Journal of Cellular Biochemistry 97:904–915 (2006)

The Significance of Telomeric Aggregates in the Interphase Nuclei of Tumor Cells

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Abstract Telomeres are TTAGGG repetitive motifs found at the ends of vertebrate chromosomes. In humans, telomeres are protected by shelterin, a complex of six proteins (de Lange [2005] Genes Dev. 19: 2100–2110). Since (Müller [1938] Collecting Net. 13: 181–198; McClintock [1941] Genetics 26: 234–282), their function in maintaining chromosome stability has been intensively studied. This interest, especially in cancer biology, stems from the fact that telomere dysfunction is linked to genomic instability and tumorigenesis (Gisselsson et al. [2001] Proc. Natl. Acad. Sci. USA 98: 12683–12688; Deng et al. [2003] Genes Chromosomes Cancer 37: 92–97; DePinho and Polyak [2004] Nat. Genetics 36: 932–934; Meeker et al. [2004] Clin. Cancer Res. 10: 3317–3326). In the present overview, we will discuss the role of telomeres in genome stability, recent findings on three-dimensional (3D) changes of telomeres in tumor interphase nuclei, and outline future avenues of research. J. Cell. Biochem. 97: 904–915, 2006.

Key words: oncogenes; 3D nucleus; genomic instability; telomeres; telomeric aggregates; chromosomes; breakage-bridge-fusion cycle; genomic instability

SIGNIFICANT EARLY WORK ON TELOMERE BIOLOGY

Müller [1938] and McClintock [1941] were the first to observe breakage-bridge-fusion (BBF) cycles. These are cycles where chromosomal end-to-end fusions contribute to the onset of chromosomal rearrangements and genomic instability. Studying broken chromosomes in *Zea mays*, McClintock [1942] observed the formation of dicentric and ring chromosomes, rearrangements, terminal deletions, and chromatin bridges at anaphase that then broke apart unequally ("non-median breaks"). She also observed continuous cycles of these events, that is cycles of new fusions followed by new breakages in the following anaphases [McClintock, 1941, 1942]. Focusing on chromosome 9 in *Zea mays*, she was able to follow distinctive types of variegation and to link them to specific rearrangements on previously broken chromosomes. Broken chromosomes were then able to fuse with sister chromatids or with other chromosomes. This affected not only kernel color but also starch formation, growth conditions, and propagation of the plants [McClintock, 1942].

The questions McClintock asked then are still valid today. "(1) Must two chromosomes or more chromosomes be in intimate contact at the time of breakage in order that fusions may occur? (2) If no intimate contact is necessary at the time of breakage, are the broken ends "unsaturated," that is capable of fusion with any other

Grant sponsor: Canadian Institutes of Health Research; Grant sponsor: Canada Foundation for Innovation; Grant sponsor: National Cancer Institute of Canada; Grant sponsor: CancerCare Manitoba Foundation; Grant sponsor: Physics for Technology program of the Foundation for Fundamental Research in Matter; Grant sponsor: The Delft Inter Faculty Research Center Life Tech; Grant sponsor: The Delft Research program Life Science and Technology.

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Received 14 November 2005; Accepted 16 November 2005
DOI 10.1002/jcb.20760

unsaturated broken end? (3) If question (2) can be answered in the affirmative, what forces are involved which lead to the contact and subsequent fusion of the two unsaturated ends? Likewise, (4) how long will these broken ends remain unsaturated, that is, capable of fusion?" [McClintock, 1942]. We found it important to use Barbara McClintock's own words to summarize some of the key questions in the field. Please note that these questions were formulated in 1942. Today, the concept of chromosomal localization is still under intense debate with respect to specific rearrangement of chromosomes. The "unsaturated ends" are indeed broken chromosomal ends that are free of telomeres and therefore able to fuse with sister chromatids or other chromosomes, and yes, chromosome ends can "be healed."

STRUCTURAL ORGANIZATION OF TELOMERES IN MAMMALIAN NUCLEI

Most studies with telomeres have been performed on metaphase chromosomes. Metaphase chromosomes reflect events that occurred prior to the metaphase being examined and, with respect to some aberrations, researchers infer from studying the metaphase chromosomes that 'telomere dysfunctions' were likely. For example, unbalanced translocations, dicentric chromosomes, and terminally deleted chromosomes suggest a defect in telomeres that may involve capping defects, DNA damage affecting the telomeric ends, oncogene activation or other stimuli [Artandi et al., 2000; Gisselsson et al., 2001; Lo et al., 2002; Deng et al., 2003; Murnane and Sabatier, 2004; Louis et al., 2005].

Advances in imaging allow us to now focus on the events that occur prior to the metaphase, namely in preceding cell cycle stages of interphase nuclei. While two-dimensional (2D) imaging of nuclei did not allow us to visualize the spatial organization of telomeres, three-dimensional (3D) and live cell imaging permit the analysis of the structural organization of telomeres in the nucleus of mammalian cells. Studies in recent years have then shown us that telomeres in normal nuclei have a dynamic cell cycle- and tissue-dependent organization. For example, in G0/G1 nuclei, telomeres are widely distributed throughout the whole nuclear space [Weierich et al., 2003; Chuang et al., 2004]. Measurements of telomere positions in the 3D space of primary mouse lymphocyte nuclei have given a precise value to telomeres in this phase of the cell cycle. The a/c ratio indicates that telomeric positions in interphase nuclei is small in G0/G1 lymphocytes, and one usually measures values of 1.4 ± 0.1 [Vermolen et al., 2005a]. This number is indicative of the distribution of telomeres throughout the entire nuclear space of primary lymphocytes, which is roughly spherical. Similarly, in S phase, the a/c ratio is small (1.5 ± 0.2; [Vermolen et al., 2005a]). The nuclear distribution of telomeres changes when cells enter into G2: telomeres align in the center of the nucleus and form a telomeric disk [Chuang et al., 2004]. At this time, the a/c ratio is large due to the organization of the telomeres in a disk-like volume, and the a/c ratio measurements usually are 14 ± 2 [Vermolen et al., 2005a]. Telomere dynamics in interphase nuclei of human osteosarcoma (U2OS), human cervical carcinoma (HeLa), and mouse MS5 cells has been carefully measured by live cell imaging approaches. Long ranging as well as short movements were observed over a time period of 20 min [Molenaar et al., 2003]. Telomere dynamics has also been observed in interphase nuclei of human keratinocytes [Ermler et al., 2004]. Telomere movement is not only dependent on cell cycle but also on cell shape [Chuang et al., 2004; Ermler et al., 2004]. Thus, we conclude that telomeres are not static in mammalian nuclei but perform cell cycle and cell-type specific movements.

Another important feature of telomeres in normal interphase nuclei is the fact that the telomeres do not overlap. Each telomere of a normal nucleus is found in its specific 3D space and does not form clusters or aggregates with other telomeres [Chuang et al., 2004]. Normal cells have a limited life span [Hayflick, 1965]. Their mitotic clock is linked to telomere length. Telomere length is known to be shortening linearly with each cell division (approximately 50–200 base pairs per division [Lansdorp, 2000]). When the telomeres become too short, normal cells will eventually stop division cycles and enter into a state of replicative arrest that is also called senescence. The senescent phenotype has been extensively studied [for review, see Campisi, 2000]. Senescence is bypassed during tumor development [Campisi, 2000; Romanov et al., 2001].

TELOMERE ORGANIZATION IN TUMOR CELLS

Telomeres in tumor cells are different from telomeres in normal cells; they are generally shorter, even critically short [Vukovic et al., 2003; Meeker et al., 2004]. However, they may also be elongated or different subpopulations of telomere lengths may be present [Meeker et al., 2004]. It was shown that telomeres in tumor cells commonly manifest telomere dysfunction, and chromosomal aberrations indicative of these defects are observed. Telomerase is activated in 85% of the tumors, while it is not present in the rest of the tumors, some of which have demonstrated alternative lengthening of telomeres (ALT) [Muntoni and Reddel, 2005].

A remarkable difference between normal and tumor cells becomes apparent when 3D imaging approaches are applied. 3D imaging revealed a specific 3D telomeric signature for tumor cells. In contrast to the non-overlapping nature of telomeres in normal nuclei, telomeres of tumor nuclei tend to form aggregates. Various numbers and sizes of such telomeric aggregates (TAs) can be found in tumor nuclei [Chuang et al., 2004]. The formation of TAs is independent of telomere length and telomerase activity [Louis et al., 2005].

There are at least two types of telomeric dysfunction in tumor cells. One type of telomere dysfunction involves critically short telomeres [DePinho and Polyak, 2004]. The other one involves the formation of TAs and is independent of telomere size or telomerase activity [Chuang et al., 2004; Louis et al., 2005]. Both types of telomeric dysfunction can lead to BBF cycles that contribute to deletions, gene amplification, non-reciprocal translocation, and overall genetic changes that are associated with tumorigenesis [Artandi et al., 2000; DePinho and Polyak, 2004; Murnane and Sabatier, 2004].

MEASUREMENT OF TELOMERE DYSFUNCTION (3D VOLUMES AND POSITIONS)

Quantitative measurement of the telomeres parameters is based on 3D data that are usually captured by acquiring many optical sections of the nucleus with a high numerical aperture oil-immersed objective lens followed by an appropriate deconvolution algorithm. The most reliable one uses constrained iterative deconvolution [Schaefer et al., 2001; Vermolen et al., 2005b]. Telomere measurements are done with a special algorithm and software package that we developed, TeloView^{TM} [Chuang et al., 2004; Vermolen et al., 2005a]. First, the position of each telomere is identified by using a threshold. Then, the center of gravity and the integrated intensity of each telomere are calculated. The integrated intensity of each telomere is the appropriate parameter for determining the length of the telomere, or the telomere copy number, which estimates the number of telomeres that are taking part in an aggregate. Aggregates are easily observed when looking at a 3D visualization of the nucleus and it can be quantitatively calculated by analyzing the integrated intensity of each telomere (Fig. 1).

IMPACT OF TELOMERE AGGREGATES ON CHROMOSOMAL ORGANIZATION

It is not just a transient aberration in the 3D organization of the nucleus when telomeres aggregate. Since some of the aggregates represent fusions, dicentric chromosomes can form. These end-to-end fused chromosomes cannot appropriately separate during cell division, but will first generate anaphase bridges and then break apart, leaving one chromosome too short (with a terminal deletion) and the other one with

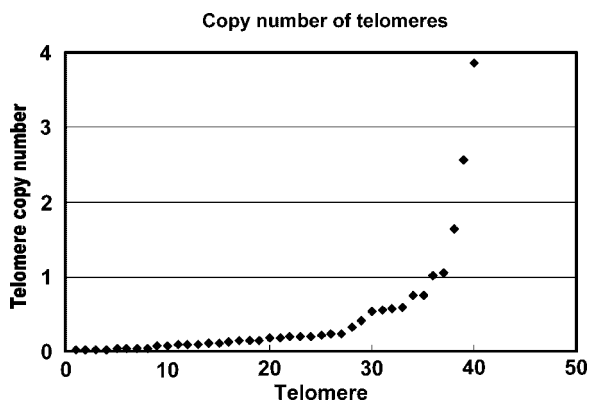

Fig. 1. Histogram illustrating the concept of telomeric aggregates (TAs) and their quantitative analysis. This histogram demonstrates how TAs are found using TeloView^{TM} [Vermolen et al., 2005a]. Each point represents the copy number of a telomere that is found in the nucleus. The intensity of an average telomere is calculated by analyzing the smaller telomeres in the nucleus (which are the majority of telomeres). See the change in the graph slope at about telomere number 37. All the telomeres smaller than telomere number 37 are interpreted as single copies while telomeres that are larger are interpreted as aggregated copies. The copy number is calculated by dividing the integrated intensity of each telomere by that of telomere number 37. The telomeres are sorted for convenience from smallest to largest (based on their integrated intensity).

a new piece (generating an unbalanced translocation). Both chromosomes are 'new' structures and both are unable to persist the way they were left after this cell division. Since both new chromosomes have telomere free ends and represent a double-strand break, they will each find a new chromosome partner, and they will fuse with it to heal their broken ends. This series of events is termed a BBF cycle and goes back to Müller and McClintock's seminal findings [Müller, 1938; McClintock, 1941]. Importantly, a BBF cycle is not a single event. One BBF cycle initiates the next and so forth until no more free ends persist to permit fusions with other chromosomes [McClintock, 1941, 1942; Louis et al., 2005].

Once aggregates form and fusions occur, BBF cycles result and with such BBF cycles, the genetic information of the chromosomes will be remodeled [Louis et al., 2005]. TAs and fusions are different from the reversible telomeric associations that have been reported for Chinese hamster embryonic cells [Slijepcevic et al., 2000]. Which events lead to such telomere-mediated nuclear remodeling? We have studied oncogenic remodeling of the 3D telomere organization. The deregulation of the oncoprotein c-Myc was able to remodel the telomeric organization from non-overlapping telomeres to TAs of various numbers and sizes [Louis et al., 2005]. A single deregulation event of c-Myc, where the oncogene was overexpressed in the nucleus for 2 h, was sufficient to initiate the formation of TAs. Moreover, TAs/fusions caused the formation of dicentric, end-to-end fused chromosomes. The latter generated anaphase bridges and broke apart as anaphase progressed, leaving behind terminal deletions and unbalanced translocations. Two hours of c-Myc deregulation initiated three BBF cycles. Twelve hours of c-Myc deregulation led to five such cycles. Thus, the time of c-Myc deregulation was directly proportional to the number of BBF cycles observed [Louis et al., 2005]. The scoring of chromosomal aberrations over a 120-h period documented the BBF cycles: from fusion to breakage with terminal deletions and non-reciprocal translocations to telomere-free ends and new fusions (ibid).

WHICH ABERRATIONS ARE GENERATED WHEN TELOMERES ARE REMODELED IN THE 3D SPACE OF THE NUCLEUS?

Two sets of parallel experiments involving chromosome painting to determine the 3D organization of chromosomes in interphase nuclei and spectral karyotyping (SKY) of metaphase chromosomes were carried out to examine the effects of TA formation on chromosomal positions and aberrations [Louis et al., 2005]. SKY data showed non-random chromosomal rearrangements affecting chromosomes $5+13$, $7+10$, $7+17$. Other chromosomes were sometimes, but not regularly involved and judged as random aberrations. When examining the positions of chromosomes $5+13$, $7+10$, and $7+17$ in interphase nuclei, we found no overlap between these pairs prior to Myc activation, while they changed their positions over the time course of c-Myc deregulation and showed substantial overlap [Louis et al., 2005].

MEASUREMENTS OF CHROMOSOMAL OVERLAPS IN THE INTERPHASE NUCLEUS

Chromosomal overlaps measurements are performed after 3D image acquisition and constrained iterative deconvolution. First, the 3D boundary of the nucleus is determined based on the DAPI counterstain image. Within this volume, a threshold level is determined for each chromosome and the total volume V_1 and V_2 of each chromosome pair is calculated (by counting only the voxels that has an intensity value above the threshold). The total volume that is occupied by both chromosome pairs is also measured (V_0). By dividing V_0 by the total volume of each one of the chromosome pairs, the relative overlap ratio is calculated, V_0/V_1 and V_0/V_2. By following the same procedure for each time point since c-Myc deregulation, we finally get the relative overlap as a function of time.

SIGNIFICANCE OF OVERLAPPING CHROMOSOMES

Chromosomal overlap is a problem for genome stability if the overlapping chromosomes fuse at their telomeric ends or are involved in illegitimate recombination events. TAs brings chromosomes into close vicinity. If TAs represent fusions, then BBF cycles will occur. This was found after experimentally-induced c-Myc deregulation [Louis et al., 2005; Mai and Garini, 2005].

There are two possibilities for the initiation of BBF cycles after TA formation and chromosome overlap. The occurrence of non-random chromosomal aberrations suggests either a non-random formation of chromosomal overlaps

resulting in end-to-end chromosomal fusions. Alternatively, one may argue that there is a non-random occurrence of TA formation resulting in chromosomal overlaps and causing the initiation of BBF cycles. At the present time, we cannot distinguish between both possibilities and both remodeling events may coexist.

TAs AND TUMORS

Genomic instability is viewed as an event through which genetic changes occur or have occurred [Hanahan and Weinberg, 2000; Gollin, 2005; Mitelman et al., 2005]. These changes can be structural and numerical, and this is the classical view of genomic instability. We would like to expand this view and include epigenetic changes that coincide with genetic alterations and/or precede them, point mutations, and alterations in nuclear organization that affect the genome. Organizational changes in the 3D space of the nucleus need to be considered as an important factor not only in tumors but also much earlier that is during the initiation of genomic instability and the establishment of tumorigenic potential.

The analysis of primary tumors revealed that TAs are common [Chuang et al., 2004]. Various cell types and tissues were examined, including primary head and neck cancer, primary mouse plasmacytoma, human neuroblastoma, and colon carcinoma cell lines [Chuang et al., 2004]. While normal cells do not show TAs, tumor cells (primary tumor cells and tumor cell lines) consistently display TAs (Fig. 2).

Importantly, being a feature of tumor cells makes one wonder if such changes in the telomeric organization of the interphase nucleus do not occur earlier, that is when cells become tumorigenic. Early data suggest that this is indeed the case. For example, in cervical cancer, non-invasive lesions, such as CIN I, show TAs in some of cells (Fig. 3). During the development of mouse plasmacytoma, early plasmacytotic foci display TAs in a subpopulation of the foci (Fig. 4). Additional analyses are ongoing and will help us understand the earliest

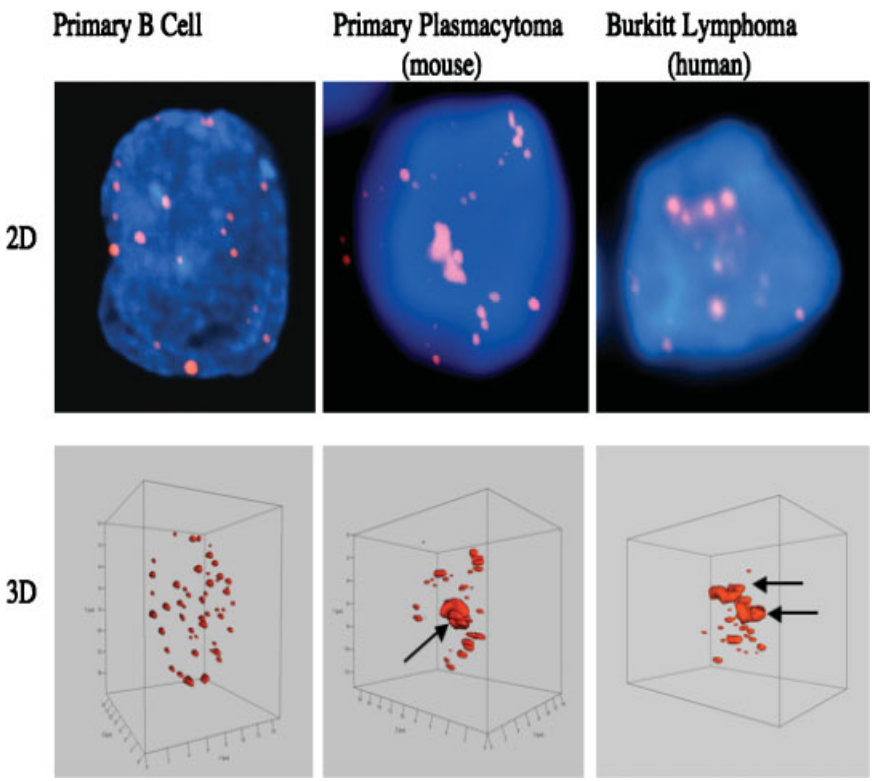

Fig. 2. Telomere organization in primary nuclei of a B cell, a primary mouse plasmacytoma and a Burkitt lymphoma line (Raji). The **top panel** shows two-dimensional (2D) representations of the above nuclei; the **bottom panel** shows the three-dimensional (3D) organization of telomeres in the above nuclei. Telomeres are shown in red, nuclei are shown in blue. Arrows point to TAs. Hybridizations were preformed as described [Chuang et al., 2004; Louis et al., 2005].

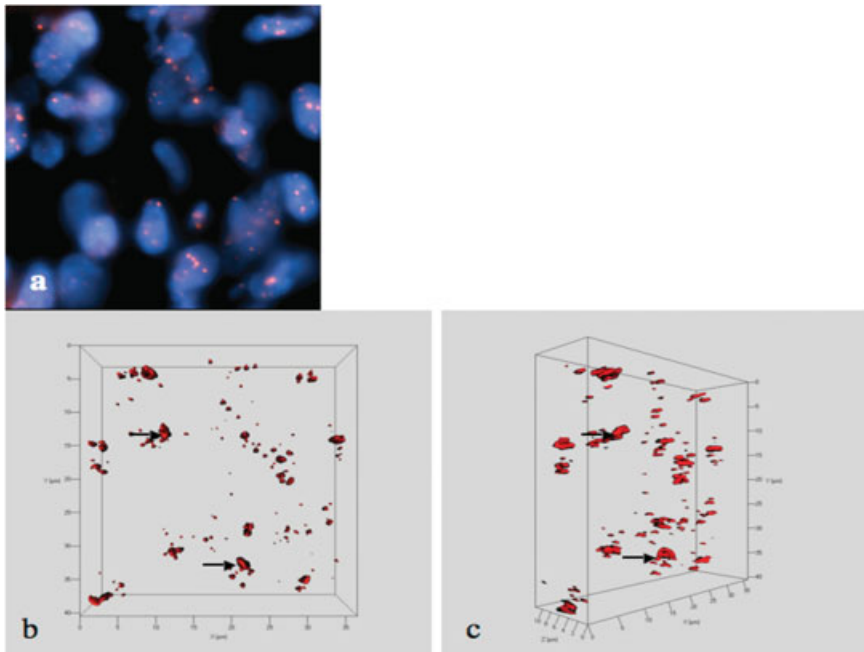

Fig. 3. Telomere organization in cervical biopsy tissue of a CIN 1 lesion. **a**: 2D image of a section showing the identical nuclei (blue) and their telomeric signals (red) that are shown in (**b**) and (**c**) as 3D images. Black arrows point to TAs that are observed in some of the cells. Frozen sections of 5-μm thickness were hybridized as described [Chuang et al., 2004; Louis et al., 2005].

time point during tumor development in vivo that show TA formation. In vitro, in a model of c-Myc-induced genomic instability in PreB and Ba/F3 mouse lymphocytes, we have shown that c-Myc deregulation elicits TA formation within 12 h [Louis et al., 2005]. Additional studies propose even earlier time points (unpublished data). Taken together, the above data indicate that the formation of TAs is an intrinsic factor in the transformation of the normal cell into a malignant one. Therefore, in the future, the knowledge of TA formation during tumor development can be used as a diagnostic tool and for monitoring of treatment success.

MECHANISMS OF TA FORMATION

How do these aggregates form? This is an area that requires intense research. At this point, nothing is known about the mechanisms that cause TA formation. One may speculate that one of the shelterin proteins [de Lange, 2005] is causally involved in TA formation. However, this has not been demonstrated in tumor models. We know from studies of de Lange and colleagues that the absence of TRF2 leads to the formation of telomeric fusions which lead to cell death and senescence [van Steensel et al., 1998; Celli and de Lange, 2005]. Whether these fusions involve TA formation and can be linked to genomic instability and cancer has not been investigated.

BOVERI'S LEGACY: IN SEARCH OF THE MECHANISMS THAT REGULATE ABERRANT NUCLEAR AND GENOMIC ORGANIZATION

Although we described the formation of TAs in tumors and after c-Myc deregulation for the first time [Chuang et al., 2004; Louis et al., 2005], the concept of the nucleus and its chromosomal order has been studied long before. Theodore Boveri (1862–1915) was the first researcher who linked nuclear organization and genome stability. Studying *Ascaris* and sea urchin eggs, he described for the first time 'chromosomal regions' ('chromosome territories' [Cremer and Cremer, 2001]). Chromosomal regions are regions within the 3D nuclear space in which chromosomes tend to be found in normal cells. Boveri also noted that an aberrant chromosome constitution leads to aberrant cell division cycles and mis-segregation of chromosomes. He found that aberrant chromosome constitution resulted in aberrant embryo development or cell death [Boveri, 1902, 1914]. From the simple organisms he studied, he inferred for tumor development that similar pathways are

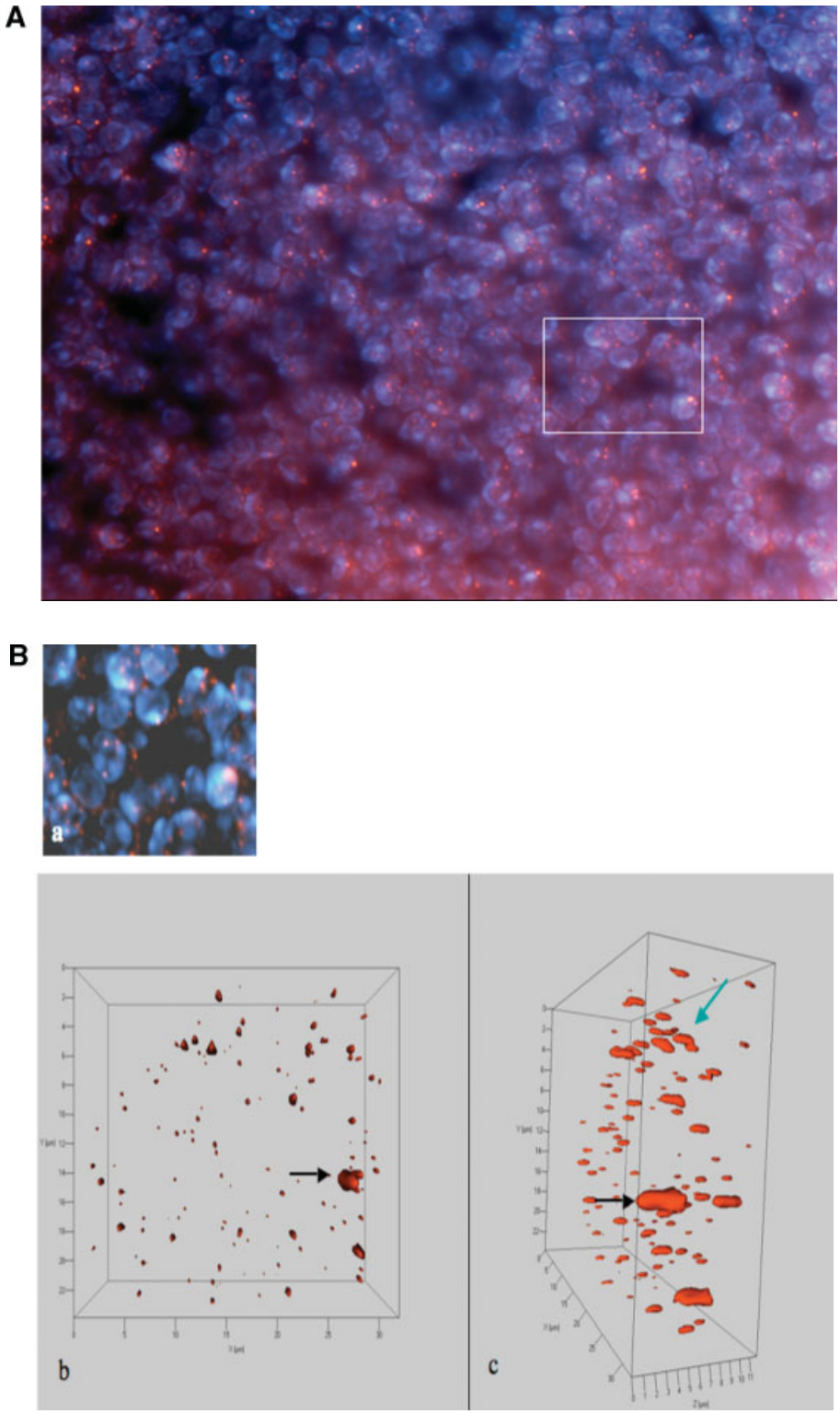

Fig. 4. Plasmacytotic focus examined by telomere hybridization. Telomere hybridizations were performed on 5-μm sections of paraffin-embedded tissues. **A**: Overview of plasmacytotic focus in 2D. Nuclei are shown in blue, telomeres in red. White box indicates area of the section that is shown in (**b**). **B**: Insert from (**a**) showing 2D and 3D organization of the telomeres. Black arrows point to TAs seen in front view (b) and in side view (**c**). The blue arrow points to a structure that appears to be a replicating telomere.

in operation [Boveri, 1914]. The centrosome cycles and aberrations thereof were also described by Boveri [1914] for the first time and later translated into English by his wife [Boveri, 1929]. Since his time, more details about the 3D organization of the nucleus and the genome have been investigated. However, the big picture that he first put forward is as valid today as it was in his time.

CURRENT CONCEPTS AND OPEN QUESTIONS

In the following paragraphs, we will discuss some of the issues that are important for future research in the area of the 3D organization of the nucleus and its alteration in the contribution to tumor development.

WHICH STIMULI LEAD TO THE FORMATION OF TAs?

Due to the impact of telomeric remodeling on genome stability, it will be important to characterize the conditions that lead to the formation of TAs. We have recently studied c-Myc-dependent TA formation and the effects of TAs on genomic instability [Louis et al., 2005]. We anticipate that other oncogenes may cause similar effects. One candidate is Ha-Ras. This oncogene was already studied with respect to its ability to alter chromatin organization [Fischer et al., 1998]. A more recent study using Balb/3T3 cells spontaneously immortalized and transfected with mutated c-Ha-Ras-1 found that Ha-Ras increased the level of chromosomal rearrangements involving telomeric sequences threefold [Peitl et al., 2002]. However, it is not known whether these rearrangements followed TA formation. It is also not known whether additional genetic changes occurred in these immortalized cells that contributed to the above results.

Other stimuli that converge at the chromosomal ends and elicit genomic instability may involve viruses that are able to immortalize or transform the host cells. Wan et al. [1997] examined telomeres after human papilloma viral infection and found a high frequency of telomeric associations and rearrangements. Using human ovarian epithelial cells immortalized by human papilloma oncoproteins, E6 and E7, the authors observed that 30–100% of all metaphases examined displayed telomeric associations (ibid). Whether these associations followed TA formation has not been investigated. However, one may postulate that this is very likely if the c-Myc-mediated remodeling of telomeres and chromosomes can be considered a general pathway to nuclear remodeling of the genome. The impact of viruses on telomeric organization and genomic instability requires further investigation. The above effects may be cell-type, host, and/or virus-specific since work by Argilla et al. [2004] demonstrates that transgenic mice expressing SV40 or HPV16 in the absence of telomerase do not exhibit telomere dysfunction or increased genomic instability.

TELOMERES AND EVOLUTION

It has been reported that human subtelomeric sequences are recombination and duplication hot spots [Linardopoulou et al., 2005]. Subtelomeric sequences are involved in interchromosomal recombinations and segmental duplications. This not only is a feature of tumor cells, but also occurs frequently during evolution. For example, half of the known subtelomeric sequences have formed recently during primate evolution. Interestingly, the subtelomeric gene duplication rate is significantly higher than the genome average. Thus, the authors conclude that this is both advantageous for evolution and may also have pathological consequences [Linardopoulou et al., 2005]. In the context of our discussion, we emphasize that telomeres and subtelomeric sequences are hot spots of evolution and genomic instability. The formation of TAs may contribute to both.

DO TAs AFFECT THE CHROMOSOMAL ORDER IN VIVO?

Since previous studies were done in established tumors or tumor cell lines [Chuang et al., 2004], one cannot say whether the chromosomal order changed due to tumor formation or due to TA formation or both. While data are emerging that TAs occur early in tumor development (Figs. 3 and 4), it is still unclear whether this is mechanistically linked to the remodeling of the nuclear order of chromosomes and to rearrangements in vivo. Thus, the cause-relationship in vivo is not yet established and needs to be examined carefully.

The closest cause-relationship study to date involved oncogenic remodeling of the telomeres and chromosomes in the nucleus [Louis et al.,

2005]. In this in vitro study using mouse lymphocytes, c-Myc deregulation led to TA formation that preceded chromosomal rearrangements via BBF cycles [Louis et al., 2005; Mai and Garini, 2005].

WHEN IS THE EARLIEST TIME POINT FOR TA FORMATION AND WHEN IS THE TUMORIGENIC POTENTIAL ESTABLISHED?

This question is critical for our understanding of the impact of nuclear remodeling in tumor development. We speculate that TAs formation may be the earliest event in tumor development and occur subsequent to oncogene deregulation. This is solely based on our in vitro studies and on studies we performed with pre-neoplastic and non-invasive lesions. More detailed studies in several tumor models will be necessary to establish this point. Is it enough for a cell to carry TAs to be tumorigenic? Are TAs and chromosomal rearrangements required before a cell becomes tumorigenic? Is a specific genetic background more susceptible to TA formation? Is the formation of TAs reversible? Can cells repair TAs? When do TAs become irreversible? Appropriate cell culture and mouse models will allow researchers to address such questions in the future.

WILL CELLS UNDERGO APOPTOSIS WHEN A CRITICAL THRESHOLD OF TAs IS REACHED?

Data on repeated c-Myc inductions suggest this may be the case. When mouse Pre B lymphocytes are stimulated to overexpress c-Myc every 12 h, >96% of all nuclei display large or several TAs. In this experimental set-up, all cells die of apoptosis within 30 h [Louis et al., 2005]. In contrast, a single activation of c-Myc deregulation for 2 h or for 12 h led to the formation of three or five TA cycles, respectively, which represent BBF cycles, without significant elevation in cell death (ibid). Thus, we propose that a critical threshold of TAs is tolerated by the cells and leads to genomic instability through chromosome remodeling by TA-induced BBF cycles. A low level of TAs allows for cell survival and cell proliferation while genomic rearrangements can occur. The latter situation is the critical one, since it contributes to the propagation of genomically unstable cells.

REMODELING OF THE NUCLEUS THROUGH TAs

In c-Myc deregulated cells, TAs form and chromosomes change their positions. Not only are there more chromosomal overlaps, but also more chromosomal ends become linked through TAs and fusions [Louis et al., 2005]. Several questions arise from these findings. Do chromosomes move normally? This is an open question, since the available data do not allow for a consensus in interpretation. While some research groups do not find substantial chromosomal movements [Abney et al., 1997; Gerlich et al., 2003], others find chromosomal reorganization during the cell cycle [Ferguson and Ward, 1992; Vourc'h et al., 1993; Bridger et al., 2000; Chubb et al., 2002; Walter et al., 2003; Essers et al., 2005], cellular differentiation [Stadler et al., 2004], and during quiescence and senescence [Bridger et al., 2000].

Whether chromosomes move normally or not, there are conditions that induce movement, such as c-Myc deregulation [Louis et al., 2005]. In the presence or absence of pre-existing movements, the potentially dynamic nature of chromosome order is a very complex issue. For example, are there specific neighborhood relationships that become established due to specific stimuli (such as oncogenic activation, viral infection, DNA damage)? Or do chromosomes that are observed in specific chromosomal neighborhoods come closer to each other diminishing the intrachromosomal space? Would this favor fusions, illegitimate recombinations, and/or non-homologous end joining?

There is evidence that a non-random nuclear order of chromosomes with specific chromosomal neighborhood relationships is important for specific rearrangements. Data by Neves et al. [1999] suggest this for *bcr/abl* in chronic myeloid leukemia. Chromosomes 9 and 22 are in close enough proximity to permit this translocation. This finding is supported by Kozubek et al. [1999] who state that the positions of chromosomes 9 and 22 have a determinative role in the induction of t(9;22) and in the development of t(9;22) leukemias. For mouse B cells, chromosomes 12 and 15 are found in a close neighborhood in lymphocytes (where they are involved in balanced translocations in mouse plasmacytoma) but are found more distant in mouse hepatocytes [Parada et al., 2004]. There are more studies that support this

chromosome neighborhood concept. Thomas and Diehl [2003] state that the proximity between translocating chromosomes is a prerequisite for their rearrangement. Roix et al. [2003] support this interpretation.

In a survey of >11,000 constitutional translocations, Bickmore and Teague [2002] concluded that the frequency of constitutional translocations depended on three main factors, and these included the chromosome positions, chromosome sizes, and specific DNA sequences.

We conclude from the above that chromosome specific neighborhood relationships exist in a cell-type specific manner and are consistent with the resulting chromosomal translocations. However, the experimental proof for this concept is lacking. For example, if a chromosome involved in translocations was moved to a new nuclear position would it still be involved in the same translocations or not? Do approaching gene loci or gene loci in the same nuclear compartment contribute to possible illegitimate recombination events? To date, these questions remain unanswered and await future investigation.

It is now possible to view all chromosomes in a nucleus [Bolzer et al., 2005]. Such 3D localization of all chromosomes needs to be combined with 3D FISH studies to assess potential gene and chromosomal region-associated movements.

CONCLUSIONS

Further research is required to fully understand the complexity of nuclear organization in normal cells and during malignancy. Studies using various approaches are required to investigate the complexity of 3D nuclear space that is crucial for understanding genome organization and stability. Geneticists, evolutionary biologists, cancer researchers, cell biologists, program developers, physicists, mathematicians, and biostatisticians are all necessary in a multidisciplinary effort to understand and model the nuclear structure and its regulation in normal and tumor cells. Only when we fully understand who the key players are, will we be able to learn how to modulate them for patient-specific treatments.

ACKNOWLEDGMENTS

We thank our lab members, especially Mrs. Cheryl Taylor-Kashton, and many colleagues for comments and discussions. We are grateful to Dr. Michael Mowat and Dr. Francis Wiener for stimulating discussions and critical reading. Work in the Mai lab was supported by the Canadian Institutes of Health Research, the Canada Foundation for Innovation, the National Cancer Institute of Canada, and CancerCare Manitoba Foundation. Work in the Garini lab was supported by the Physics for Technology program of the Foundation for Fundamental Research in Matter, the Delft Inter Faculty Research Center Life Tech, Cyttron, and the Delft Research program Life Science and Technology (Delft, The Netherlands).

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