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Genomic stability and instability in different neuroepithelial tumors. A role for chromosome structure?

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Summary

Selected childhood and adult neoplasm exemplify fundamental differences in their propensity for genomic change. DNA replication is essential for the formation of neuroepithelial tumors, probably because the genome can be remodeled. Nonetheless, several differentiated and stable childhood neoplasms retain their nuclear controls for differentiation. In contrast, rapidly arising gliomas often show a variety of phenotypic changes. Genomic plasticity and instability allow gliomas to flexibly adapt to new environments. Gene changes (in DNA) can be limited in childhood tumors whereas more widespread genetic changes in malignant gliomas indicate a fundamental alteration in many chromosome regions. Can such regions be defined? We used one repeated DNA sequence (TTAGGG)n, present at the end of all normal human chromosomes, to investigate chromosome termini in more detail. Pulsed-field gel electrophoresis showed this region can be unusually variable, as several other multilocus probes did not reveal comparable changes. Because telomeres form unique chromosomal structures, and are thought to provide essential signals to position chromosomes in the interphase nucleus, it was pertinent to assess these regions by in situ hybridization. Many telomeric domains localized at variable as well as interior nuclear positions in glioma cells. These positions, which are presumably abnormal, may be generated by the DNA variants observed. Such position changes may contribute to the more general 'disorder' observed in glioma nuclei. Other chromosome domains with a unique DNA-protein structure may define additional genomic loci that are preferentially modified in neoplasia. A fundamental understanding of chromosome structure should clarify the problem of multilocus instability in glioblastoma.

Introduction

Neurons and macroglia derive from the same germinal layers of the neuroepithelium yet have vastly different neoplastic potentials. Larger neurons develop from neuroblasts defined by their precise time of exit from the germinal matrix. Although some of these new neurons may be selectively discarded during early mammalian development, most are committed to an extraordinarily long lifespan without recourse to mitotic self-renewal. Because the germinal matrix disappears in early life, there is no new source of larger neurons. In contrast, other germinal epithelia throughout the body continue to divide throughout life. Basal cells of the skin for example, continuously replenish their rapidly discarded terminally differentiated progeny. One obvious reason why there are essentially no 'tumors' of larger neurons (excluding the relatively rare collections of developmentally altered cells), is that terminally differentiated neurons are incapable of cell division. Part of the solution to neoplasia may be clarified by focusing on the cellular, developmental and chromosomal controls that *prevent*

DNA synthesis and/or mitotic segregation of chromosomes in larger neurons. Because neurons in some non-mammalian species are capable of DNA synthesis, and maintain multiple copies of their genome in a single interphase nucleus without cell division, the restriction on neuronal division is likely to be controlled by more than a single enzyme or gene product.

Macroglia (astrocytes and oligodendroglia), as well as small neurons, have a different developmental history that prepares them for variable cycles of cell division, and more flexible forms of differentiation in response to stimuli outside of the germinal matrix, i.e., in the substance of the developing central nervous system (CNS). They can respond to the sequential developmental changes of larger neurons, such as those associated with synapse formation. They often undergo at least one round of cell division before they settle into their terminal lineage, and macroglia differentiation is linked in time with the development of synapses [1]. Nonetheless, at least in young animals and humans, these embryonic glial precursor cells continue to coexist with their more differentiated progeny. For example, in viral infections of young animals, remyelinating oligodendrocytes probably derive from remaining precursor cells after at least one round of replication. Notably such developmentally 'late wave' cells, which remain mitotically capable, have a greater neoplastic potential than larger neurons in both an experimental and natural setting.

I discuss a few human neuroepithelial tumors with respect to mechanisms of genomic change that are likely to require a mitotic event. The word genome is used in the broadest sense, and encompasses all the complex proteins and structural elements of the interphase nucleus [see 2 for a review]. The neuroepithelial tumors briefly discussed here have been chosen to represent a spectrum of neoplastic change. In some of these tumors changes are limited, whereas in others they are extraordinarily complex. The reasons for these variable propensities remain unclear. In this context, I will present some experimental evidence for complex changes that underscore a severe genomic instability in malignant gliomas. In the following overview I stress selected mechanisms and concepts that may be useful for particular tumors, as well as several paradoxes of morphology and biology to elicit further discussion.

Tumors with an earlier age of onset

Embryonic precursor cells with a growth advantage

What is the consequence of having mitotically capable precursor cells? First, if cell differentiation can be imprinted [3] during DNA replication, then a reversal of this process can lead to abnormal growth of a relatively normal cell, provided genes specifying a growth advantage are affected. For example, DNA methylation, one mechanism for imprinting which can stabilize the repressed state of specified genes and gene regions, can be erased by new DNA synthesis. Demethylation changes would be subtle at the molecular level, especially if only one or two small loci are affected. Moreover, as a random and correctable event, a demethylation change would infrequently define a perpetuated growth advantage. Likewise, new methylation of certain DNA sequences, as well as other chromosomal protein changes that result in gene silencing, could have similar consequences. For example, methylation or inactivation of a suppressor gene [reviewed in 4] may also lead to a reversible growth advantage by releasing a cell from its normal growth constraints. In all the above cases one would predict that 1) the tumor formed would be extremely slow growing because revertant cells might be continuously produced, and 2) the tumor would express virtually all of its original biological properties.

This type of mechanism typifying non-permanent change may be operative in examples of midline and cerebellar spongioblastoma polare, a relatively rare tumor of the CNS. The cells of a spongioblastoma polare are highly uniform, extremely slow growing, and resemble glioblasts (i.e., 'spongioblasts') formed during embryonic life and maintained as recruitable astrocytic precursor cells in young animals. Remarkably, such slow growing tumors display the characteristic and unusual ability to grow in an alien species [5], a feature confined to embryonic cells (including neurons) and highly ma-

lignant cells [6]. Clearly these tumors are not highly malignant because the incompletely removed tumor can take over 15 years to regrow in the same locus, with the same features. Several of these have been documented in our collection.

The concept that some CNS tumors may derive from embryonic precursor cells with subtle growth advantages is therefore useful. Although a 'primitive' cell phenotype is often equated with rapid growth, the growth of normal precursor cell variants need not follow this rule. Indeed, treatment of such tumors with radiation or chemotherapy may more permanently destabilize the genome, adding undesirable new growth advantages for the activated cell.

More permanent gene alterations: retinoblastoma

Although a reversible modification of suppressor genes in tumors as postulated above is not generally considered, more permanent nucleic acid base changes in suppressor genes and activator genes have been amply documented in a number of tumors. One of the most informative examples involves retinal precursor cells of the neuroepithelium. Because the suppressor Rb (retinoblastoma) gene is defective, the phosphoprotein Rb, which is involved in both transcription and cell cycling, is unable to exert its growth suppressive effects in the interphase nucleus. In inherited cases of retinoblastoma, both Rb alleles are defective, and tumors are expressed at an early age. Nonetheless, there are cases in which mutations develop in normal alleles with similar consequences [7]. Such mutations presumably occur in selected cells during DNA synthesis, either as a random event, or for predisposing reasons that remain unknown. This simple permanent genetic change appears to be sufficient for defining both growth and malignancy in retinoblastomas possessing only this single gene alteration. In such genetically simple examples of retinoblastoma, the growth of cultured cells can be completely reversed by insertion of a normal Rb allele [8]. This remarkable reversal, effected by insertion of a single gene, appears to be unique.

Neuroepithelial tumors commonly exhibit more complex genetic changes that have not been cured by similar means. In the more common neuroepithelial gliomas for example, it has been difficult to define simple initial genetic events that underlie a plethora of secondary or progressive alterations (vide infra). Moreover, an inherited loss of both Rb alleles only *predisposes* selected cells to form tumors, *but does not inevitably lead to tumor formation*. This, as well as the restricted tissue expression of Rb-associated tumors, should give pause to those with a monolithic view that limited DNA changes *alone* (in suppressor or enhancer genes) cause cancer. Tumorigenesis is also likely to rest on as yet undefined exquisite differences between individual cells as well as environmental factors.

Neuroblastomas: differentiation, malignancy and amplification

Neuroblastomas provide another example of tumors formed from precursor cells with mitotic capability. Post-natally, a few neurons of the peripheral sympathetic-parasympathetic nervous system continue to divide. Thus these neurons contrast with larger neurons of the central nervous system. In neuroblastomas, characteristic and limited genetic changes are observed. Neuroblastomas can display a numerically normal 46 chromosome complement, unlike the more common adult tumors of the CNS. However, they typically contain an amplified chromosome domain (amplicon). This unit contains multiple gene copies of the N-myc oncogene (which codes for a nuclear transcription protein), as well as large stretches of adjacent DNA. Amplified genes, first observed as homogeneous staining regions in neuroblastoma chromosomes, can also exist as extrachromosomal DNAs (double minutes) of ~ 3 million base pairs (Mb) in length as shown by pulsed-field gel electrophoresis (PFGE) [9].

Genetic amplification requires DNA synthesis, and in the case of mammalian tumors, underscores an archaic mechanism of cellular adaptation. In non-mammalian species such as Xenopus, an increased synthetic capacity is achieved by ribosomal gene amplification. Although DNA amplification is not normally utilized by mammalian cells to in-

crease synthetic products, local environmental changes, or specific stimulatory growth factors, may provoke this archaic mechanism in selected target sympathetic neurons that are still dividing. The amplification of the N-myc gene may reflect an aberrant response to a normal growth factor or other stimulus, or, a compensatory mechanism for an ineffectual regulatory protein produced by a non-neoplastic cell. This response driven mechanism is appealing because gene amplification in mammalian tumors is frequently provoked by chemical treatments. (For example, amplification of the dihydrofolate reductase gene is a well-known response to a chemotherapeutic agent.) This concept also has relevance for glioblastomas that can express a variety of different amplified genes (vide infra). Because some glioblastomas can have a highly variable phenotype, selected tumor cells producing a stimulatory factor (e.g., PDGF) may elicit genetic amplification in a variant tumor cell of the population, with consequent secondary growth advantages.

In neuroblastomas, amplification of the N-myc gene by itself does not explain the malignant or invasive capacity of these cells, although N-myc amplification and its consequent expression may enhance cell cycling (replication). Possibly the invasiveness of this childhood tumor may relate to maintenance of an embryonic capacity. On the other hand, some neuroblastomas have also shown deletion of a region on chromosome 1p32-36 thought to encode a suppressor gene [reviewed in 10]. Moreover, invasive neuroblastoma cells can maintain a highly differentiated neuronal appearance, even without the stimulus of nerve growth factor [11, 12]. Thus the amplification of the N-myc transcription factor only vitalizes gene regions that are already determined, i.e., those in an accessible and responsive state.

Neuroblastomas exemplify three instructive features for the problem of neuroepithelial neoplasia. First, external factors may ultimately initiate permanent genetic changes. Second, biological capacities such as invasion may involve cellular responses not directly related to the visibly altered gene region. And third, genetic changes covering a locus as large as 3 Mb does not inevitably lead to a more general genomic instability. N-myc amplification units are typically maintained with few rearrangements in each neuroblastoma line [13]. Moreover, individual clones of neuroblastoma cells can maintain both their neuronal phenotype as well as a stable chromosome complement with extended passage *in vitro* for more than 10 years [11]. In such cells the only chromosomal change thus far detected has been the morphological form of the amplicon. The amplicon in daughter cells stabilizes either as an integrated homogeneous staining region, or as extrachromosomal double minutes (dm, 9). In both cases cells appear identical, except for a slower growth rate in dm cells (unpublished data). The reason for a *lack* of de-differentiation and/or aneuploidy in these neuroblastoma cells may rest on the extensive but as yet uncharacterized molecular controls associated with committed neuronal differentiation, as suggested in the introduction. Some of these controls may be a consequence of structural changes in the interphase nucleus as discussed below.

Viral oncogenesis, temporal susceptibility and medulloblastomas

The importance of developmental cell fate for phenotypic tumor expression in the cerebellum has been recognized for a long time. At the turn of the century, Shaper postulated childhood medulloblastomas derived from 'indifferent cells' of the external granule cell layer of the cerebellum. These cells divide post-natally and are precursors for both astrocytes and small granule neurons. Although this idea fell from disfavor with many neuropathologists, more refined immunocytochemical markers have recently substantiated this concept. Medulloblastoma cells can exhibit both neuronal and glial markers [14, 15].

I bring up this example because the experimental creation of medulloblastomas emphasizes the temporal limits for tumor formation and susceptibility. Additionally, it serves as an example of virally induced genetic change. The human papovavirus (JC and comparable isolates) from adult cases of multifocal leukoencephalopathy, was considered to have a neoplastic potential because bizarre astrocytes were observed. Papovaviruses, such as SV-40, are

known to require DNA synthesis for genomic integration, and characteristically lead to many types of experimental tumors, hence the original subgroup name *poly*oma. It is therefore not entirely surprising that the human JC isolate, when injected into hamsters, caused the formation of a variety of brain tumors. What was more remarkable, however, was that specific and different tumor phenotypes were obtained in animals inoculated at different ages. And medulloblastomas were produced only when young animals were so challenged [16]. Thus this human virus is sufficient to cause a tumor in an experimental setting, and potentially could have a role in human neoplasias. Presumably the virus targets residual dividing (precursor) cells of the cerebellum. Alternatively, it might cause reinitiation of DNA synthesis in an incompletely differentiated post-mitotic cell. This raises several interesting issues for human medulloblastomas and other tumors.

First, these findings emphasize the exquisite nuclear controls that limit genomic expression. Some of these controls may be surpamolecular, or structural, i.e., involve chromosome folding. Second, recent estimates indicate that over 60% of humans carry papovaviral sequences in a latent integrated form. These viral sequences are increasingly detected in a variety of non-neoplastic CNS diseases with PCR techniques. Yet over the last 20 years the incidence of medulloblastomas has declined at Yale and other hospitals. This is a perplexing paradox. One simple explanation of this discrepancy might be that papovaviruses are rarely present when dividing external granule cells of the cerebellum are susceptible to tumor formation, i.e., at a young age. Alternatively, one might argue that human papovaviral variants with a reduced tumorigenic capacity have recently become more prevalent. Humans may also be evolving selective defense mechanisms against these endemic viruses. Although it is difficult to explore this latter possibility experimentally, it remains an intriguing question. Finally, the latent integration of papovaviruses brings up the issue of tumor susceptibility as a function of one or more common latent viruses. In this context prevalent Herpes viruses (e.g., EBV) and retroviruses (e.g, HTLV-I) have been linked to tumor formation in some populations. These viruses create complex changes on the nuclear level, by their residence as epigenetic structures in the nucleus, or as transcriptionally competent genomic integrants that can target multiple genomic sites [17]. Rather than emphasize the many growth enhancing oncogenes, transcriptional proteins and specific viral products that have been extensively studied and well reviewed in the context of neoplasia [e.g., 18–20]. I would like to underscore two more general concepts that may have some use. Although viruses can help to initiate neoplastic processes, viruses may also kill nascent tumor cells. Viruses can selectively and completely destroy neoplasms in animals [21], and more recently, engineered viruses have been used to target several types of tumors. Second, *latent viruses may create additive genomic stresses that cause new patterns of nuclear organization and response*. These new patterns may ultimately define the neoplastic cell. This brings me to the problem of adult (supratentorial) gliomas where genomic instability is pivotal.

Adult gliomas

In the adult mammalian nervous system astroglia have the highest capability for cell division. Although the detailed functions of astroglia are not entirely understood, hyperplasia of these cells is clearly part of a response to injury. Because mitosis occurs in well differentiated astrocytes [1], some remodeling of glial functions is likely to occur continuously in low numbers of normally dividing astrocytes. Thus these more differentiated cells provide a potential ground for neoplastic transformation. The very low-grade astrocytomas can sometimes be difficult to distinguish from a normal cellular response to gross injury, or to more subtle environmental changes. Such low grade astrocytomas may be biologically distinct from astrocytomas that develop malignant changes over a period of years. In the adult, evolving astrocytomas frequently show the characteristics of progression, i.e., they go 'from bad to worse'. Nonetheless, experimental studies point out progression is not an inevitable fate for all new growths, and abortive or regressive tumors are often ignored [22].

Similarly, oligodendrogliomas also often progress, and display aggressive characteristics and morphological phenotypes that are indistinguishable from the more malignant astrocytomas. In the adult, oligodendrogliomas may derive from mitotically capable cells with molecular markers specific for oligodendroglia rather than less differentiated precursor cells. Such phenotypically marked cells can synthesize DNA after trauma or other insults [23, 24]. However, the mixed cellular phenotype of these tumors (with astrocytoma cells) indicates they are less rigidly differentiated than some of the childhood neoplasms described above, i.e., the controls on their genomic expression are flexible.

The phenotypic flexibility of the glioma group is often quite apparent in later biopsies of the same tumor. Increasing cellularity indicates the selective advantage of more rapidly dividing cells, and when more uniform cells predominate, it is likely that a clonal variant has been selected. Nonetheless, anaplasia (variations in cell size, shape, and expression of different phenotypic markers) is often seen. This underscores the adaptability of these cells to various humoral and environmental factors, some of which may be secreted by tumor variants. Several secreted factors may contribute to necrosis, an indicator of poor prognosis. More uniform appearing glioblastomas can also provoke regional necrosis in the surrounding parenchyma. Although rapid growth may explain this necrosis, variant cells that are not yet recognizable on a morphological basis, may also secrete necrosis-inducing factors.

The clonal hypothesis, environmental modulators and genetic markers

The paradigm of progressive stepwise changes in a clonal variant is appealing in the case of evolving and more uniform tumors. Recent studies of p53 mutations indicate that some more malignant gliomas can be clonal [25]. However, anaplastic tumors are less readily explained by this clonal mechanism, and p53 clonal expansion was detected in only 4 of 7 'higher grade' gliomas. Indeed, the case for clonal selection may be even less compelling for understanding the primary glioblastoma multiforme, the most common neuroepithelial tumor of adults. These more common glioblastomas ($\sim$ 57% of all gliomas at Yale) appear suddenly, often have pleomorphic features, cannot be related to a small indolent growth (a very rare finding in most autopsy brains), and rapidly lead to a patient's demise. Moreover, these highly malignant primary tumors, unlike evolving gliomas, are capable of heterologous growth in the brain and eye [5, 26]. The pleomorphism and biological variety of such glioblastoma cells are also demonstrable experimentally. For example, in different environments such as tissue culture and heterologous transplants, human glioblastomas produced different characteristic morphological patterns, including epithelial (carcinoma-like) tumors [26]. A variety of more permanent changes, including distinctive chromosomal patterns, were also induced by different environmental conditions (sites of transplantation). These were most consistent with selection of different variants.

The selection of variants does not necessarily indicate selection of more malignant cells. Remarkably, more malignant properties of these glioblastomas could be lost, or more permanently repressed [26]. For example, after *in vitro* propagation, virtually all glioblastoma cell lines lost their ability to grow in the brain unless first transplanted to a subcutaneous site in cortisone treated animals. Even in cortisone treated animals, subcutaneous tumors were often abortive.

Despite the biological malignancy of primary human glioblastomas, they do not metastasize to other visceral organs except when seeded iatrogenically by surgical procedures such as shunting. This seems especially perplexing because glioblastomas frequently show neovascularization, a result of several different tumor associated factors [e.g., 27]. Thin-walled vessels in these invasive tumors should increase the likelihood of spreading tumor cells to other organs. However, subcutaneously inoculated glioblastoma cells often elicit a lymphocytic and necrotizing inflammatory response [26]. More recent transfection studies also indicate lymphocytic surveillance is effective in eradicating experimental rat gliomas [28]. Thus in humans, cell mediated immunological surveillance may play a role in effectively destroying most of the widely variant cells

that are likely to seed the body in small numbers. Other local environmental or tissue specific factors may also play a role in inhibiting the metastatic growth of glioblastomas. Many extra-neural tumors similarly show a strong preference for specific tissues, i.e., a supportive environment. Perhaps one can exploit extra CNS tissue products to limit the growth of glioblastomas by recombinant means.

More recent genetic and chromosomal studies also attest to the variety of changes that can take place in glioblastomas. Even in evolving gliomas, the path of progression is neither simple, nor is it characteristic for this class of neoplasms. Molecular genetic studies have shown various amplifications in oncogenes or their receptors [e.g. 29–31]. However, none of these are universal in glioblastomas, and in most cases it is not known if they are invariably present in all of the tumor cells. Additionally, a large number of different gross chromosomal changes, including translocations, deletions, and over-representation of entire chromosomes have been detected, and these have been partially catalogued [e.g., 32]. More subtle cytogenetic changes in gliomas have also been delineated by in-situ suppression hybridization [33], and by comparative genomic in situ hybridization [34]. The molecular application of newly isolated DNA sequences from the human genome project to these tumors, is likely to extend even further the list of genetic rearrangements, amplifications, and deletions that can be found in glioblastomas.

How informative will such a list be for understanding the biological propensities of these cells and their malignant properties? Will one or two limited DNA sequence changes define both the neoplastic initiative, and the nature of the subsequent changes that can be latent, slowly progressive, or rapid? Moreover, will they lead to a unified understanding of progressive change? The observation that even clones of an individual glioblastoma cell can give rise to heterogeneous variants at the chromosomal level [33, 35], might indicate that DNA sequence changes are not the entire answer. Rather, the fundamental problem of genetic instability in glioblastomas needs to be addressed. The variable patterns of phenotypic expression and invasiveness may ultimately rest on more general (supramolecular) features of genomic organization.

The experimental data briefly presented below emphasizes large molecular structures and genomic change at multiple sites. Although this work is in its infancy, it suggests that some genomic sites are differentially susceptible to change. It also contrasts the relative sequence stability of a malignant neuroblastoma with variations seen in different glioma cells that are comparably passaged. Specific DNA sequence motifs may define hypervariable loci. However, differences among tumors may also depend on higher levels of chromosome structure (or folding) at defined loci. In this context I return to the underexplored area of supramolecular nuclear organization because it is likely to have a role in limiting or expanding the repetoire of tumor cells.

Selected hybridization studies

PFGE patterns assessed by hybridization of defined sequences

Pulsed-field gel electrophoresis (PFGE), which can resolve very large restriction fragments of DNA (50 kb to 5 Mb in length), has been used extensively in the human genome initiative. It has not been extensively applied to the study of genetic changes in tumor cells. However, PFGE blots can provide a view of DNA sequence changes that are too subtle to detect on metaphase chromosomes. Additionally, non-coding DNA domains, which constitute $\sim 95\%$ of the genome, can be assessed. Such 'extragenetic' DNA domains include transcriptional control regions, as well as those that have a propensity for alterations linked to pathological breakage, recombination and deletion. We have studied the hybridization pattern of several non-coding repeated sequences in order to define those that may be more generally involved in the genomic instability of gliomas. In such studies our ultimate aim is to understand more general rules of DNA sequence organization that can define regions expecially prone to damage. In the following example I show one repeat sequence associated with variability, as well as

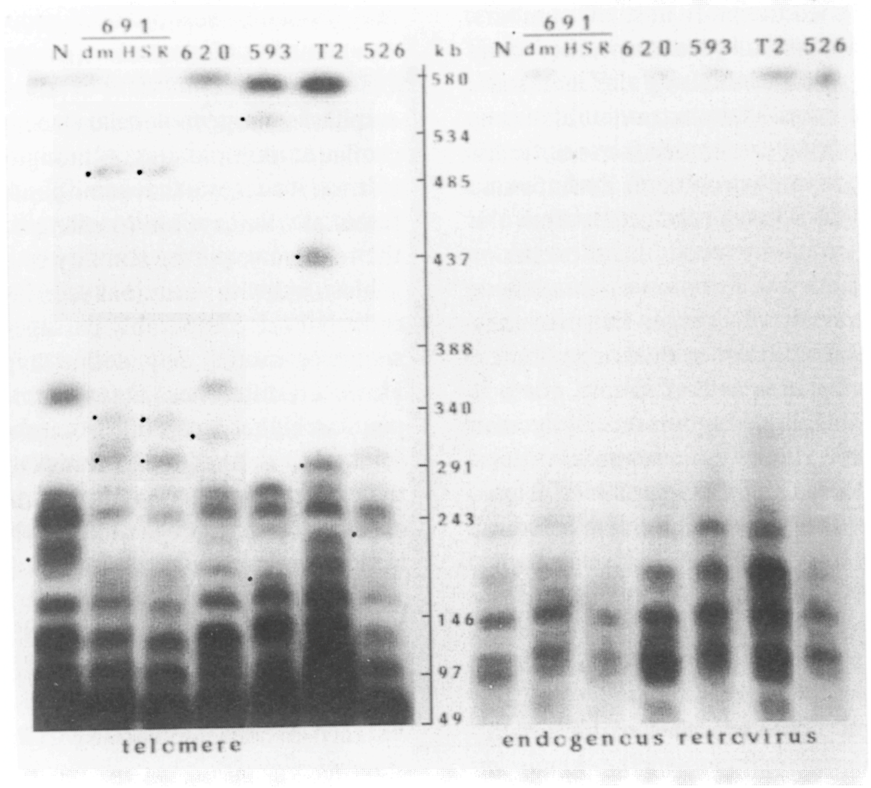


Fig. 1. PFGE blot of different transformed cells (see text, 11). The left panel shows hybridization to a ^{32}P labeled oligonucleotide with the canonical telomere repeat (24 mer). Variable bands are detected (see dots) in each cell line. The right panel shows the same blot, hybridized to a retroviral insert [17]. Note the uniform size of bands in each cell line. This equivalent banding pattern also indicates the digests were complete in each sample. PFGE was done as previously described with minor modifications [44].

another that is relatively invariant in neuroepithelial tumor cells.

McClintock originally found chromosome ends were especially important in both 'healing' and recombination [36]. Specific DNA sequence motifs in these terminal regions have now been determined. Studies in tetrahymena revealed telomeric repeats of a canonical DNA sequence that could be added to chromosome ends by an RNA dependent (reverse transcriptase-like) telomerase [37]. Another canonical (TTAGGG)n repeat is conserved in mammalian evolution, and is present on the ends of all human chromosomes [38]. More recently, a reduction in the overall number of these telomeric repeats has been related to cell senescence, i.e., a failure to divide [39, 40]. On the other hand, the stabilization of longer telomeric repeats has been correlated with transformation (immortality) and chromosome instability [41]. Because telomeres represent specialized chromosome structures that may be especially prone to alteration, and also can be involved in transformation, it was pertinent to study these repeats in neuroepithelial tumors. We used PFGE to resolve inidividual variants, and were able to delineate changes in several individual human chromosomes. In such studies, the enzymes used to fragment the DNA cut outside of the telomeric repeat. Thus the variations detected can be assigned to sequences that are more proximally located, i.e., toward the centromere. Several of these more proximal sequences are also confined to a few chromosomes. The intensity of resolved bands additionally gives an indication of the number of telomeric repeats in each large DNA fragment.

The variation in telomere patterns are obvious in the different cell lines examined as shown in Fig. 1. This blot included the following cells: 1) lymphocytes derived from normal blood samples (N), 2 &

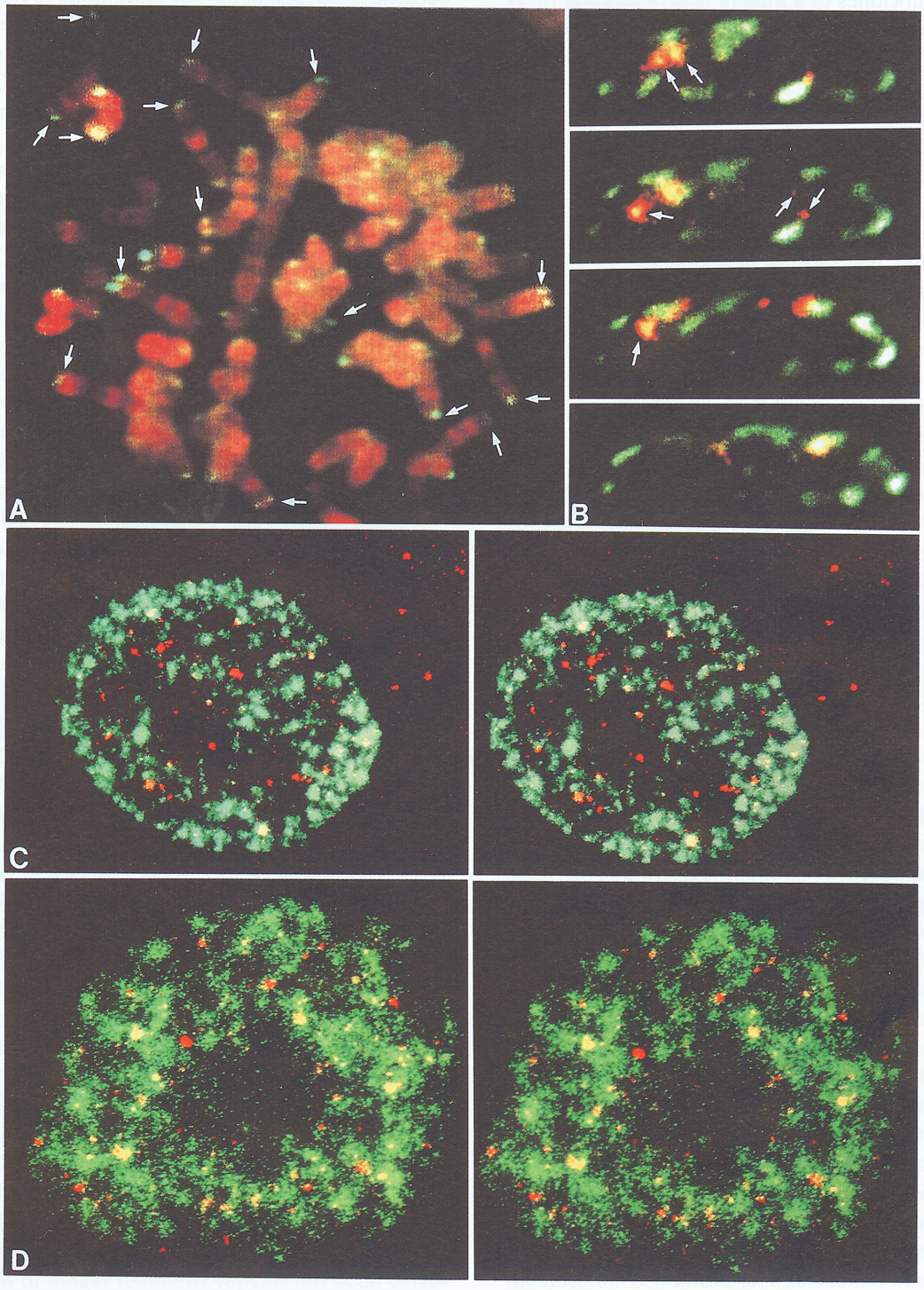
3) two subclones of a human neuroblastoma (691) from the same parental cell, one with a double minute (dm) and the other with a homogeneous staining region (HSR). In both cases there are ~ 50 N-myc copies in each amplicon (9), 4) an oligodendroglioma line (620), 5) a glioblastoma propagated only in tissue culture, 6) an embryonic teratocarcinoma (T2) that is pluripotent and capable of neuronal differentiation (42), and 6) a glioblastoma cultured after heterotransplantation in the anterior chamber of the guinea pig eye (526). The teratocarcinoma line was included because germinal cells contain unusually long telomere repeats [see 37].

Each tumor shows its own individual pattern of telomeric DNA bands. Each of the distinctive DNA fragments in each cell line are noted by dots in this Sfi I digest. These differences are not simply a consequence of *in vitro* propagation because the dm and HSR variants of TC 691 show an almost identical pattern of telomere bands, although separately subpassaged for over 10 years. Only one extra DNA fragment at ~ 310 kb is detected in the dm line. Therefore this line retains a remarkable stability in this most telomeric region. Additionally, each of the well resolved fragments marked by dots represents a specific change that is limited to only one or two chromosome telomeres, as judged by the relative signal intensity of these fragments. One minor band at ~ 240 kb (see dots) was present only in the glioma lines 593 and 526 despite their different histories of propagation. This may indicate a change common to gliomas. However, human telomeric repeats have not been previously resolved by PFGE, and there is little comparative data available for different somatic cells, tumors and individuals. Therefore at this point it is only possible to conclude that many variants are readily detectable in these different tumor cells. Presumably these exceed the polymorphic variation from individual to individual.

Several other probes that define specific chromosome domains were also used to probe these same blots. LINE sequences, which cluster in most Giemsa-dark chromosomal bands and can define another subgroup of PFGE fragments [43, 44], showed negligible pattern variation in different cell lines (data not shown). This may be due to inadequate resolution of the many individual LINE-rich domains, or because these regions are *less* susceptible to change. However, these LINE hybridizations (as well as ethidium bromide profiles) did confirm there were comparable amounts of DNA in each lane. Thus there are variations in the *total number* of telomeric repeats in each line.

PFGE telomere fragments of equal length show differences in intensity in each line. Therefore the number of telomere repeats can vary at different chromosomal sites. Notably, TC 691 and TC 526 both show a generally reduced intensity of telomeric bands (compare lanes N, dm and 526). Thus findings in these two lines capable of permanent growth *in vitro* do not support the general assumption that a global reduction in telomeric repeats causes senescence. A contrasting view, that telomere loss underlies transformation and chromosome instability [45], is also not supported in these studies. Telomere loss is not readily apparent in gliomas 620 and 593, both of which show multiple gross chromosomal changes [33, 35]. These two neuroepithelial tumors are unlike most other tumor lines examined, where loss of TTAGGG repeats were repeatedly found.

Hybridization of an endogenous retroviral sequence further demonstrated telomeric domains were unusually susceptible to variation. Retroviral sequences were chosen for comparison to telomeric DNA because retroviruses also enter the genome after RNA dependent transcription and DNA ligation. Moreover, endogenous retroviruses can continuously reintegrate into the genome [46], and potentially provide new sites of genomic instability. Reintegration may also delineate 'hot spots' susceptible to further damage. The human endogenous retroviral sequence chosen for study has preferentially integrated in a limited number of telomeric Giemsa-light chromosome bands associated with recombination [17]. The right panel in Fig. 1 shows the PFGE pattern obtained using this C type human proviral sequence as a probe. The pattern of autoradiographic bands is essentially identical in each cell line. Darker films, revealing longer fragments in this restriction digest, as well as studies with different restriction enzymes, showed a remarkably invariant pattern in each cell line with this probe (data not shown). Some differences in band intensity were observed, and this may reflect differ-


A
B
C
D

ences in representation of specific chromosomes harboring these retroviral sequences in each of the tumor lines. In summary, chromosome regions defined by this retroviral sequence appear to be *less* susceptible to change than sequences most adjacent to chromosome termini.

In-situ studies of interphase chromosomes, dynamic organization and flexibility of response

In highly differentiated neurons, centromeres and some telomeres maintain highly conserved and specific patterns of nuclear organization [47, 49]. Adult astrocytes also show distinct patterns of centromeric placement in the nucleus, but are less rigidly defined. At the other end of the spectrum are glial tumor cells. These display highly variable patterns of centromere and nucleolar organization [12, 35]. Such variable patterns are part of a flexible or adaptive response to changing environmental stimuli. More recent experiments on glioma lines using probes that 'paint' an entire individual chromosome further document the extreme variability of 'disorder' of interphase chromosome positions in individual glioma cells [33]. A fundamental plasticity in interphase chromosome organization can provide glioma cells with a growth advantage.

In order to delineate a large number of variable telomeres in the interphase nucleus, several glioma cultures were studied in situ using telomere specific repeats. Decorated loci were evaluated using confocal microscopy and three-dimensional reconstruction. Two representative examples are shown below, and demonstrate the following in these neoplastic cells: 1) telomeres can be at a distance from the nuclear membrane, 2) telomeres are not in a 'Rabl orientation' in interphase (at one pole of the nucleus), and 3) the overall pattern of telomere position is not a consequence of DNA synthesis. These structural studies provide a morphological correlate for the polymorphisms detected at the DNA sequence level.

Figure 2A shows the decoration of metaphase chromosome ends by the TTAGGG telomeric repeat. (Telomeric sequences in A are shown in green, and when they overlap a red signal on the chromosome, they are yellow.) In this example normal metaphase spreads were simultaneously hybridized with a relatively infrequent Alu sequence that correlates with a Giemsa-light banding pattern [48]. In A, these Alu hybridization sites are depicted in red. In accord with the PFGE studies, some of the telo-

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Fig. 2. (A) Normal human metaphase chromosomes hybridized to the telomere oligonucleotide (green) and to a subset of Alu repeats (red) to delineate chromosomal banding patterns. Some telomeric signals show differences in intensity and size (arrows) but all are limited to telomeric domains. (Overlapping chromosomes are not well visualized in this spread and yellow signals indicate telomeres overlie Alu-rich chromosome domains, i.e., green plus red gives a yellow signal.) (B) XZ sequential confocal sections (top to bottom panels) from the central portion of a TC 620 cell. This cell shows a pattern of BrdU incorporation (green signals) consistent with late S. Note several interiorly positioned telomeres (red to yellow, arrows). The larger signals probably represent a collection of several telomeres, and are overexposed (yellow tint) to bring out the smaller (red) telomeres. These 620 cells were pulsed with BrdU for ten minutes prior to fixation with paraformaldehyde. (C) XY stereo pair shows another cell from the same preparation in tomographic 3D reconstruction of confocal slices 0.3 microns apart. Stereo pair is at 0 and 7 degrees. A nucleus with a green BrdU signal shows a pattern of labeling consistent with early S phase. This nucleus shows many interior telomeres (red to yellow) with no connection to the nuclear envelope. The central portions of the nucleus with reduced signals correspond to nucleolar regions. At the upper right, 7 isolated red telomeres are seen. These are in a G1 nucleus that has not incorporated BrdU. (D) A TC 526 cell shows hybridization of Alu repeats (green), used to define all nuclei. Numerous telomeres (red and yellow spots) are in the interior portion of the nucleus. There is no Rabl orientation (clustering of telomeres at one pole of the nucleus). Stereo pairs in C and D are presented for cross-eye convergence. The top- and bottommost sections of the nucleus in C were omitted to provide a clearer view of interior telomeres.

Hybridization and simultaneous detection of digoxigenin and biotin labeled probes was done as previously described [17] with modifications for 3-dimensionally preserved cells (in preparation). Serial optical sections were collected on a Leica confocal microscope equipped with a pizoelectric (z-stepping) stage and a variable pinhole. A small pinhole ($\sim$ 75 setting) was generally used (except in XZ sections) to avoid photobleaching of serial sections. The data sets for each fluorochrome were simultaneously collected for each slice to assure convergence in the z-plane. Stereo movies in two or three colors were examined for individual sections, and for whole three-dimensionally reconstructed sets (in preparation), to confirm the relative positions and relationships of different signals.

meres are larger than others, i.e., the telomere repeats can be more numerous on individual chromosomes.

The same conditions of hybridization were used to detect telomeric sequences in three-dimensionally preserved cells in TC 620, an oligodendroglioma line. Sequential confocal sections on an xz, or xy plane, were evaluated in a number of cells. XZ sections are equivalent to perpendicular sections through the cell, where the image bottom represents the coverslip surface, and the top represents the cell surface facing the feeding medium. Cells were briefly pulsed with BrdU to define cells in DNA synthesis. Nuclear boundaries are apparent with the BrdU detector (shown in green). In these individual optical sections, taken at a sequential distance of 0.4 microns in the central portion of the cell, it is obvious that the telomeres are frequently at a distance from the nuclear envelope (Fig. 2B). Although there is no Rabl orientation of telomeres, the larger red to yellow signals indicate close apposition of several telomeres. These are considerably larger than the individual telomeres seen in the metaphase spread. The above data were substantiated in xy serial reconstructions. Figure 2C shows a three dimensional view in the xy plane (looking at the cell's bottom surface, stereo pair) in another typical 620 cell undergoing DNA synthesis (green signal). Telomeres (red signal) can be seen in interior portions of the nucleus at different levels. Part of an adjacent cell that is in G1 (not synthesizing DNA, and therefore without a green signal) is also seen at the right; although only telomeres are detected, they are at the same focal level as those in the S phase cell. 3-D reconstruction of several other G1 nuclei (counterstained with Dapi to illuminate all nuclear DNA) confirmed the interior position of telomeres in G1 620 cells (data not shown).

Double-label studies using the Alu repeat and the telomeric probe were also used to evaluate many G1 cells in more detail. A typical example of TC 526, a glioblastoma with more chromosomes and therefore more telomeres, again showed an interior distribution for many telomeres (Fig. 2D). In this second glioma line there is again no Rabl or polar orientation of telomeres (at one end of the nucleus). Some of the telomeres in this cell are arranged in a circular fashion at a distance from the nuclear envelope. Because many telomeric domains are rich in Alu sequences, many of the telomeric signals appear yellow in this stereo pair.

In-situ data from non-mammalian studies, are often cited to support the conclusion that all eukaryotic chromosome ends are anchored at sites juxtaposed to the nuclear envelope [see 37]. Our previous in-situ data using human minisatellite probes adjacent to single telomeres [49], did not support this view. The data above further contradicts this generalization, because many telomeres can be far from the nuclear envelope. However, because these are tumor cells, the interior position of telomeres can be a consequence of some loss of telomere structure and/or function. The apparently random position of telomeres in these glioma cells may additionally signify a loss of chromosome order in these interphase nuclei. Given the caveat that there is still insufficient comparative data for normal and reactive astrocytes, the positional variations shown here for glioma telomeres are striking. Positional changes, for telomeres as well as other chromosome domains previously described, probably reflect fundamental molecular and structural alterations that create genomic instability. Such changes may release constraints on chromosome condensation and position that are essential for neoplastic flexibility and survival.

Several key features have been highlighted by these telomere studies. First, they show that primary DNA features may provide signals for change. Second, although primary DNA sequence features may define chromosome regions that are preferentially susceptible to change, they do not do so in isolation. In the interphase nucleus of a functioning cell, DNA is continuously interacting with proteins. Because telomeric DNA tightly associates with the nuclear matrix [50] as well as other highly specialized proteins (e.g., RAPI), uniquely folded domains can be defined. Third, these structures ultimately must be considered in susceptibility to damage or progressive change. Fourth, it is likely that such specialized structures can define some aspects of nuclear organization. This concept has precedence because other specialized DNA motifs, for example in the centromere, can be organized in a limited way

[47, 51]. Fifth, other specialized structures on chromosome arms may similarly define regions especially prone to variation, and these may be critical for a more generalized genomic instability. Although few studies have been done on specific matrix proteins in neoplasia, at least a few tumors, as well as virally infected cells, show remarkable differences in a limited number nuclear matrix proteins [52, 53 and Penman, personal communication]. Such unique proteins may specify a set of unstable chromosomal sites that are recruited in evolving neoplasms.

Conclusions

There are many informative and elegant experiments on the nature of specific genetic changes that can occur in neoplasia, and I have tried to point to a few that are most pertinent for neuroepithelial tumors. Although I have always argued for the centrality of genetic change in tumorigenesis, which can be propagated by a variety of known mechanisms (e.g., amplification, viral insertion, direct DNA damage and recombination), the detailed data is enormously complex, with specifics most relevant for selected tumors. In this sense, one must ask if these findings alone make us wiser about the more general roots of malignancy.

In revisiting some of the biological and experimental properties of neuroepithelial tumors, several features are most relevant, and suggest different approaches to genomic stability and plasticity may be both fruitful and unifying. Continuing nuclear controls for normal differentiation are operative in examples of childhood neoplasms such as spongioblastoma and neuroblastoma. Such examples provide one reason for emphasizing chromosome structure in the interphase nucleus. Clearly the spongioblastoma can fail to progress with time, indicating there are underlying molecular controls for maintaining the integrity of the genome. Several of these are likely to operate during DNA synthesis and mitosis, as these events are prerequisites for neoplastic vulnerability.

At the other end of the spectrum, rapid or apparently sudden glioblastomas would indicate a profound and very basic change. The data on anaplasia and heterotransplantation additionally indicates multiple sites on chromosomes may be remodeled, with consequent flexible responses to the surrounding environment. These can result in a growth advantage for the tumor cell. The various phenotypes in such tumors may reflect a random instability in a subset of more *susceptible or plastic* genomic sites. Environmental factors, including new growth factors produced by some tumor cells, are likely to impose a force for selecting certain tumor populations. These can lead to a more uniform cellular phenotype. Nevertheless, such cells experimentally display a remarkable adaptability that underscores their genomic plasticity.

The clues that higher order genomic structure is central to this problem includes the following: 1) high rates of cell division, where the genome is remodeled for differential expression, is linked to subsequent structural changes in interphase chromosomes. For example, DNA methylation and other gene silencing changes stabilize heterochromatin formation and/or nuclease insensitivity. 2) Refolding of newly synthesized DNA into transcriptionally functional interphase chromosomes involves protein-DNA interactions that are known to result in differences in gene accessibility (e.g., to nucleases). 3) Permanent structural changes, such as deletions and translocations, frequently have functional consequences not limited to a single gene. 4) Finally, nuclear 'disorder' (or highly variable chromosome positions) often signify more profound or global changes. Although the basis for positional changes in nuclei is not understood, they appear to be part of the adaptive neoplastic response. Structural studies of the interphase nucleus to date, as well as relevant DNA sequence data discussed above, indicate more differentiated childhood tumors (e.g., neuroblastomas) do not show the global structural changes of the malignant gliomas. The alteration of only one gene (such as Rb) or the creation of an amplicon (as seen in the neuroblastoma) apparently does not typically or inevitably lead to a more global genomic instability.

I have used the example of telomeres to show that different non-coding DNA regions can be very stable, or can vary. Although differences in the

number of telomeric repeats and/or adjacent restrictions site polymorphisms do not readily support either senescence or malignancy hypotheses in a straightforward way, telomeres provides a paradigm for uniquely folded structures at multiple chromosome sites. The specific DNA-protein conformation at telomeric loci may be especially prone to change. Presumably variations in telomeric DNA may lead to altered binding by the nuclear matrix, with dynamic consequences for interphase chromosome positions. Local changes in folding may affect nearby genes, or provide signals for further change. Moreover, general chromosome positions may also be altered, possibly with functional consequences such as enhanced adaptability. Other specific noncoding DNA motifs, as well as minor structural proteins of the nucleus, may likewise interactively fold chromosomes to provide multiple sites that are unusually bent or kinked [54, 55]. These regions can specify multiple domains especially prone to damage and/or instability. Such susceptible structural elements of the chromosome may be defined by general rules based on special DNA sequence motifs as well as the binding properties of a few proteins. Although these rules are not yet apparent, they can have relevance for sudden as well as progressive instability. Structural and molecular studies that address these issues are likely to clarify the fundamental problem of genome plasticity in neoplasia.

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References

1. Manuelidis L, Manuelidis EE: An autoradiographic study of the proliferation and differentiation of glial cells *in vitro*. Acta Neuropath 18: 193–213, 1971
2. Manuelidis L: A view of interphase chromosomes. Science 250: 1533–1540, 1990
3. Reik W, Surani MA: Genomic imprinting and embryonal tumors. Nature 338: 112–113, 1989
4. Weinberg RA: Tumor suppressor genes. Science 254: 1138–1146, 1991
5. Manuelidis EE: Heterologous transplantation of cerebral and cerebellar astrocytomas. Acta Neuropath 20: 160–170, 1972
6. Greene HSN, Arnold H: The homologous and heterologous transplantation of brain and brain tumors. J Neurosurg 2: 315–331, 1945
7. Friend SH, Bernards R, Rogelj S, *et al.*: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323: 643–646, 1986
8. Huang H-JS, Yee J-K, Shew J-Y, *et al.*: Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242: 1563–1566, 1988
9. Chen TL, Manuelidis L: Neuroblastoma double minutes isolated by pulsed field gel electrophoresis without prior strand cleaving treatments. Genomics 4: 430–433, 1989
10. Solomon E, Borrow J, Goddard AD: Chromosome aberrations and cancer. Science 254: 1153–1160, 1991
11. Manuelidis L, Manuelidis EE: Surface growth characteristics of defined normal and neoplastic neuroectodermal cells *in vitro*. Prog Neuropath 4: 235–266, 1979
12. Manuelidis L: Active nucleolus organizers are precisely positioned in adult central nervous system cells but not in neuroectodermal tumor cells. J Neuropath Exp Neurol 43: 225–241, 1984
13. Zehnbauer BA, Small D, Brodeur GM, Seeger R, Vogelstein B: Characterization of N-myc amplification units in human neuroblastoma cells. Mol Cell Biol 8: 522–530, 1988
14. Trojanowski JQ, Tohyama T, Lee VM: Medulloblastomas and related primative neuroectodermal brain tumors of childhood recapitulate molecular milestones in the naturation of neuroblasts. Mol Chem Neuropath 17: 121–135, 1992
15. Valtz NL, Hayes TE, Norregaard T, Liu SM, McKay RD: An embryonic origin for neuroblastoma. New Biologist 3: 364–371, 1991
16. Zu Rhein GM: Studies of JC virus-induced nervous system tumors in the Syrian hamster: a review. In: Sever JL, Madden DL (eds) Polyomaviruses and Human Neurological Diseases. Alan R. Liss, New York, p. 205
17. Taruscio D, Manuelidis L: Integration site preferences of endogenous retroviruses. Chromosoma 101: 141–156, 1991
18. Bishop JM: Cellular oncogenes and retroviruses. Ann Rev Biochem 52: 301–354, 1983
19. Bishop JM: The molecular genetics of cancer Science 235: 305–311, 1987
20. zur Hausen H: Viruses in human cancers. Science 254: 1167–1173, 1991
21. Pond AR, Manuelidis EE: Oncolytic effect of poliomyelitis virus on human epidermoid carcinoma (HeLa tumor) heterologously transplanted to guinea pigs. Am J Path 45: 233–249, 1964

22. Rous P, Kidd JG: Conditional neoplasms and subthreshold neoplastic states. J Exp Med 739: 365–389, 1941
23. Ludwin SK: Proliferation of mature oligodendrocytes after trauma to the central nervous system. Nature 308: 274, 1984
24. Manuelidis L, Manuelidis EE: Proliferation and response of oligodendrocytes. Lab Invest 52: 1–2, 1985
25. Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, *et al.*: Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature 355: 846–847, 1992
26. Manuelidis EE: Experiments with tissue culture and heterologous transplantation of tumors. Ann NY Acad Sci 159: 409–431, 1969
27. Ariza A, Fernandez LA, Inagami T, Kim JH, Manuelidis EE: Renin in glioblastoma multiforme and its role in neovascularization. Am J Clin Path 90: 437–441, 1988
28. Trojan J, Johnson TR, Rudin SD, Ilan J, Tykocinski J, Ilan J: Treatment and prevention of rat glioblastomas by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. Science 1993
29. Libermann TA, Nusbaum HR, Razon N, Kris R, *et al.*: Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumors of glial origin. Nature 313: 144–147, 1985
30. Wong AJ, Bigner SH, Bigner DD, Kinzler KW, *et al.*: Proc Natl Acad Sci (USA) 84: 6899–6903, 1987
31. Yamazaki H, Fukui Y, Ueyama Y, Tamaoki N, *et al.*: Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-erbB) in human brain tumors. Mol Cell Biol 8: 1816–1820, 1988
32. Mittelman F: Catalogue of chromosome aberrations in cancer. Second edition, Liss, New York, 1985
33. Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L: Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genetics 80: 235–246, 1988
34. du Manoir S, Speicher MR, Joos S, Schröck E, *et al.*: Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization. Hum Genet 90: 590–610, 1993
35. Cremer T, Tesin D, Hopman AHN, Manuelidis L: Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumor cells by in situ hybridization with chemically modified DNA probes. Exp Cell Res 176: 199–220, 1988
36. McClintock B: The stability of broken ends of chromosomes in Zea mays. Genetics 26: 2 34–282, 1941
37. Blackburn EH: Structure and function of telomeres. Nature 350: 569–573, 1991
38. Moyzis RK, Buckingham JM, Cram LS, Dani M, *et al.*: A highly conserved repetitive DNA sequence (TTAGGG)n, present at the telomeres of human chromosomes. Proc Nat Acad Sci USA 85: 6622–6626, 1988
39. Lundblad V, Szostak JW: A mutant with a defect in telomere elongation leads to senescence in yeast. Cell 57: 633–643, 1989
40. Harley CB, Futcher AB, Greider CW: Telomeres shorten during aging of human fibroblasts. Nature 345: 458–460, 1990
41. Counter CM, Avillon AA, LeFeuvre CE, Stewart NG, *et al.*: Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11: 1921–1929, 1992
42. Andrews PW, Damjanov I, Simon D, Banting GS, *et al.*: Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Lab Invest 50: 147–162, 1984
43. Manuelidis L, Ward DC: Chromosomal and nuclear distribution of the Hind III 1.9 kb repeat sequence. Chromosoma 91: 28–38, 1984
44. Chen TL, Manuelidis L: SINEs and LINEs cluster in distinct DNA fragments of Giemsa band size. Chromosoma 98: 309–316, 1989
45. de Lange T, Shiue L, Myers RM, Cox DR, *et al.*: Structure and variability of human chromosome ends. Mol Cell Biol 10: 518–527, 1990
46. Heidmann O, Heidmann T: Retrotransplantation of a mouse IAP sequence tagged with an indicator gene. Cell 64: 159–170, 1991
47. Manuelidis L: Different CNS cell types display distinct and non-random arrangements of satellite DNA sequences. Proc Natl Acad Sci USA 181: 3123–3127, 1984
48. Matsera AG, Ward DC: Oligonucleotide probes for the analysis of specific repetitive DNA sequences by fluorescence in situ hybridization. Hum Mol Genet 1: 535–539, 1992
49. Manuelidis L, Borden J: Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three-dimensional reconstruction. Chromosoma 96: 397–401, 1988
50. de Lange T: Human telomeres are attached to the nuclear matrix. EMBO J 11: 717–724, 1992
51. Manuelidis L: Heterochromatic features of an 11 Mb transgene in brain cells. Proc Natl Acad Sci USA 88: 1049–1053, 1991
52. Greenfield I, Nickerson J, Penman S, Stanley M: Human papilloma virus 16 E7 protein is associated with the nuclear matrix. Proc Natl Acad Sci USA 88: 1217–1221, 1991
53. Hoffman M: The cell nucleus shapes up. Science 259: 1257–1259, 1993
54. Manuelidis L, Chen TL: A unified model of eukaryotic chromosomes. Cytometry 18: 8–25, 1990
55. Kim Y, Geiger JH, Hahn S, Sigler PB: Crystal structure of a yeast TBP/TATA-box complex. Nature 365: 512–520, 1993

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