Movement of the X Chromosome in Epilepsy

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The position of selected chromosomes was assessed in samples of normal and epileptic human cortex with biotinylated probes specific for individual chromosome domains. Optical sectioning provided a rapid method for three-dimensional resolution of in situ hybridization signals in interphase cells, and solid models were reconstructed from digitized images for detailed rotational studies. There was a dramatic repositioning of the X chromosome in neurons of both males and females in electrophysiologically defined seizure foci. Other chromosomes (1, 9, and Y) showed more subtle positional changes. Specifically altered nuclear patterns involving the X chromosome may become established and create the genetic memory for intractable seizure activity.

WHEN MAJOR CHANGES IN GENE expression occur, as during terminal differentiation, one might predict there would be concomitant alterations in nuclear structure. Although some structural changes can be part of a global activation that is submicroscopic, other alterations, such as the changes in heterochromatin patterns that occur during lymphocyte differentiation may be appreciable at the light microscopic level (*1*). Furthermore, studies of developing post-mitotic neurons indicate that specific chromosome domains can be reorganized and moved during differentiation (*2*). We considered that individual chromosomes might be dynamically rearranged in pathological states. To test this hypothesis we have used cloned DNA probes that specifically decorate domains on four individual chromosomes to study potential rearrangements in human epileptic foci. Seizure activity has been considered to cause both acute and long-term changes in cellular function (*3*) and recent studies indicate changes in genetic expression (*4*).

Relatively little is known about the spatial organization of individual chromosomes in interphase. In situ hybridization offers a powerful tool for marking individual chromosomes, as each chromosome contains specific DNA sequences. Recent work on the human genome has provided a large resource of DNA sequences that have been cytogenetically mapped, and probes that delineate individual chromosomes have been identified. We have previously used high resolution in situ hybridization techniques to define the principal features of interphase chromosome organization in normal neurons of the cerebral cortex and cerebellum. Such studies indicate that there are reproducible arrangements of specific chromosome domains that delineate different classes of mature neuroectodermal cells (*5*). For example, many centromeres are cohesively arrayed on the nucleoli of larger neurons, but in small granule neurons and astrocytes these arrays are intimately associated with the nuclear membranes. These unique patterns have been conserved during evolution despite significant DNA sequence variations in centromere motifs among species (*6*). Nucleolus organizing regions also show defined and distinct patterns of spatial organization in different types of neuroectodermal cells (*7*) and, more generally, different heterochromatin-euchromatin features characterize various neuroectodermal cell types (*8*). In larger neurons, such as those in the cerebral cortex, many chromosome arms are found in an interior compartment that is spatially resolved from both the nucleolus and the nuclear membrane; this interior compartment is likely to contain many transcriptionally active genes as judged by deoxyribonuclease I sensitivity, the presence of many chromosome arms, and localization of the very Alu-rich 1p telomeric domain (*6*). Alu sequences are clustered in band-like arrays on chromosomes (*9*) and are associated with early replicating chromosome domains that contain many housekeeping genes transcribed in all cell types (*10*).

The chromosomes studied here represent a range of sizes, and do not include the ribosomal gene–containing acrocentric chromosomes, which should remain anchored to the nucleolus. The "normal" position of each human chromosome domain was first evaluated in samples of apparently normal cerebral cortex (*11*). No tumors, infectious processes, or significant pathology were detected in these specimens. Multiple blocks from ten patients (22 to 55 years old) were evaluated by in situ hybridization to chromosome-specific probes. The normal spatial residence of the 1q12, 9q12, and Yq12 domains, the specificity of the probes, and the hybridization methods used were as described (*6, 12*). We have now additionally evaluated the spatial residence of the X centromere region in interphase neurons, and confirmed the specificity of this probe (*13*) by in situ hybridization to metaphase chromosomes simultaneously banded with bromodeoxyurdine (BrdU); as expected only two signals were present in female cells, and one signal was seen in male cells (*14*). Whole chromosomes occupy discrete territories in interphase nuclei, as shown previously in rodent-human hybrid cells (*15*) and more recently by decoration of entire individual chromosomes in human cells with libraries of cloned probes from sorted chromosomes (*16*). Three of the probes used here (1q12, 9q12, and X centromere region) reasonably define the center of each these metacentric chromosomes in the decorated interphase territory, as shown in examples of double label/color hybridizations (*17*).

In normal cortical tissue the 1q12 and 9q12 neuronal signals are found only on the nuclear membrane or abutting the nucleolus (*6*). In both instances each homolog occupies a separate nuclear territory; for example, one homolog is on the nucleolus and the other is on the membrane, two distinct

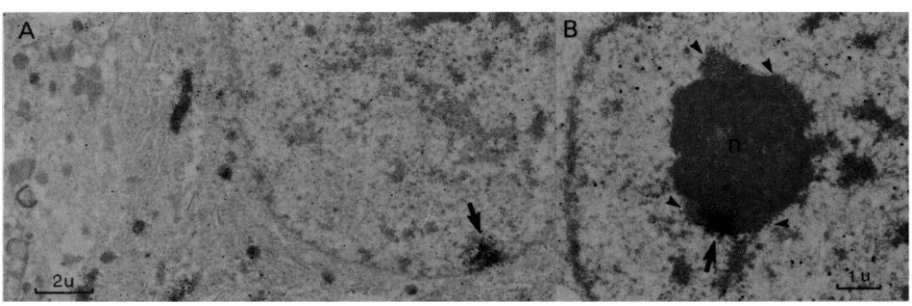

Fig. 1. Representative examples of 9q12 signals by electron microscopy. (**A**) Arrow shows a signal in contact with the nuclear membrane of a cortical neuron. There is only a small amount of dense heterochromatin adjacent to the nuclear membrane. Note reasonable preservation of structure after in-situ hybridization. (**B**) Typical example of neuronal perinucleolar signal (arrow). This signal is embedded in one of several large heterochromatic arrays (arrowheads) on the nucleolus (n). Vibratome slices were embedded for electron microscopy (*5*), and signals here intensified with silver (*9, 12*) yielding silver grains in peroxidase reaction product.

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perinucleolar signals are seen, or two separated membrane signals are observed. Each of these homolog arrangements may relate to neuronal size (*18*) and possibly to specific functional subsets of neurons. We here confirmed the accuracy of these assignments which had been made by light microscopy, by use of the higher resolving power of the electron microscope (Fig. 1). The perinucleolar signals were embedded in dense heterochromatic aggregates (Fig. 1B), typical of those present in many species (*5, 7*) and contained a majority of the centromeric domains; this region showed very bright fluorescence when counterstained with DAPI (diamido phenyl indole) or propidium iodide (*6*) and was often distinguised by its enhanced contrast in differential interference microscopy. Generally, membrane-associated signals were not associated with large heterochromatic aggregates (Fig. 1A) and were often more extended in three-dimensional reconstructions.

In normal specimens, less than 5% of 1q12 or 9q12 signals were found in interior and predominantly euchromatic regions of the nucleus, with presumed transcriptional activity (a distance of >1 μm from the nucleolus or membrane was counted as an interior signal). Typically, neither the two 1q12 nor the 9q12 signals were repositioned interiorly in seizure foci. Fewer than 10% of neurons had an interior signal in the seizure focus with these probes, although there was an appreciable increase in interior 9q12 signals in the focus of one patient (Fig. 2A). In the few neurons from the seizure focus in which a 1q12 or 9q12 domain was interior, increased numbers of surrounding astrocytes were observed. This astrocytosis was found beside ~50% of interior signal neurons, equivalent to <5% of sampled neurons, and numerically accounted for the small additional population of interior-signal nuclei in the seizure focus. Thus, additional pathological effects may be responsible for the relatively infrequent interior repositioning of these two chromosomes in the seizure foci. Figure 3A shows a solid model reconstruction of numerous astrocytic nuclei surrounding such a neuron, with one interior and one membrane signal for each neuronal 9q12 homolog depicted in red.

The position of the X chromosome in cortical pyramidal neurons of nine species has been studied by means of Nissl and other classical staining techniques; these techniques highlight the inactive X, or Barr body (*19*). Such studies indicate only a perinucleolar or a membrane residence for the Barr body in female samples; in other words, it is compartmentalized together with transcriptionally inactive centromeric/heterochromatic chromosome domains. The position of the X chromosome in male nuclei, and the non-heterochromatic X homolog in female nuclei has been ambiguous, as these chromosomes do not form large, dense, Nissl-staining domains in the interphase nucleus.

When X-specific probes were used, almost all normal male cortical neurons showed a single signal only on the nuclear membrane regardless of the cortical region sampled (Fig. 3, B and C). These in situ results are clearly different than those of Barr *et al.* (*18*) who suggested that the X chromosome in cerebral cortex of normal males is perinucleolar; possibly they were detecting perinucleolar centromeres. Unlike the X, the constitutive heterochromatin of Yq12 was normally aggregated with other centromeres on the nucleolus (Fig. 3C) in ~95% of neurons by in situ hybridization. When a seizure focus was compared either to a normal sample from another male patient, or to a non-focus control from the same patient, there was a dramatic increase in the percent of interior X chromosome signals (Fig. 2, A and B, inset). An example of this repositioning is shown in Fig. 3E. In contrast, the Yq12 domain, a sex chromosome control, was not moved interiorly in four seizure specimen examined (<10% interior signal neurons). The 9q12 signals were also repositioned interiorly much less frequently than the X chromosome, as counted in parallel, ~40-um vibratome slices from the same tissue blocks (Fig. 2A). This indicates that the euchromatic X chromosome on the membrane of male neurons was specifically repositioned in the epileptic focus. Since control cortical blocks from a patient with severe uncontrollable seizures did not show the robust positional X changes of the seizure focus, it is most likely that these changes in focal epilepsy are not simply a consequence of secondary stimulation events.

In normal female neurons, X homologs were typically either on the nucleolus or on

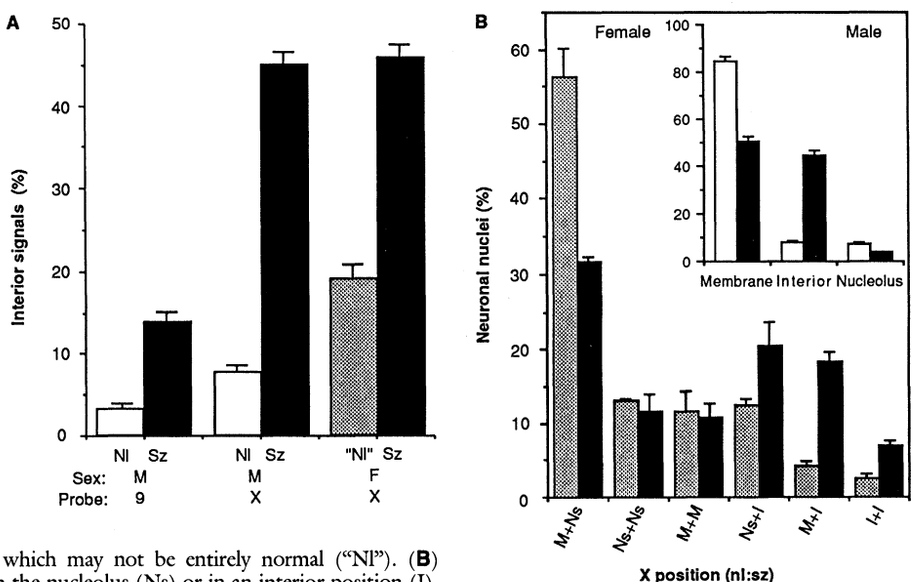

Fig. 2. Representative cases were used to systematically count chromosome positions in neurons from non-seizure and seizure foci (n = 1763). Several vibratome slices of each case were counted [error bars = SEM, n = 282 to 487 neurons for each bar shown in (A)]. Seizure focus samples are shown as solid bars; corresponding control samples shown as open bars (for males) or lightly shaded bars (for females). (**A**) Percent interior X signals are dramatically increased in seizure foci (Sz) as compared to normal samples (Nl). In the male frontal cortex seizure specimen, adjacent vibratome slices from the same tissue block were hybridized to the 9q12 probe and X probes; interior 9q12 signals were considerably less numerous than interior X chromosome signals in the seizure focus. Two seizure foci (parietal and temporal cortex) from females show similar percentages of interior X signal nuclei as male seizure specimen. Each of the two female seizure foci were also not significantly different from each other (*14*). These two specimen are here compared to parallel hybridizations from a female control block only 3 cm distant from the focus, which may not be entirely normal ("Nl"). (**B**) Distribution of X signals: on the membrane (M), on the nucleolus (Ns) or in an interior position (I), where solid bars represent the arrangement of X signals in nuclei of the seizure focus. Inset shows single male X signal is normally positioned on the membrane, and is moved interiorly in the seizure focus. In female neurons, the majority of control neurons show one homolog on the membrane and one on the nucleolus. Both of these X homologs can be repositioned interiorly in the seizure focus (I + I). However, the most conspicuously rearranged cells in seizure foci are M + I, that is, the nucleolar signal is preferentially repositioned. The altered arrangement of signals in seizure foci as compared to controls were highly significant by χ^2 tests ($P < 0.0005$ for male samples, $P \leq 0.001$ for female samples).

the nuclear membrane of cortical neurons, which is similar to the arrangement of the 1 and 9 homologs. The majority of neurons displayed one homolog on the nucleolus with the other on the nuclear membrane (Fig. 2B, lightly shaded bars), although other arrangements were also present. Figure 3 (F and G) shows a normal but less prevalent arrangement of two perinucleolar signals in a neuronal nucleus in relevant optical sections and by three-dimensional reconstruction.

One or both X chromosome signals were moved interiorly in seizure foci from females. There was a numerically significant increase in interior X signals in the seizure foci of two representative female patients where all neurons in the slices were system-

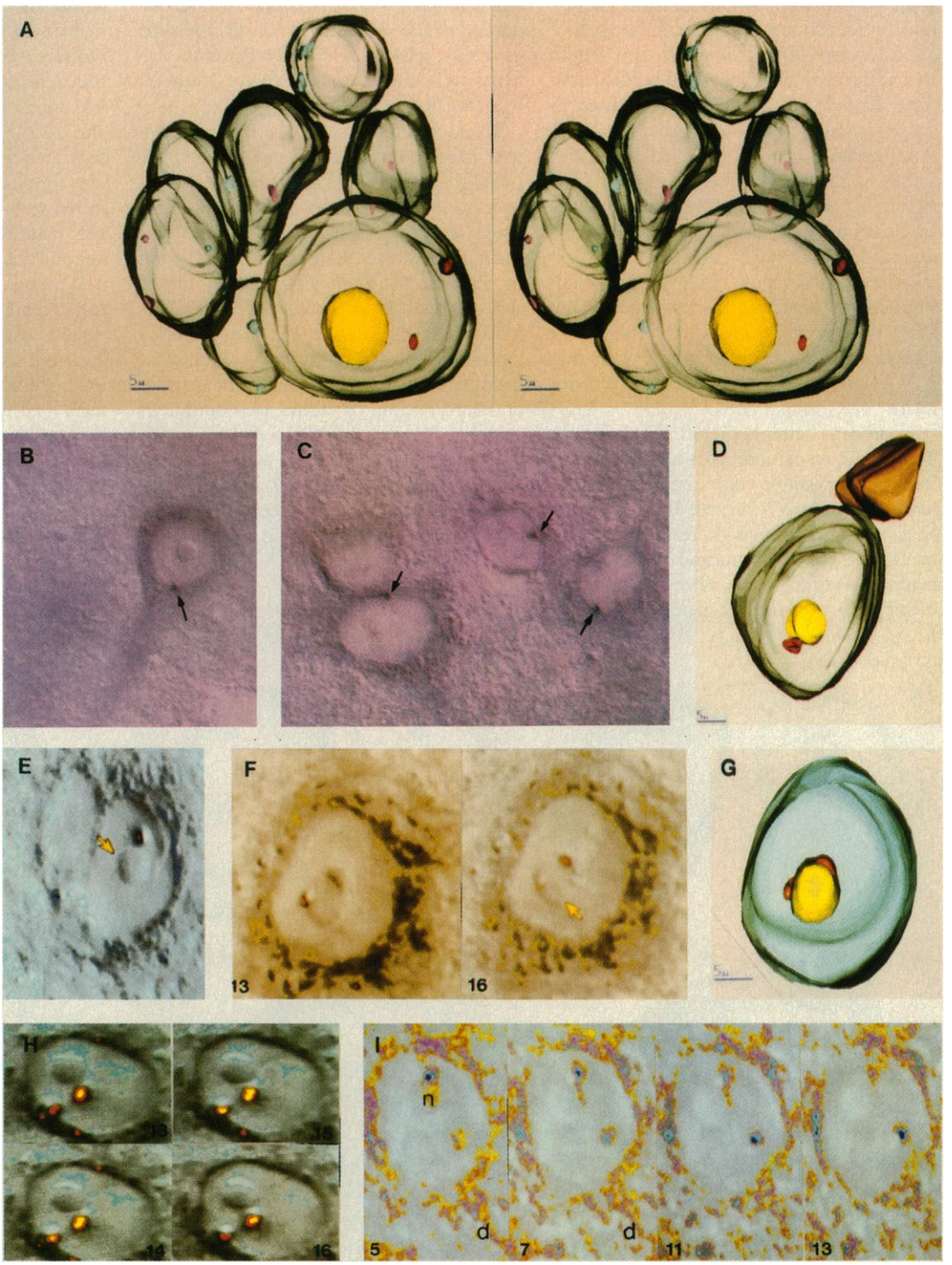

Fig. 3. (**A**) Solid model reconstruction from temporal cortex hybridized to the 9q12 probe; stereo pair. A neuronal nucleus with a large nucleolus (yellow) shows one homolog is interior and the other is on the membrane (red signals). This neuron is surrounded by many astrocytic nuclei; 9q12 signals are depicted in magenta (pink) or cyan (turquoise) for each of these astrocytes. (**B** and **C**) Male frontal and temporal cortex respectively showing signals of the X chromosome on the membrane (arrows) of neurons with different morphology and size. Namorski optical section of vibratome slice by conventional photography; pink color due to storage for several months prior to photography. (**D**) In contrast to X, the Y signal was almost always positioned on the nucleolus, as shown here in a solid model reconstruction where the nucleolus is yellow, the Y chromosome signal is red and the major dendrite orientation is shown in brown. (**E**) Single X signal in a male neuron was repositioned interiorly in the seizure focus; the most intense center of the signal in this digitized optical section is readily discriminated by gray scale (pseudocolored in red) and the arrow points to the nucleolus, which begins to show the perinucleolar heterochromatin. (**F**) Digitized optical sections (frames 13 and 16 of 33 frames with standard 0.3-u steps) from a normal female neuron with two perinucleolar X signals (pseudocolored red). Arrow shows region of perinucleolar heterochromatin. (**G**) Solid model reconstruction of nucleus shown in (F), with two perinucleolar X chromosome signals in red, nucleolus in yellow. (**H** and **I**) Digitized optical sections showing neurons (32 frames each) from female seizure foci with interior X signals, n denotes nucleolus, numbers denote level of optical section within in each series. An interior + membrane arrangement of X signals is seen in (**H**). The interior signal in this nucleus shows an extended doublet motif (greatest signal intensity in yellow, with lower intensity pseudocolored in red in all four frames) and a more compact membrane signal (only frames 14 and 15). I shows an example of a neuron with a perinucleolar (frames 5 and 7) and an interior (frames 11 and 13) X signal (dark blue shows most intense region of signal, surrounded by cyan). Orientation of the major dendrite (d) highlighted by pink-yellow pseudocolor of less dark greys. In all digitized frames the darkest feature in the field was the signal(s), and signals were captured in the linear camera range (80–110 grey scale value of 0–256 total scale) as described (*6, 12*).

atically counted (Fig. 2A). The somewhat higher proportion of interior X signals in control slices from females, as compared to the male cells above, probably relates to the fact that the slices counted for comparison in this analysis were from blocks closely adjacent to the seizure focus (~3 cm apart). Thus there appears to be a gradient of X positional changes spreading from the seizure focus, although other secondary pathological factors, not histologically apparent in this adjacent region, may also be contributory. Unlike the few additional interior-signal nuclei detected with the 1q12 and 9q12 probes, neurons with an interior X signal in the seizure focus did not show significant adjacent astrocytosis. Typical examples of these altered neurons are shown in digitized optical sections (Fig. 3, H and I) and in reconstructions (Fig. 4). As shown in Fig. 4, both large and smaller neurons were affected. Interior X signals were often more extended (Figs. 3H and 4B), a feature that may suggest more genetically active domains (*6*).

In order to better appreciate if there was a preferential repositioning of one X chromosome, each possible combination of homolog positions was analyzed. In seizure foci (Fig. 2B) there was a decrease of 25% in membrane (M) plus nucleolar (Ns) signal neurons as compared to more normal specimens, and a concomitant increase of 27% of cells with interior (I) signals in seizure foci. Neurons with a membrane plus interior signal were most conspicuously increased in the seizure focus (Fig. 2B). It is apparent that both nucleolar and membrane signals can be repositioned to the interior compartment of the nucleus (for example, I + I) although, unlike neurons of the male, there seems to be a slight preference for repositioning of the perinucleolar X chromosome in female cells. This could suggest that in female cells the normally inactive X can be recruited and repositioned.

Thus, chronic uncontrolled seizure activity was linked to profound and specific positional changes of the X chromosome in both males and females. Since these changes occurred in both sexes it was not due to simple recruitment of facultative heterochromatin of the inactive X chromosome in female cells. Remarkably, in 1949 Barr and Bertram found the female X chromosome is repositioned from its normal nucleolar or membrane position in a similar proportion of neurons after 8 hours of continuous electrical stimulation (*20*). Although the Barr body can be confused with other heterochromatic arrays, and has also been mistakenly associated with dense RNA-rich bodies (*8, 21*), the present in situ methods unambiguously define the X chromosome domain.

We have previously proposed that centromeric and constitutive heterochromatic regions may function in interphase and act as organizing centers for the coalescence of facultative heterochromatin during global transcriptional overhauls, such as during differentiation (*6*). The present results support that theory, since the facultative X chromosome was intimately associated with constitutive heterochromatic compartments, defined by paracentromeric, repeated DNA sequence arrays. However, the changes in the X chromosome position in epilepsy were less subtle than we would have predicted, and clearly the X centromere region can be separated from this compartment. The present results also indicate that substantial movements of chromosomes in interphase can occur in the absence of DNA replication and mitosis.

We have shown that these X chromosome movements are essentially limited to epileptic foci and thus they cannot be simply the consequence of generalized seizure activity. With experimental stimulation, the movement of the Barr body in the hypoglossal nucleus of cats took 2 to 3 days to be manifest, and a considerably longer time (~16 days) to show reversion to a normal pattern (*20*). Clearly many of the X chromosomes in this study have not reverted to a normal pattern, and the changes appear to be less acute and transient than recently described changes in *fos* transcripts (*4*). Fu-

Fig. 4. Representative three-dimensional reconstructions of interior X signals in seizure foci from females; (A) and (B) each represent stereo pairs. (**A**) Two smaller neurons, one with interior + perinucleolar signals (magenta) and the second with interior + membrane signals (red). (**B**) A large neuronal nucleus with an extended doublet interior signal. (**C**) Small cortical neuron with two interior X signals (red), and perinucleolar centromere-rich heterochromatin depicted in cyan. Adjacent single glial nucleus is shown in gray (without transparency).

ture studies with animal models of epilepsy and seizure may provide additional insights into the mechanisms, factors, and detailed temporal events that are important in nuclear reorganization, when specific chromosomal probes for these species become available. Although the X chromosome appears to be selectively altered in the present study, additional chromosomes should be sampled.

The current studies provide a novel structural approach for the delineation of nuclear changes in disease processes. Some of the observations here suggest different mechanisms than previously considered in human epilepsy. In the kindling model of focal epilepsy, repeated subclinical stimulation is thought to result in functional alterations in an epileptic focus which then assumes an independent capability to initiate seizures (*22*). Many studies have focused on long-term neuronal membrane potentiation effects and on synaptic modulation to explain kindling. We propose that specific nuclear patterns involving specific chromosome rearrangements may be more or less permanently established from a variety of causes (for example, trauma, developmental abnormalities, scarring, toxins and membrane/seizure activity itself), and that such nuclear changes underlie or give rise to intractable foci of neuronal activity. In this context, relevant genes on the X chromosome, possibly those arrayed near the centromere, deserve further attention.

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11. Tissue biopsies were obtained from human subjects undergoing resective procedures for control of medically intractable focal epilepsy. "Normal" specimens were removed to access a seizure focus. In large parietal, temporal, occipital, and frontal cortex resections, regions >7cm from the focus were also operationally defined as normal. Specimens 3 to 5 cm adjacent to the mapped seizure focus were also evaluated. Foci were localized by continuous EEG telemetry and video monitoring, by depth electrodes as described [S. S. Spencer and D. D. Spencer, *Neurol. Clin.* **3**, 313 (1985)], by MRI (magnetic resonance imaging) and a battery of other tests as part of ongoing investigative projects on epilepsy by D. Spencer and his colleagues. One parietal focus was localized by subdural extraoperative grid stimulation and recording [R. P. Lesser *et al.*, *Epilepsia* **22**, 240 (1981)]. Electrode tracts were not within the samples used for in situ hybridization and one normal sample with no depth electrode exploration was additionally evaluated as a normal control. The five patients whose samples were reported had the following age (sex): 27(M), 28(M), 55(M), 22(F), and 30(F). All patients except one had complex partial seizures, no parental or sibling history of seizures, no history of febrile seizures, and onset of seizures in childhood or adolescence, that is, >4 years of documented medically intractable seizures. Frequency of clinical seizures ranged from 4 to greater than 15 per month. Further details on patients are available on request. Complete neuropathological workup of all specimen was also done in standard paraffin sections.
12. Briefly, samples were placed in picric acid–paraformaldehyde fixative in the operating room, and multiple blocks were cut under fixative 30 minutes to 1 hour later. Vibratome slices of ~35 μm were freeze-thawed (*5*) to increase penetration of DNA probes. Plasmids were labeled with bio 11 dUTP by nick translation to an average length of ~300 bp. Tissue slices and labeled DNA were denatured together and hybridized overnight at relatively high stringencies (60% formamide–2× SSC, 39°C) as described in (*6*), and T. C. Cremer *et al.*, *Exp. Cell Res.* **176**, 199 (1988). Hybrid molecules were detected either with strepavidin and biotinylated alkaline phosphatase, or alternatively with rabbit antibody to biotin (anti-biotin) followed by goat anti-rabbit peroxidase. Representative slices of the latter were flat embedded in Epon for thin sectioning; silver intensifications of peroxidase signals (*9*) prior to embedding increased the minor cytoplasmic signals disproportionately (Fig. 1), probably due to more rapid penetration of the cytoplasm in these ~3-minute reactions; BrdU was used to label late replicating DNA for banding of lymphocyte metaphase chromosomes in studies of probe specificity; BrdU bands were detected with fluoroscein isothiocyanate in an antibody sandwich technique, and hybridized sites were simultaneously detected with alkaline phosphatase (*6*). A Hamamatsu video camera attached to a digitizing board (512 by 512 by 8 bits pixel resolution, 256 gray levels) on a Vax Graphics Workstation II was used to examine and store 0.3-μm step optical sections for pseudocoloring, volume determinations, and solid model reconstructions. Programs have been described in detail (*6, 16, 17*) and source codes [J. Borden, M.D. thesis, Yale Medical School (1988)] are available with consent of the authors.
13. Probe (pBAM X7-E) was obtained from H. Willard and has been described [J. S. Waye and H. F. Willard, *Nucleic Acids Res.* **13**, 2731 (1985)]. Other cloned probes are: pUC1.77 for 1q12 [H. J. Cooke and J. Hindley, *ibid.* **6**, 3177 (1979)]; pHY2.1 for Yq12 [H. J. Cooke, J. Schmidke, J. R. Gosden, *Chromosoma* **87**, 491 (1982)]; pHuR 98 for 9q12 [R. Moyzis *et al.*, *Chromosoma* **95**, 375 (1987)].
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Complementary Hemispheric Specialization in Monkeys

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Twenty-five split-brain monkeys were taught to discriminate two types of visual stimuli that engage lateralized cerebral processing in human subjects. Differential lateralization for the two kinds of discriminations was found; the left hemisphere was better at distinguishing between tilted lines and the right hemisphere was better at discriminating faces. These results indicate that lateralization of cognitive processing appeared in primates independently of language or handedness. In addition, cerebral lateralization in monkeys may provide an appropriate model for studying the biological basis of hemispheric specialization.

IT IS IMPORTANT TO KNOW WHETHER nonhuman primates have complementary specialization of the cerebral hemispheres corresponding to the well-known differences described for human beings (*1–5*), in whom some types of information are typically processed better by the left side of the brain and other types better by the right (*2*). For example, such information would help to determine whether the lateralization of handedness or language in humans led to more global hemispheric specialization for cognitive processing, as is frequently stated (*3, 4*), or whether hemispheric differences in cognition are independent of handedness

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