

The Dynamics of Acentric Chromosomes in Cancer Cells Revealed by GFP-Based Chromosome Labeling Strategies

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Abstract Autonomous replicons, such as viral episomes and oncogene containing double minute chromosomes (DMs), lack centromeres and consequently should be lost rapidly when the nuclear membrane breaks down at mitosis. Surprisingly, they are not. This raises the important question of the mechanisms that enable their efficient transmission to daughter cells. We review recent developments in GFP-based chromosome labeling strategies that enable real time analyses using high resolution light microscopy to provide insights into this issue. The results reveal that episomes and DMs both adhere to host chromosomes, a process referred to as "chromosome tethering". Such association enables acentric molecules to use the chromosomal centromere in trans, thereby achieving efficient transmission to daughter cells. This unique mechanism of mitotic segregation also raises the possibility of developing a new class of anti-cancer drugs that work by selectively eliminating growth enhancing genes from cancer cells. *J. Cell. Biochem. Suppl.* 35: 107–114, 2000. © 2001 Wiley-Liss, Inc.

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Chromosomes undergo dynamic changes during the cell cycle including intranuclear movements during interphase, DNA replication and sister chromatid cohesion, chromosome condensation, and segregation during mitosis. Extensive microscopic and genetic analyses have now begun to uncover the molecular basis of these fundamental cell cycle regulated alterations. Light microscopic observation using fluorescent labeling of chromosomes *in vivo* has become an indispensable tool for studying the dynamic behavior of chromosomes and interphase chromatin. A variety of novel fluorescent labeling strategies invented recently (reviewed in [Zink and Cremer, 1998]) offer the promise of more sensitive and precise description of each process, and the molecules that mediate them. The recent introduction of the jellyfish *Aequorea*

victoria green fluorescent protein (GFP) into the armamentarium of reagents for fluorescent visualization of intracellular components has resulted in the development of novel strategies to enable their analysis in real time without perturbing cellular structure (reviewed in [Misteli and Spector, 1997]). The following represent just some of the many advantages of using GFP fusion proteins to analyze chromosome dynamics. First, GFP fusion proteins can be expressed constitutively or can be induced in cell lines with stable transgenes (see [Kanda et al., 1998] for an example), which enables long-term analyses. Use of GFP fusion proteins also ensures an endless supply of the reagent for multiple experiments. Second, special reagents, such as fluorochrome-labeled nucleotides that are incorporated into DNA during replication, or fluorescent dyes that directly stain DNA *in vivo*, are not required. Use of GFP-tagged proteins instead of these reagents is advantageous for minimizing photodamage to which DNA is especially susceptible. Third, it is often important to be able to analyze multiple proteins along with analyzing chromosome dynamics in a single time lapse experiment. It is now relatively straightforward to perform multi-color imaging analyses using different GFP spectral

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TABLE I. GFP Based Chromosome Labeling Techniques in Higher Eukaryotic Cells

	References
Nonspecific chromosome labeling	
Mitotic chromosome dynamics	[Bunz et al., 1998; Kanda et al., 1998]
Interphase chromatin dynamics	[Verschure et al., 1999; Monier et al., 2000]
Histone protein dynamics	[Phair and Misteli, 2000]
Tracking apoptotic chromatin	(Goldstein J et al., unpublished communications)
Transfection marker for FACS analyses	[Furnari et al., 1998]
Mouse xenograft models	(Kanda T, Siegle I et al., unpublished observation)
His2AvD-tagged GFP [Clarkson and Saint, 1999]	
Specific chromosome labeling	
CENP-B tagged GFP [Shelby et al., 1996]	
Lac operator/lac repressor-GFP system [Robinett et al., 1996]	[Robinett et al., 1996; Li et al., 1998; Tumber et al., 1999]
Interphase chromatin structure	[Kanda et al., 2001]
Mitotic behavior of acentric chromosomes	

variants, such as CFP and YFP [Ellenberg et al., 1999].

GFP-Based Chromosome Labeling Strategies

GFP-based chromosome labeling techniques can be divided into two categories, (1) which labels entire chromosomes and (2) which labels specific chromosomal regions (Table I). Histones tagged with GFP have been used to visualize chromosomes in *Drosophila* [Clarkson and Saint, 1999] and human cells (H2B-GFP) [Kanda et al., 1998]. The H2B-GFP chimeric protein was shown to be incorporated efficiently into nucleosomes, despite the addition of a C-terminal GFP tag that is almost twice the mass of H2B itself [Kanda et al., 1998]. The H2B-GFP protein did not produce measurable effects on long-term cell viability or the kinetics of mitosis [Bunz et al., 1998; Kanda et al., 1998]. It also enabled sensitive detection of chromosome condensation states in interphase nuclei [Kanda et al., 1998]. Human, mouse, and hamster cell lines expressing H2B-GFP have been established by many groups (unpublished communications), providing useful sources for analyzing various aspects of chromosome dynamics in mammalian cells in a variety of genetic backgrounds and in response to diverse experimental conditions (Table I). For example, chromosome territories have been studied in relation to sites of transcription [Verschure et al., 1999], dynamic changes in nuclear morphology have been studied following virus infection [Monier et al., 2000]. It has also enabled rapid screens of apoptosis through detection of chromatin hypercondensation after UV-irradiation (Goldstein J et al., unpublished communications). Since H2B-GFP staining survives after ethanol fixation, it has also been

used as a convenient transfection marker for FACS analyses [Furnari et al., 1998]. Another application is using H2B-GFP labeled tumor cells, instead of GFP labeled tumors [Chishima et al., 1997], for making mouse xenograft models (Kanda T, Siegle I, and Wahl GM, unpublished observation). The frequency of mitotic as well as apoptotic cells can be readily determined by simply analyzing tumor sections.

Another category of GFP-based technology involves labeling of specific chromosomal regions using GFP fusion proteins that bind specific DNA sequences (Table I). The target DNA sequences can be endogenous chromosomal sequences or artificially-synthesized molecules introduced into chromosomes via gene transfer. The former approach was used to visualize centromeric regions using a CENP-B-GFP fusion protein [Shelby et al., 1996]. This study provided convincing evidence that centromeric regions exhibit little movement except Brownian motions, and that significant translocation is rare in interphase nuclei. The gene transfer approach used thus far has employed an array of lac operator repeats (256 copies = 10.1 kb) which are recognized by a lac repressor-GFP fusion protein [Robinett et al., 1996]. As an initial application of this system, a CHO cell line with a large amplified region (~90 Mbp) of lac operator repeats (called homogeneously staining region, HSR) was established [Robinett et al., 1996]. The intranuclear localization and higher order structural change of this heterochromatic, late-replicating HSR was successfully visualized from G1 phase through G2 phase using lac repressor-GFP staining [Li et al., 1998]. The same cell line was also used to investigate the effect of transcriptional activation on higher order chromatin structure in

interphase nuclei [Tumbar et al., 1999]. Dramatic unfolding of the heterochromatic HSR into an extended chromonema fiber was observed to start within 15 min after induction of GFP/lac repressor/VP16 fusion protein.

Acentric Chromatin Bodies in Cancer Cells

These novel technologies can also be applied to study the chromosomal consequences of the genetic instability that typifies a majority of solid tumors. Aneuploidy, chromosome deletions, translocations, inversions, and gene amplifications are found in a significant fraction of human solid tumors. However, little work has been done to assess the impact of abnormal numbers or structures of chromosomes on nuclear dynamics, or to assess whether aberrant chromosomes behave differently than a wild type counterpart. As an example, the dynamics of mitotic segregation of acentric chromosomes, called DMs, has not been extensively analyzed due to a lack of adequate methodology.

DMs are structurally acentric, atelomeric, circular chromatin bodies, known to harbor amplified oncogenes and drug resistance genes [Alitalo and Schwab, 1986], and they are present in a significant fraction of human cancers [Benner et al., 1991] (Fig. 1A). DMs replicate once per cell cycle using the cellular replication machinery since they contain one or more chromosomal replication origins (see [Carroll et al., 1991] for an example). They range in size from submicroscopic size (several hundred Kbp) in which case they can be detected using molecular strategies [Ruiz et al., 1989], to 30 Mbp or more [Hamkalo et al., 1985]; those that are 1–2 Mbp are barely detectable by light

microscopy. The paired minute chromosomes visible in metaphase spreads represent replicated sister chromatids.

The mechanisms by which DMs are formed remain to be discussed, but it is very likely that chromosome breakage plays an important role [Windle et al., 1991; Hahn, 1993; Toledo et al., 1993; Singer et al., 2000]. Their copy number varies from metaphase to metaphase, even in a single cell line, and such heterogeneity is due to uneven mitotic segregation of acentric DMs, rather than due to their uncontrolled replication (reviewed in [Hahn, 1993]). However, a mathematical model based on the analysis of DM segregation in individual cells early during drug selection revealed a surprisingly high fidelity of mitotic segregation [Kimmel et al., 1992]. As described below, the basis for the unexpectedly high efficiency of mitotic segregation of DMs is starting to be revealed through the use of powerful GFP labeling strategies.

Mitotic Behavior of DMs Analyzed by GFP-Based Chromosome Labeling Strategies

As DMs lack functional centromeres, they cannot segregate by the same mechanism used by normal chromosomes. If DMs lacked a mechanism to segregate into daughter nuclei, they would disperse into the cytoplasm upon nuclear membrane disassembly at mitosis. Early clues to the mitotic behavior of DMs came from simple Giemsa staining experiments of mitotic figures in cells prepared in the absence of colcemid [Barker and Hsu, 1978; Levan and Levan, 1978]. These studies indicated that DMs could associate with each other in clusters and could tether to mitotic chromosomes. Nucleolar

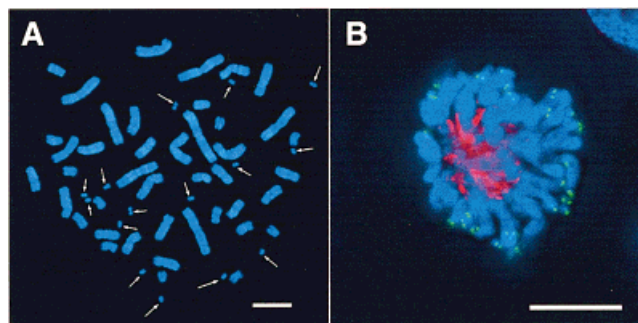


Fig. 1. DMs in cancer cells: conventional and recent views. **A:** Mitotic chromosome spreads of COLO320DM cells pretreated with colcemid. Chromosomes were stained with DAPI. DMs (arrows) are identified as double dots ($\sim 1 \mu\text{m}$ in diameter) scattered around chromosomes. The scale bar is $10 \mu\text{m}$. **B:** DMs were specifically tagged with lac operator repeats and detected

in vivo expression of lac repressor-GFP fusion protein (green). Cells were directly fixed without colcemid treatment, and processed for immunostaining using an anti- α -tubulin antibody to visualize microtubules (red). Chromosomes are stained with DAPI. The scale bar is $10 \mu\text{m}$.

material was observed to surround DMs, and was proposed to contribute to the unusual chromosomal associations [Levan and Levan, 1978].

Giemsa-staining of fixed samples cannot provide a kinetic view of the process by which DMs associate with chromosomes, nor can it elucidate the molecules that may be involved. We have begun to develop methods to enable acquisition of high resolution images of DMs during the cell cycle in real time to gain insight into these issues. One strategy involved introducing an H2B-GFP expression vector into cancer cells containing DMs [Kanda et al., 1998]. Time-lapse microscopy demonstrated that segregating DMs “piggybacked” onto segregating chromosomes from anaphase to telophase, validating the “chromosome tethering model” of DM segregation.

More recently, we devised a strategy to visualize DMs specifically. This was achieved by introducing a vector containing 256 repeats of the 40 bp lac operator sequence [Kanda et al., 2001]. The lac operator repeats were specifically introduced into the DMs using an Epstein-Barr virus (EBV) vector, which we found to integrate into DMs and not into normal chromosomes [Kanda et al., 2001]. The lac operator-tagged DMs behaved like native DMs and could be readily detected subsequent to expression of the lac repressor-GFP fusion protein. We used a combination of lac repressor-GFP and immunostaining of microtubules to demonstrate that DMs do not associate with microtubules, but they do associate with the periphery of prometaphase chromosome rosettes and metaphase chromosomes (Fig. 1B). DMs always localized far from the spindle poles, suggesting that they may be subjected to a force that propels them away from each spindle pole (see below for further discussion). DM movement was tracked from mitosis into G1 phase using H2B-CFP to visualize chromosomes and lac repressor-YFP to reveal DMs, and the result is summarized in Figure 2. Sister minutes were readily visualized as double fluorescent dots that remained associated throughout mitosis. The paired dots then dissociated during G1, as previously described using fixed cells [Takayama and Uwaike, 1988]. These studies reveal a difference in the behavior of the sister chromatids of chromosomes, in which cohesion is disrupted at the onset of anaphase, and DMs, which remain paired well into G1. It remains to be determined whether

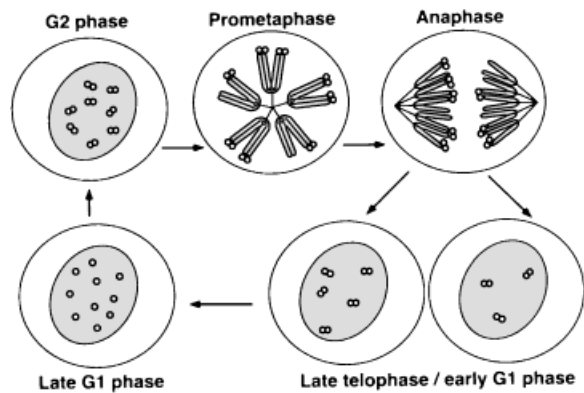


Fig. 2. Schematic representation of DM dynamics during the cell cycle. DMs attach to the periphery of prometaphase chromosome rosettes and segregate into daughter nuclei by hitchhiking onto normal chromosomes. Most of the DMs are still identified as double dots in early G1 phase, but they are no longer paired in late G1 phase, suggesting that the separation of sister minutes occurs during G1 phase.

this reflects a difference in the way that cohesive forces are disrupted in acentric molecules, or whether the association of sister minutes results from catenation that is resolved in G1.

Similarity Between DMs and Extrachromosomally Replicating Viruses

Two aspects of DM behavior remain to be explained. The first concerns the peripheral localization of DMs in prometaphase and metaphase cells. One explanation for this behavior is that DMs, but not EBV vectors, are subjected to the microtubule-mediated antipolar force (Fig. 3), which is known to push chromosomal arms away from the poles [Heald, 2000]. In support of this idea, disrupting microtubule organization diminished the peripheral localization of DMs [Kanda et al., 2001]. Interestingly, we have also found that Kid protein [Tokai et al., 1996], which is a kinesin-like motor protein implicated in the anti-poleward force, localizes to DMs as well as to chromosome arms.

The second issue concerns the molecular mechanisms that enable DMs to tether to mitotic chromosomes. We have begun to gain insight into potential answers to this intriguing problem by studying the DNA sequences and trans-acting factors involved in the mitotic segregation of extrachromosomally replicating viruses, such as EBV. EBV-based plasmids containing the viral origin of replication, oriP, and the virally-encoded protein, EBNA-1, also

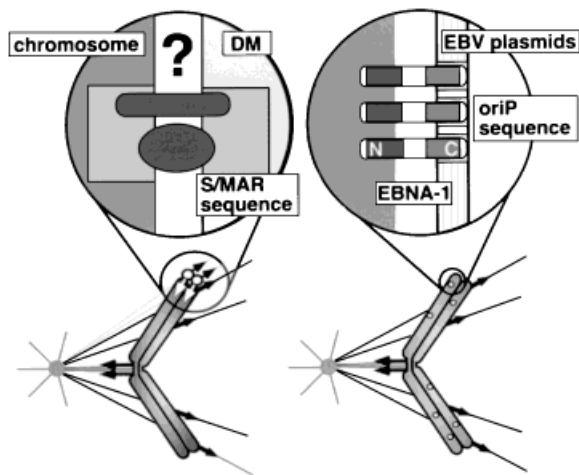


Fig. 3. Models of chromosome tethering of DMs and EBV vectors. The mode of chromosomal association of DMs (white circles in bottom left) and EBV plasmids (smaller circles in bottom right) are shown. Chromosomal association of EBV plasmids is mediated by EBNA-1 protein, which serves as a bridging molecule between mitotic chromosomes and oriP-containing plasmids (top right). In case of DMs, there may be cellular counterparts acting as cis-acting sequence(s) and transacting factor(s) for their chromosomal association (top left). A spindle pole is shown as a gray circle, and microtubules are shown as solid lines. Microtubule-mediated forces are shown as black arrows, while interacting forces between DMs and chromosomes are shown as white arrows. EBV plasmids are not subject to microtubule-mediated antipolar force due to their small size.

tether to mitotic chromosomes, which has been demonstrated by FISH analyses (see [Simpson et al., 1996] for an example). EBNA-1 has a modular domain structure comprising a C-terminal region that binds to specific sequences in oriP, while its N-terminus contains sequences that bind to mitotic chromosomes [Marechal et al., 1999]. Therefore, EBNA-1 appears to serve as a bridging molecule between mitotic chromosomes and oriP-containing plasmids (Fig. 3). Interestingly, analysis of mutants in either oriP or EBNA-1 showed that chromosomal association correlates with replication competence (Kanda et al, unpublished observation). One implication of this result is that the association between cellular chromatin and EBV plasmids is established during DNA replication.

It is tempting to speculate that DMs mimic the chromosome tethering strategy of EBV plasmids. DMs may also have cis-acting sequences that recruit cellular transacting factors to them to mediate chromosome association.

Scaffold/matrix attachment regions (S/MAR), which are frequently found near cellular replication origins [Pemov et al., 1998], and putative S/MAR associated cellular protein(s) may also be candidates for cis-acting sequence(s) and trans-acting factor(s) that mediate tethering (Fig. 3). Consistent with this hypothesis, a recent study showed that an episomal vector containing a human S/MAR sequence and an SV40 origin associates with mitotic chromosomes [Baiker et al., 2000]. Further experiments are required to validate such a model. The relationship between DM-specific integration of EBV plasmids [Kanda et al., 2001] and analogous chromosome tethering strategies employed by both DMs and EBV plasmids is currently unclear.

DM Behavior in Interphase Nuclei

Since DMs lack centromeres and telomeres, their intranuclear dynamics is expected to be very different from normal chromosomes during interphase. For example, DMs have a far greater propensity to localize to micronuclei than do chromosomes [Snapka and Varshavsky, 1983; Von Hoff et al., 1992; Shimizu et al., 1996]. DM-enriched micronuclei may arise in several ways. First, if chromosomal tethering fails, lagging DMs should be separated from groups of segregating chromosomes and incorporated into micronuclei at the end of mitosis. An additional, or alternative model proposes that DMs may be excluded from interphase nuclei during DNA replication [Shimizu et al., 1998]. The model is based on the FISH analyses of DM dynamics using synchronised cell populations in which a good correlation was noted between the number of DM-enriched micronuclei and the progression of DNA replication. Subsequent analyses showed that DMs move from the nuclear periphery to the nuclear interior just before the replication of peripheral heterochromatin, and that such dramatic movement is not observed for intrachromosomally amplified regions [Itoh and Shimizu, 1998]. However, the biological significance of S-phase micronucleation and intranuclear DM movement remain to be clarified. Recently, FISH and three dimensional image analyses were used to study the nuclear topology of DMs in comparison to other chromosome territories [Solovei et al., 2000]. The result revealed that DMs are typically located at the periphery of complexly folded chromosome territories, raising the idea that

DMs are located within the interchromosomal domain space so that the amplified genes have an easy access to protein complexes for transcription and splicing [Solovei et al., 2000].

Eliminating DMs, a Novel Cancer Therapy?

Since DMs encode growth promoting genes, such as oncogenes and drug resistance genes, eliminating DMs from cancer cells could be a novel chemotherapeutic strategy. The feasibility of such approach was first demonstrated by *in vitro* studies showing that DM loss from tumor cells is greatly accelerated by low concentration of hydroxyurea [Snapka and Varshavsky, 1983]. Other studies showed that treating DM-containing cells with hydroxyurea resulted in decreased plating efficiency in soft agar and reduced tumorigenicity in mouse xenograft models [Von Hoff et al., 1992; Canute et al., 1998]. The concentration of hydroxyurea required to accelerate DM loss (50~100 μM) is at least an order of magnitude lower than its cytotoxic concentration. It is possible that this concentration of hydroxyurea interferes with DM replication in such a way as to impede its association with chromosomes during interphase, resulting in a failure to hitchhike during mitosis. Consistent with this proposal, FISH analysis of the mitotic distribution of DMs after hydroxyurea treatment revealed that the chromosome hitchhiking of DMs was indeed inhibited [Tanaka and Shimizu, 2000]. Another study demonstrated that fractionated radiation accelerated DM loss through radiation-induced micronucleation [Sanchez et al., 1998]. Since replication inhibitors such as hydroxyurea and ionizing radiation can induce DNA damage in S-phase, it is possible that this either directly interferes with DM-chromosome association, or sends a signal to prevent such association, resulting in an increased rate of DM loss. The new methods for real time visualization of individual DMs and chromosomes during the cell cycle should help to clarify the mechanisms by which DM association with chromosomes, DNA replication, and DNA damage are linked.

FUTURE DIRECTIONS

Although DMs were first described almost 50 years ago, their analysis has been hampered by their structural complexity, lack of methods to visualize their behavior due to their small size,

and their tendency to integrate in to chromosomes during *in vitro* culture. Recent progress of GFP-based chromosome labeling strategies enabled us to re-examine the dynamics of DMs in living cells and highlighted their unique mode of mitotic segregation. Future research will be oriented to enable an understanding of the molecular mechanisms governing their mitotic segregation, interphase dynamics, and ability to tether to chromosomes. The finding that viral and cellular acentric molecules both tether to chromosomes suggest that the viruses may have acquired a cellular protein and adapted it for this purpose. The molecular tractability of viral systems should enable significant progress to be made in understanding the precise molecular mechanisms governing this interaction that is crucial for efficient viral maintenance, and perhaps for replication of those viruses that utilize cellular replication machinery, such as EBV.

The dynamics of DMs in interphase nuclei must be examined in relation to DNA replication and subsequent mitotic chromosome tethering of DMs. Theoretically, as DMs have been successfully labeled using the lac operator/lac repressor system [Kanda et al., 2001], it should be possible to analyze DM dynamics in interphase nuclei. However, several technical hurdles must be overcome including the photo-damaging effects of long-term observation and z-axis motion of DMs inside the nuclear volume. Technological improvements such as development of highly sensitive cameras and rapid scanning of multiple z-series images should enable one to make multi-color, 4 dimensional movies (3 dimensions + time). Such analyses should eventually provide a very clear view of DM dynamics in interphase nuclei.

Once the rules for chromosome tethering and replication are sorted out, it should be possible to construct artificial DMs, autonomously replicating plasmids that segregate efficiently in the absence of a centromere. Instead of using viral origins and transacting factors, cellular origins and cellular transacting factors could be tested for the ability to enable episomal plasmid maintenance. Cellular S/MAR sequences and cellular S/MAR binding protein(s) are attractive materials to start with for such purposes.

It is also possible that understanding the molecular interactions that mediate chromosome tethering might provide new approaches for the design of drugs that interfere with

this process. Small molecules that specifically inhibit DM or viral tethering could comprise a new class anti-cancer or antiviral therapies, and owing to the restriction of DMs to cancer cells, perhaps afford a very favorable therapeutic index. GFP-based chromosome labeling strategies described in this review would serve as indispensable tools to assay for the effectiveness of such agents.

Finally, GFP-based chromosome labeling will be applied for in vivo studies in the future. For example, intravital videomicroscopic observation [Borgstrom et al., 1996] of implanted chromatin-labeled tumor cells in mice will enable visualization of chromosome dynamics, such as formation of aneuploidy or apoptosis, under tumor microenvironment. Another in vivo application should be producing transgenic mice in which chromosomes are prestained with GFP. Such mouse will be a useful source for variety of "chromosome-prestained cells", which facilitates studying various aspects of chromosome dynamics in a broad spectrum of tissues. The generation of transgenic flies expressing histone variant His2AvD tagged with GFP [Clarkson and Saint, 1999] suggests that analysis of developmental processes in other species may also benefit from approaches using fluorescent chromosome labeling. Thus, the future seems brighter with the advent of an expanding array of strategies to analyze chromosome behavior in vivo, and in real time.

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