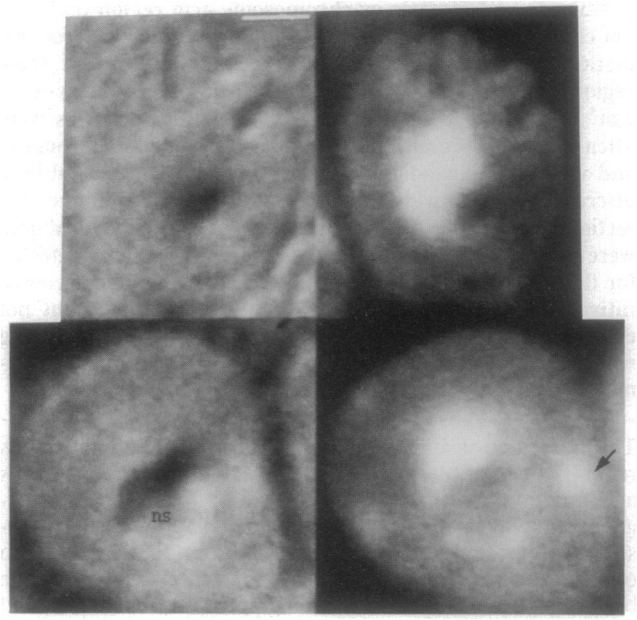

FIG. 2. (*Upper* and *Lower*) Two spinal cord neurons of similar size. (*Upper*) This neuron shows florid invagination of the nuclear membrane. At this *z* plane, both neurons show one transgene on the nucleolar surface, adjacent to bright centromeres. (*Lower*) This neuron, representing a subset of these cells, also has brightly staining satellite DNA on the nuclear membrane (arrow). ns, Nucleolus. (*Left*) Nomarski images. (*Right*) Corresponding epifluorescent images. (Bar = 2 μm.)

large neurons, at least one transgenic domain always contributed to the perinucleolar aggregate of brightly staining centromeres but tended to be at the periphery rather than in the center of the centromeric aggregate (Fig. 2). Double hybridization experiments with digoxigenin-labeled β-globin–pBR and biotin-labeled mouse satellite DNA (see *Materials and Methods*) gave comparable results to those shown.

Whereas transgenic domains on the nucleolus were always closely associated with constitutive heterochromatin, membrane-localized signals in large neurons were not invariably associated with intensely fluorescent centromeric heterochromatin. This feature, however, is also characteristic for pericentromeric or centromeric satellite DNAs that decorate individual chromosomes; ≈50% of large neurons show one isolated homolog on the membrane, whereas the other homolog is aggregated with other centromeres in perinucleolar heterochromatin (6, 7). In no case were the transgenic signals found in more euchromatic "interior" regions of the nucleus, which contain most chromosome arms and are devoid of constitutive heterochromatin under normal circumstances. Many extended, ≈0.25-μm-wide fibers (chromosome arms) have been visualized by confocal fluorescent microscopy (9) and LINE hybridization (6) in this interior region of the nucleus. Volume determinations of three-dimensionally reconstructed transgenic domains (Fig. 3) gave values of 1–1.3 μm^3 for each 11-Mb region, in accord with volumes determined for other C-bands of similar size (6). The volume of these domains was similar in all cell types examined (larger neurons, small granule cell neurons, astroglia, oligodendrocytes, and pericytes). The amount of transgenic DNA packed in this volume indicates its high degree of condensation in interphase, one that is close to that achieved in the very heterochromatic metaphase state (9), and represents a linear distance of ≤2.5 μm between 11 Mb of DNA in interphase.

Ultrastructurally, a high degree of tissue preservation is apparent (Figs. 4 and 5), and structural details of the transgenic domain and its intimate association with heterochromatin are highly resolved. In Fig. 4*A*, two representative densely labeled transgenes in cortical neurons are cut approximately through their centers. The labeled membrane and perinucleolar domains are both intimately associated with unlabeled dense constitutive heterochromatin. Without *in situ* detection these domains would be unrecognizable as individual chromosome elements. Both domains are compact and consistent in size with measurements from optical section studies. A supercoil of an ≈0.25-wide fiber is apparent in the perinucleolar transgene (Fig. 4*A Inset*). In Fig. 4*A*, a heterochromatic, presumably single unlabeled membrane-bound satellite DNA centromere, estimated to be ≈7 Mb (9), shows similar structural features as the labeled transgenes and substantiates the fidelity of the *in situ* detections.

Transgene positions were characteristic for each cell type and mimicked the characteristic positions for C-band DNA in each of these cell types. Euchromatic cerebellar Purkinje neurons characteristically had two perinucleolar signals attached to perinucleolar heterochromatin (Fig. 5*A*). The vast majority of centromeres in this cell type are characteristically collected on the nucleolus (5). In small cerebellar granule neurons, groups of centromeres are clustered in four to six discrete heterochromatic bodies in the nucleus and do not always attach to the nuclear membrane (5). In this cell type, the transgene was always attached to these bodies and, as C-band DNA, did not always display either a membrane attachment site or an intimate association with a nucleolus (Fig. 5*B*). In astrocytes, which unlike adult neurons retain a DNA synthetic capacity, each homolog was closely associated with the nuclear membrane and was not invariably associated with the nucleolus (Fig. 4*B*). In small pericytes, as in granule

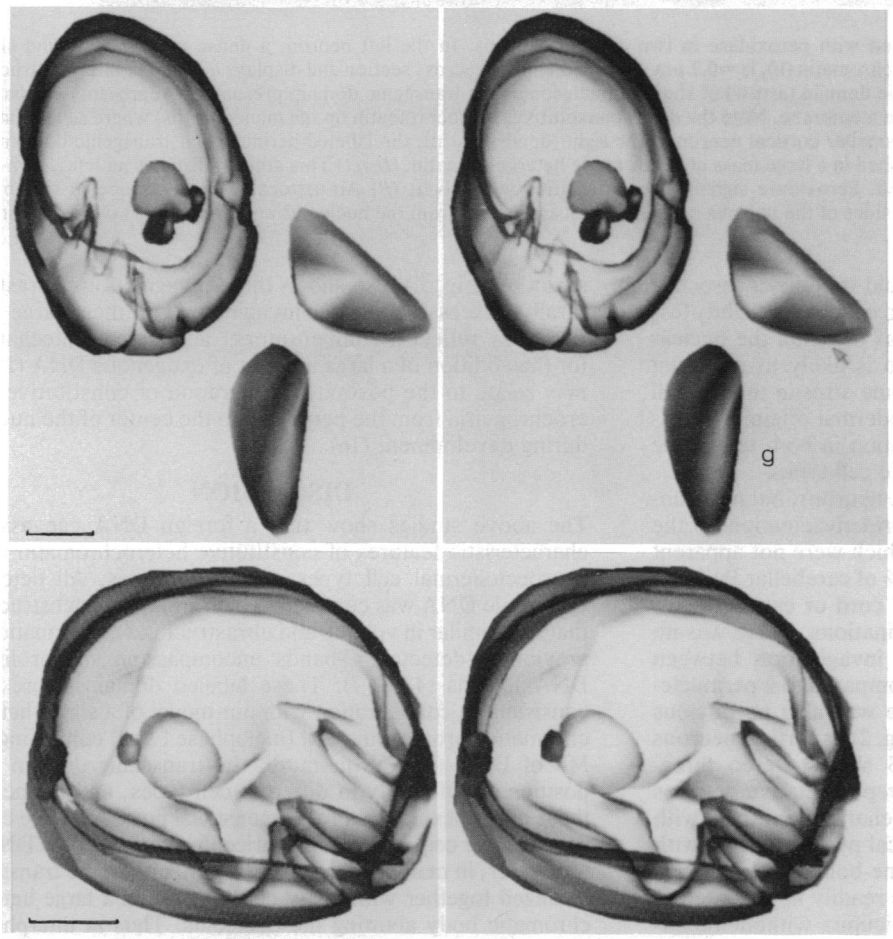

FIG. 3. Three-dimensional stereo pair reconstructions. (*Upper*) Cerebellar Purkinje cell with two signals impinging on the nucleolus (light grey). The membrane has many folds, and the gray arrow points to part of the extending dendrite of this cell. g, Glial nucleus. (*Lower*) In a cortical pyramidal neuron with extensive membrane invaginations, one signal loops from the nuclear membrane (white arrow), and the other is on the nucleolus. (Bars = 5 μm.)

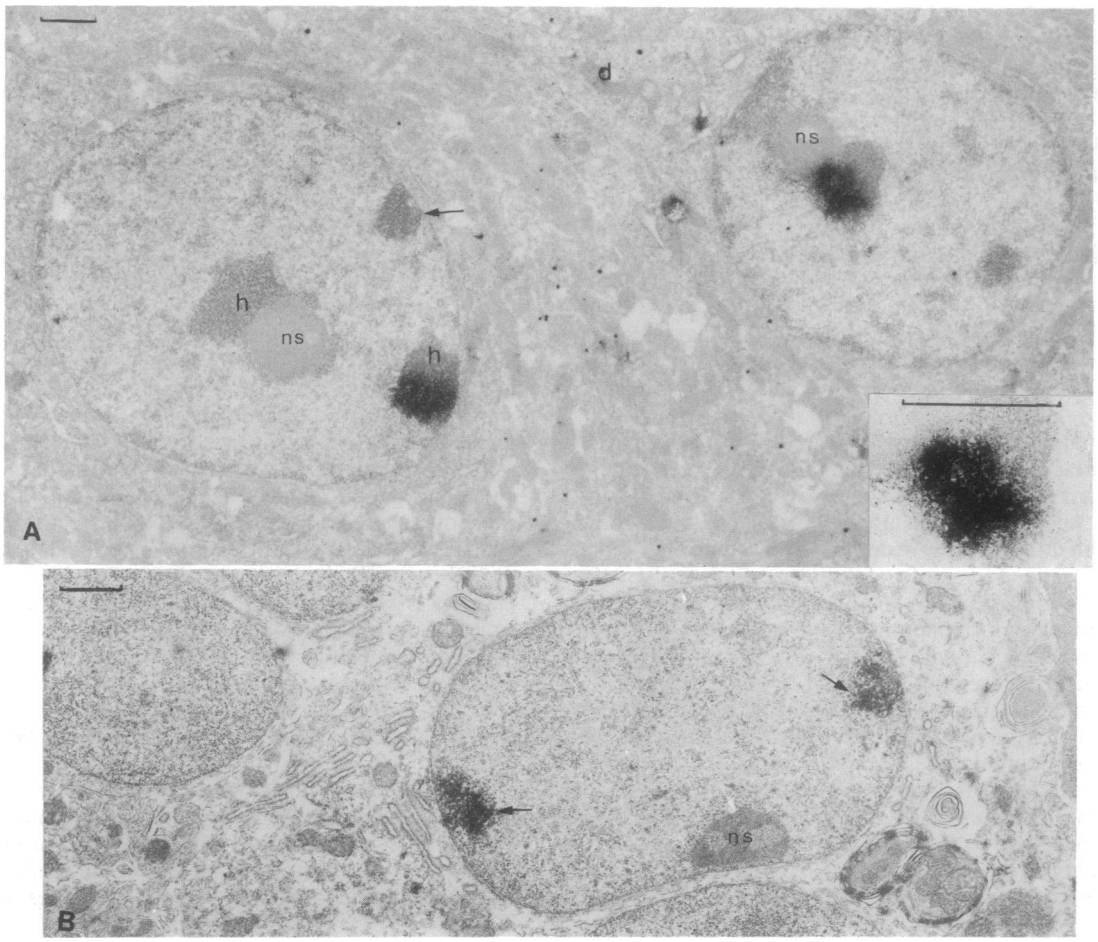

FIG. 4. (*A*) Transgenic signals detected with peroxidase in two cortical neurons. In the left neuron, a dense membrane-bound signal, intimately associated with compact heterochromatin (h), is ≈0.7 μm wide in longitudinal cross section and displays a complex internal structure. Another unlabeled curved heterochromatic domain (arrow) of similar dimension as the transgenic domain presumably represents a C-band of a single chromosome and is attached to the membrane. Note the dense constitutive heterochromatin on the nucleolus (ns) where several mouse C-bands are known to collect (5). In the smaller cortical neuron at the right (dendrite at d), the labeled perinucleolar transgenic domain, cut obliquely through its center, is also enmeshed in a large mass of centromeric heterochromatin. (*Inset*) This domain displays an ≈0.25-μm-wide labeled fiber that is looped or supercoiled. Peroxidase signals were intensified with silver. (*B*) An astrocyte with two labeled membrane-associated transgene domains at opposite sides of the nucleus (arrows). Both are distant from the nucleolus and one appears curved. (Osmium intensification; bars = 1 μm.)

cell neurons, the transgene domain could straddle or associate with both very dense C-band heterochromatin and slightly less dense heterochromatin. The latter was often on the nuclear membrane of pericytes (Fig. 5*C*) and is likely to represent facultative condensation of chromosome arms in these small nuclei. Pericytes are not of neuroectodermal origin, and it is likely that a high degree of condensation in both transgenic domains would be found in all somatic cell types.

Interestingly, some transgenic large neurons, but not granule neurons or astroglia, displayed florid invaginations of the nuclear membrane (Fig. 2 *Upper*), which were not apparent in normal C57b mice. As many as 30% of cerebellar Purkinje cells, as compared to ≈7% of spinal cord or cortical pyramidal neurons, displayed these invaginations. There was no obvious difference with respect to invagination between neurons that had two perinucleolar compared to a perinucleolar/membrane signal pattern. There was also no obvious difference in the size of spinal cord (Fig. 2) or cortical neurons that displayed invaginations. Fig. 3 shows stereo three-dimensional reconstructions of two representative neurons with membrane invaginations: a cerebellar Purkinje cell (with two perinucleolar signals) and a cortical pyramidal cell (with one perinucleolar and one membrane-bound signal). The extent of the nuclear invaginations is readily appreciated in the stereo pairs and was less conspicuous without reconstruction. Fig. 5*A* also shows these invaginations ultrastructurally. Excessive nuclear invaginations in these large neurons may reflect a compensatory "adjustment" mechanism for the addition of a large amount of exogenous DNA (15) or may relate to the postmitotic migration of constitutive heterochromatin from the periphery to the center of the nucleus during development (16).

DISCUSSION

The above studies show that a foreign DNA can assume characteristic features of constitutive heterochromatin in all neuroectodermal cell types and in pericytes. All detected transgenic DNA was confined to a discrete compact structure that was similar in volume and ultrastructural conformation to previously detected C-bands encompassing similar linear DNA lengths (4, 6, 7). These labeled domains were also consistent in configuration with our model of a single heterochromatic chromosome coil (metaphase band) containing ≈9 Mb of DNA (9). Furthermore, the transgenic domain was positioned differently in different cell types, and in each of these cell types, these three-dimensional positions were characteristic for centromeric and pericentromeric satellite DNAs. Moreover, in many euchromatic large neurons, the transgene localized together with many centromeres in a large heterochromatic body abutting the nucleolus. Thus in interphase,

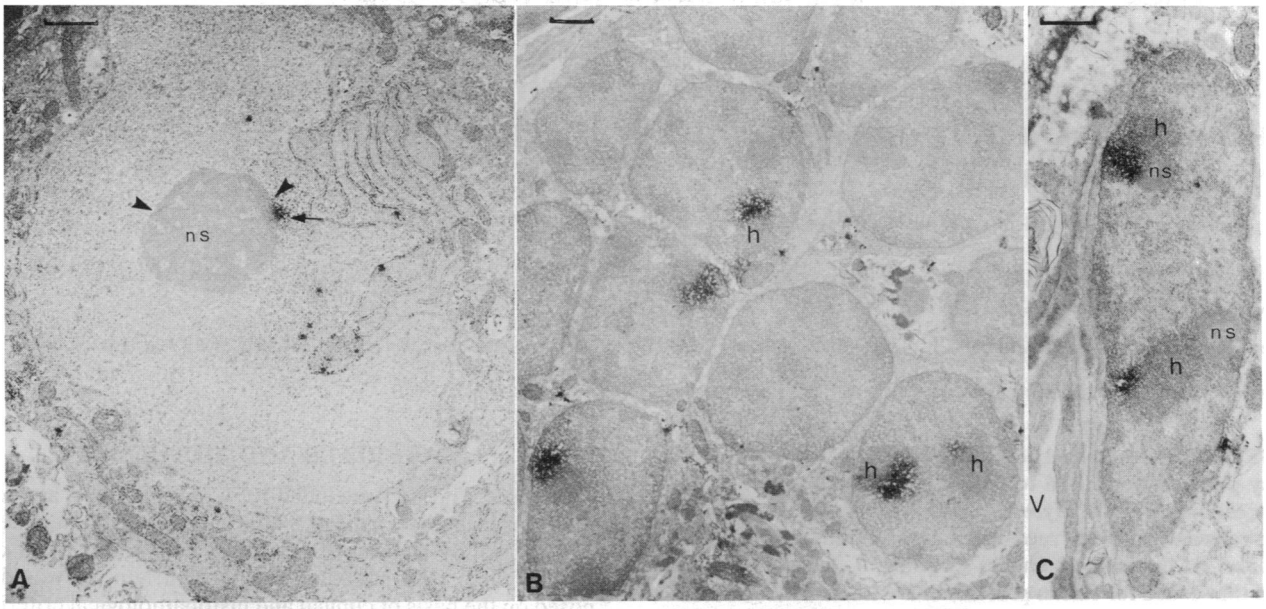

FIG. 5. (*A*) Cerebellar Purkinje cell with multiple membrane invaginations. The edge of a transgenic domain (arrow) adheres to a thin rim of heterochromatin (between arrowheads) on the nucleolus (ns). (*B*) Several small granule neurons and astrocytes are seen. Unlike astrocytes, where transgenic domains contact the nuclear membrane, some granule neurons show centrally placed transgenes adjacent to very dense round heterochromatic bodies (h), which contain satellite DNA (5). (*C*) A pericyte beside a vessel (V) shows two dense heterochromatic bodies adjacent to nucleoli. The upper signal adheres to three structures: dense perinucleolar heterochromatin, the nuclear membrane, and the nucleolus. Heterochromatin that is likely to be facultative is on the nuclear membrane and extends into the interior of the nucleus. (Bars = 1 μm.)

this transgene was positionally segregated, or "compartmentalized" three-dimensionally, in a manner indicative of negligible transcriptional activity. These structural data are in accord with other relevant molecular data on this transgene as well as pBR and β-globin studies (2).

Although no probes specific for the telomeric region of mouse chromosome 3 are currently available, it is unlikely that the telomeric arm region of chromosome 3 is normally associated with this heterochromatic compartment (see the Introduction). Presumably, the introduced transgenic domain has been recognized and segregated from euchromatin in a novel manner. This positioning is likely to ultimately depend on DNA sequence information contained in the transgene. Because an ≈0.5-Mb minisatellite in a telomeric region did not segregate with constitutive heterochromatin, but was found within interior euchromatic regions of the nucleus (6), the length of the transgenic insert studied here may be critical. Structural condensation alone also does not seem to be sufficient for a similar three-dimensional position effect since this minisatellite domain was also quite condensed (0.18 μm^3). The human Y chromosome is reasonably informative in this context and bears comparison with the transgenic locus. This chromosome is unique in that it contains a telomeric, tandemly repeated A+T-rich satellite DNA of similar length as the transgene. This Y telomere is also positioned together with perinucleolar C-bands in large neurons (6) and in astrocytes is attached to the nuclear membrane. It remains to be determined whether (*i*) tandem repetition in the transgene or (*ii*) exclusion of specific regulatory sequences from this large domain is essential for a heterochromatic conformation. It is, however, clear that G+C-richness does not preclude a high degree of condensation in interphase.

The present study raises the intriguing question of whether centromeres may act in interphase as organizing centers for heterochromatin. In postmitotic Purkinje neurons, mouse centromeres are dynamically moved from the periphery to the center of the nucleolus and positioned on the nucleolus (16). The finding of transgene domains at the periphery of centromeric aggregates may indicate a temporal order of events—namely, that centromeres may first coalesce and subsequently attract other heterochromatic domains that share DNA methylation and protein-binding characteristics. C-bands are known to bind special proteins (16, 17). Additional nuclear matrix components (e.g., refs. 18–21) may also be involved in both the interphase condensation and cell type-specific positioning of these domains. The systematic evaluation of other transgenic inserts, combined with antibody markers for nuclear proteins, may help to further elucidate additional determinants of various forms of heterochromatin (14, 22) in interphase.

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1. Grosveld, F., vanAssendelft, G., Greaves, D. & Kollias, G. (1987) *Cell* **51**, 975–985.
2. Lo, C. W., Coulling, M. & Kirby, C. (1987) *Differentiation* **35**, 37–44.
3. Katsumata, M. & Lo, C. W. (1988) *J. Cell Sci.* **90**, 193–199.
4. Chen, T. L. & Manuelidis, L. (1989) *Chromosoma* **98**, 309–316.
5. Manuelidis, L. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3123–3127.
6. Manuelidis, L. & Borden, J. (1988) *Chromosoma* **96**, 397–401.
7. Borden, J. & Manuelidis, L. (1988) *Science* **242**, 1687–1691.
8. Manuelidis, L. & Ward, D. C. (1984) *Chromosoma* **91**, 28–38.
9. Manuelidis, L. & Chen, T. L. (1990) *Cytometry* **11**, 8–25.
10. Manuelidis, L. (1985) *Focus* **7**, 4–8.
11. Cremer, T., Tesin, D., Hopman, A. H. N. & Manuelidis, L. (1988) *Exp. Cell Res.* **176**, 199–220.
12. Manuelidis, E. E., Kim, J. H. & Manuelidis, L. (1986) in *Unconventional Virus Diseases of the Central Nervous System*, eds. Court, L. A., Dormont, D., Brown, P. & Kingsbury, D. (Abbaye de Melleray, Brittany, France), pp. 221–230.
13. Spring, K. R. (1985) *Pflügers Arch.* **405**, Suppl. 1, S23–S27.
14. Manuelidis, L. (1990) *Science* **250**, 1533–1540.
15. McClintock, B. (1984) *Science* **226**, 792–801.
16. Manuelidis, L. (1985) *Ann. N.Y. Acad. Sci.* **450**, 205–220.
17. Moroi, Y., Hartman, A. L., Nakane, P. K. & Tan, E. M. (1981) *J. Cell Biol.* **90**, 254–259.
18. Nelson, W., Pienta, K. J., Barrack, E. R. & Coffey, D. S. (1986) *Annu. Rev. Biophys. Biophys. Chem.* **15**, 457–475.
19. Heck, M. M., Hillelman, W. N. & Earnshaw, W. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1086–1090.
20. Ankenbauer, T., Kleinschmidt, J., Walsh, J. A., Weiner, O. H. & Franke, W. W. (1989) *Nature (London)* **342**, 822–825.
21. He, D., Nickerson, J. A. & Penman, S. (1990) *J. Cell Biol.* **110**, 560–580.
22. Reuter, G., Giarre, M., Farah, J., Gausz, J., Spirer, A. & Spirer, P. (1990) *Nature (London)* **344**, 219–223.