Mitotic segregation of viral and cellular acentric extrachromosomal molecules by chromosome tethering

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SUMMARY

Mitotic chromosome segregation is mediated by spindle microtubules attached to centromeres. Recent studies, however, revealed that acentric DNA molecules, such as viral replicons and double minute chromosomes, can efficiently segregate into daughter cells by associating with mitotic chromosomes. Based on this similarity between viral and cellular acentric molecules, we introduced Epstein-Barr virus vectors into cells harboring double minute chromosomes and compared their mitotic behaviors. We added lac operator repeats to an Epstein-Barr virus vector, which enabled us to readily identify the transgene in cells expressing a fusion protein between the lac repressor and green fluorescent protein. Unexpectedly, we found that Epstein-Barr virus vectors integrated into the acentric double minute chromosomes, but not into normal chromosomes, in all of the six stably transfected clones examined. While transiently transfected Epstein-Barr virus vectors randomly associated with wheel-shaped prometaphase chromosome rosettes, the chimeras of double minute chromosomes and Epstein-Barr virus vectors in stably transfected clones always attached to the periphery of chromosome rosettes. These chimeric acentric molecules faithfully represented the behavior of native double minute chromosomes, providing a tool for analyzing their behavior in living cells throughout the cell cycle. Further detailed analyses, including real-time observations, revealed that double minute chromosomes appeared to be repelled from the spindle poles at the same time that they attached to the chromosome periphery, while centromeric regions were pulled poleward by the attached microtubules. Disrupting microtubule organization eliminated such peripheral localization of double minute chromosomes, but it did not affect their association with chromosomes. The results suggest a model in which double minute chromosomes, but not Epstein-Barr virus vectors, are subject to the microtubule-mediated antipolar force, while they both employ chromosome tethering strategies to increase their segregation to daughter cells.

Movies available on-line:
http://www.biologists.com/JCS/movies/jcs1941.html

Key words: Acentric chromosome, Mitosis, Epstein-Barr virus, Microtubule

INTRODUCTION

The replication and subsequent faithful segregation of duplicated chromosomes are crucial for the proper transmission of the cellular genome to daughter cells. In higher eukaryotes, the nuclear membrane breaks down at the beginning of mitosis, and subsequently spindle microtubules attach to centromeric kinetochores to assure the even distribution of sister chromatids. At the end of mitosis, the nuclear membrane reassembles around each group of chromosomes to form two daughter nuclei. Therefore, a reasonable assumption would be that acentric DNA molecules should not be maintained stably in nuclei as they do not attach to microtubules and should be dispersed throughout the cytoplasm subsequent to nuclear membrane breakdown.

In many cases, acentric DNA molecules, lacking functional centromeres, exhibit a surprisingly high stability in dividing cells. Examples of such stably transmitted acentric DNA molecules in human cells include cellular acentric chromosomes called double minute chromosomes (DMs) and extrachromosomally replicating viral DNAs. DMs are cancer-specific genomic anomalies known to harbor amplified oncogenes and drug resistance genes (Alitalo and Schwab, 1986; Hahn, 1993; Wahl, 1989). They are autonomously replicating, acentric, atelomeric, circular chromatin bodies, and usually 1-2 megabase pairs in size. Although they apparently lack functional centromeres (Barker and Hsu, 1978; Levan and Levan, 1978), their segregation efficiency is much higher than expected (Kimmel et al., 1992; Pauletti et al., 1990). Clues to the mechanisms underlying the efficient segregation came from light and electron microscopic observations showing that DMs frequently associated with mitotic chromosomes (Barker and Hsu, 1978; Hamkalo et al., 1985; Jack et al., 1987; Levan and Levan, 1978). We extended these observations using a fusion protein of human histone H2B and *Aequorea victoria* green fluorescent protein (H2B-GFP) to reveal DM clusters tethered to segregating daughter chromosomes in living cancer cells (Kanda et al., 1998). Time-lapse microscopy demonstrated that DMs could ‘hitchhike’ on segregating chromosomes from anaphase to

telophase, indicating how chromosome tethering could contribute to increased segregation efficiency.

Recent studies revealed that chromosome tethering may be a common mechanism for enhancing the transmission of extrachromosomally replicating viruses into daughter nuclei (Bastien and McBride, 2000; Ilves et al., 1999; Lehman and Botchan, 1998; Marechal et al., 1999; Skiadopoulos and McBride, 1998). One of the best characterized episomal vectors is based on the Epstein-Barr virus (EBV) replicon, which utilizes the cis-acting oriP sequence and the virally encoded EBNA-1 protein (Mackey and Sugden, 1999). OriP is composed of two clusters of EBNA-1 binding sites, referred to as the family of repeats and the dyad symmetry element (Reisman et al., 1985). It has been shown that EBNA-1 both enables autonomous replication of oriP-containing plasmids in human cells (Yates et al., 1985) and mediates the nuclear retention of the plasmids (Krysan et al., 1989; Middleton and Sugden, 1994). Since EBNA-1 protein localizes on mitotic chromosomes (Grogan et al., 1983; Marechal et al., 1999; Petti et al., 1990), it is reasonable to infer that EBNA-1 could recruit oriP plasmids to mitotic chromosomes. Consistent with this, fluorescence in situ hybridization (FISH) previously demonstrated that EBV vectors did associate with mitotic chromosomes (Simpson et al., 1996; Westphal et al., 1998). Such chromosome tethering should facilitate the efficient segregation of EBV vectors into daughter nuclei when the nuclear membrane reforms at the end of mitosis.

The observation of viral association with host chromosomes, 'hitchhiking', raised the intriguing question of whether DMs achieve efficient segregation by a similar mechanism. We explored this possibility by introducing EBV vectors into DM-harboring cells. Unexpectedly, we found that EBV vectors integrated into DMs, but not into normal chromosomes, in all of the six stably transfected clones examined. This observation suggested that EBV vectors preferentially integrate into acentric autonomously replicating structures, and it enabled us to devise a method to tag DMs with exogenous DNA. Cell lines with DM-EBV chimeras were derived in which lac operator (lacO) repeats were introduced as part of the EBV vector. These lacO-tagged DMs were readily detected using a fusion protein between the lac repressor (lacR) and green fluorescent protein (GFP), as previously demonstrated for visualizing homogeneously stained regions in CHO cells (Robinett et al., 1996). This provided a powerful tool for analyzing the mitotic behavior of DMs. We found different distributions of free EBV vectors and DM-EBV chimeras, although they both hitchhiked onto mitotic chromosomes. Possible molecular mechanisms governing the behavior of these acentric molecules are discussed.

MATERIALS AND METHODS

Plasmids

EBV oriP and EBNA-1 coding sequences derived from pCEP4 (Invitrogen) and a blasticidin resistance gene (Izumi et al., 1991) derived from pYN3215-bsr (kindly provided by Dr Fumio Hanaoka, Osaka University) were subcloned into pMBL19 to make pMBL19-EBVbsr. pMBL19, which has a bacterial p15A ori, was chosen for its ability to subclone unstable inserts (Nakano et al., 1995). Lac operator (lacO) repeats (256 direct repeats) derived from pSV2-dhfr 8.32 (Robinett et al., 1996) were subcloned into the pMBL19-EBVbsr to make EBV-lacO vector using STBL2 competent cells (Life Technologies, Grand Island, NY, USA) (Belmont et al., 1999). pCLMFG-lacR-GFP was constructed by subcloning a gene encoding EGFP (Clontech, Palo Alto, CA, USA) fused to lac repressor-nuclear localization signal (p3′SSdimerClonEGFP) (Robinett et al., 1996) into a splicing retroviral vector pCLMFG-MCS (kindly provided by Dr Nikunj Somia, Salk Institute), a derivative of the pMFG vector (Dranoff et al., 1993). pCLMFG-lacR-YFP was constructed in the same way using EYFP gene (Clontech). A histone H2B-CFP fusion gene was made by swapping the GFP gene of H2B-GFPN1 (Kanda et al., 1998) with the ECFP gene (Clontech). The H2B-CFP gene was subcloned into a pCLNRX vector (Naviaux et al., 1996). Cloning details are available upon request. Production of VSV-G pseudotyped retroviruses was performed by cotransfection of each retroviral vector and pMD.G (the plasmid encoding the envelope protein VSV-G) into 293 gp/bsr cells as described (Miyoshi et al., 1997).

Establishing cell lines with lacO-tagged DMs

COLO320DM cells harboring DMs containing an amplified c-*myc* gene were grown as described (Kanda et al., 1998). Exponentially growing cells (1×10^7) were transfected with 5 μg of the EBV-lacO vector using electroporation (BioRad, Hercules, CA, USA), resuspended in 10 ml of culture medium, and plated into two 10 cm dishes (8 ml, 2 ml for each dish). Blasticidin (15 μg/ml, Calbiochem, San Diego, CA, USA) was added to the transfected cells 24 hours after transfection, and cells were selected for 14 days. Drug resistant cells were further grown under reduced blasticidin concentration (5 μg/ml). Blasticidin resistant colonies were isolated 4 weeks after transfection and then replated into 48-well dishes. Cells were expanded in medium containing blasticidin (5 μg/ml) and 12 fast growing clones were selected for infection with the lacR-GFP retrovirus. Punctate staining in nuclei was observed in all clones analyzed, and three independent clones that exhibited the brightest fluorescent dots by lacR-GFP staining were chosen for further FISH analyses, as we expected these clones to contain the highest number of EBV-lacO vectors. This strategy was repeated independently twice (total six clones) to confirm the reproducibility of the experimental data.

Fluorescence in situ hybridization (FISH)

Cells were treated with colcemid (100 μg/ml) for 50 minutes, and chromosome spreads were prepared by conventional fixation. For dual color FISH, c-*myc* cosmid DNA was labelled with biotin, while the lacO repeat (*Sal*I-*Xho*I fragment of pSV2-dhfr 8.32) (Robinett et al., 1996) was labelled with digoxigenin using random prime labeling. Denaturation, hybridization and washing were performed as previously described (Shimizu et al., 1996). Signals were detected using FITC-avidin (10 μg/ml, Vector Laboratories, Burlingame, CA, USA) and rhodamine-conjugated sheep anti-digoxigenin antibody (4 μg/ml, Boehringer Mannheim, Indianapolis, IN, USA). Chromosomes were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/ml) in VectaShield (Vector).

Chromatin fibers were prepared on slide glasses as previously described for fiber-FISH (Parra and Windle, 1993). Signals were detected using three sequential steps of a signal amplification protocol as follows: (1) FITC-avidin (5 μg/ml) and anti-digoxigenin monoclonal antibody (1 μg/ml, Boehringer Mannheim); (2) biotinylated goat anti-avidin (1 μg/ml, Vector) and digoxigenin-labelled sheep anti-mouse IgG (2 μg/ml, Boehringer Mannheim); (3) FITC-avidin (5 μg/ml) and rhodamine-conjugated sheep anti-digoxigenin antibody (4 μg/ml).

Visualizing DM-integrated and free EBV vectors by in vivo lacR-GFP staining

One of the established cell lines, containing DMs tagged with lacO repeat, was infected with lacR-GFP retrovirus. The infected cells were subcloned by limited dilution to obtain sublines in which DMs are more uniformly labelled with lacR-GFP. For time-lapse imaging, the

same cell line was infected with H2B-CFP and lacR-YFP viruses simultaneously, and the double-labelled cells were subcloned by limited dilution.

For visualizing EBV vectors in transiently transfected cells, a subline of COLO320DM cells stably expressing lacR-GFP protein was established by retrovirus infection and subcloning of lacR-GFP positive cells. The established cells were transfected with the EBV-lacO vector by electroporation, and the transfected cells were harvested for immunofluorescence analyses at 3 days posttransfection.

Immunofluorescence staining

Cells were harvested by gentle pipetting, attached to slide glasses by cytospin (500 rpm (25 ***g***), 1 minute, Shandon, Pittsburgh, PA, USA), and fixed with 3.7% formaldehyde for 10 minutes. When indicated, cells were treated with either taxol (10 μM paclitaxel, Sigma Chemical Co., St. Louis, MO, USA) or vinblastine (10 μg/ml, Sigma) for 3 hours prior to harvesting them in order to disrupt microtubules. Slides were washed with PBS three times, and treated with blocking buffer (2.5% BSA, 0.2M glycine, 0.1% Triton X-100) for 30 minutes.

Primary and secondary antibodies were diluted in the blocking buffer. Primary antibodies were rabbit anti-EBNA-1 serum K67-3 (1:1000, kindly provided by Dr Jaap Middeldorp, Free University Hospital, Amsterdam, the Netherlands), human CREST autoantiserum hACA-M (for detecting centromeres, 1:2000 dilution) (Sullivan et al., 1994), and monoclonal anti α-tubulin (for detecting microtubules, 1:2000 dilution, Sigma). Following incubation for 60 minutes at room temperature, slide glasses were washed three times with PBS. Secondary antibodies were Texas Red-conjugated anti-rabbit IgG (1:500, Jackson ImmunoResearch, West Grove, PA, USA), Cy5-conjugated goat anti-human IgG (1:1000, Amersham, Piscataway, NJ, USA), and rhodamine-conjugated anti-mouse IgG (1:1000, Boehringer Mannheim), respectively. Following incubation for 60 minutes at room temperature, slides were washed three times with PBS, and chromosomes were counterstained with DAPI (1 μg/ml). Fluorescence of lacR-GFP was preserved well by this protocol.

Microscopy

All images appearing in this article were collected using a DeltaVision microscope system (Applied Precision Inc. Issaquah, WA, USA) with either a 63×/NA 1.4 or a 100×/NA 1.35 oil immersion objective. For fixed specimens (except for the images of Fig. 2), three-dimensional data sets were collected to visualize EBV vectors and DMs as they distributed in multiple focal planes. Optical sections were collected at 0.2-μm focal intervals; pixel size was 0.111 μm for 63× objective and 0.0669 μm for 100× objective. Out-of-focus contamination was removed from each optical section via deconvolution processing and two-dimensional images were created by projecting the three-dimensional data stacks using the software supplied with the DeltaVision system.

For observation of living specimens, cells were grown on 40 mm coverslips pretreated with fibronectin (25 μg/ml in PBS) and placed in an FCS2 chamber system (Bioptechs, Butler, PA, USA) with prewarmed medium (containing 20 mM Hepes, pH 7.3). Special filter sets required for CFP and YFP detection (Ellenberg et al., 1999) were installed into the DeltaVision microscope system. Single-slice images were collected every 2 minutes using a 100× objective equipped with an objective heater (Bioptechs). Pixel size was 0.1338 μm and a binning factor of 2 was used to minimize the total exposure time during observation. Pseudo-color images were created using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

RESULTS

DMs are preferred targets for EBV vector integration

In the initial stage of this study, we transfected EBV vectors into COLO320DM cells, which contain DMs encompassing c-*myc* loci, and isolated drug-resistant clones after blasticidin selection. We examined the localization of EBV vectors by FISH analyses in three different clones, and found that transfected EBV vectors colocalized with DMs in three out of three clones (data not shown). One explanation for this finding was that EBV vectors preferentially integrated into DMs. To facilitate the tracking of transfected EBV vectors, we added 256 direct repeats of the lac operator (lacO) to the EBV vector (EBV-lacO) (Fig. 1). If the EBV-lacO vectors recombine with DMs, DMs should be tagged with lacO repeats and should be rapidly detected in cells expressing a fusion protein between the lac repressor and GFP (lacR-GFP) (Fig. 1) (Robinett et al., 1996). We transfected the EBV-lacO vector into COLO320DM cells and selected for blasticidin resistance. Drug resistant colonies were obtained at frequencies of approximately 1×10^{-4}-10^{-5}. Importantly, both the oriP sequence and the EBNA-1 gene were required for obtaining transformants, as no colonies arose in transfections employing vectors lacking either element (transformation efficiency $<1\times10^{-7}$). Three drug resistant colonies were expanded into cell lines and analyzed by dual-color FISH analyses using a c-*myc* cosmid probe (to detect DMs) and a lacO repeat DNA (to visualize EBV-lacO vectors). The result revealed that, in all three clones, the signals generated by both probes frequently overlapped in metaphase chromosome spreads (a representative metaphase spread from one clone is shown in Fig. 2A). We repeated the entire process (transfection, drug-selection, colony isolation, and FISH) independently and found colocalization of DMs and EBV-lacO vectors in three additional clones (total 6/6 clones examined). It is noteworthy that lacO signals never overlapped with intrachromosomal c-*myc* signals (see Fig. 2A for an example), and no evidence of EBV-lacO integration into chromosomes was observed in >50 metaphase spreads in three different clones. Although we cannot exclude the possibility that the

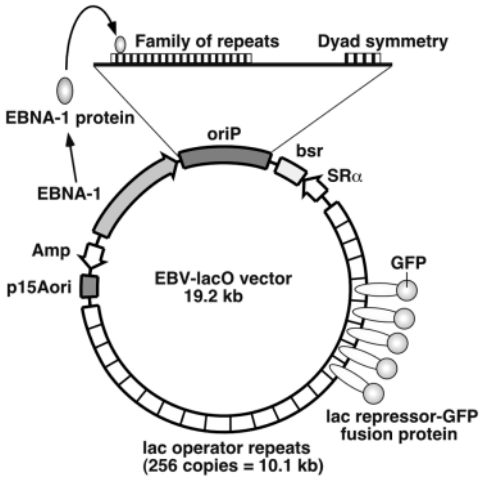

Fig. 1. EBV vector used for specific labeling of DMs. The EBV-lacO vector contains the EBNA-1 gene and oriP sequence, which has total of 24 EBNA-1 binding sites in two distinct regions (Family of repeats and Dyad symmetry) (Reisman et al., 1985). The vector has 256 tandem repeats of the lac operator (lacO), to which lac repressor (lacR)-GFP fusion protein binds with high affinity. The vector also has a blasticidin resistance gene (bsr) driven by SRα promoter as a drug selection marker.

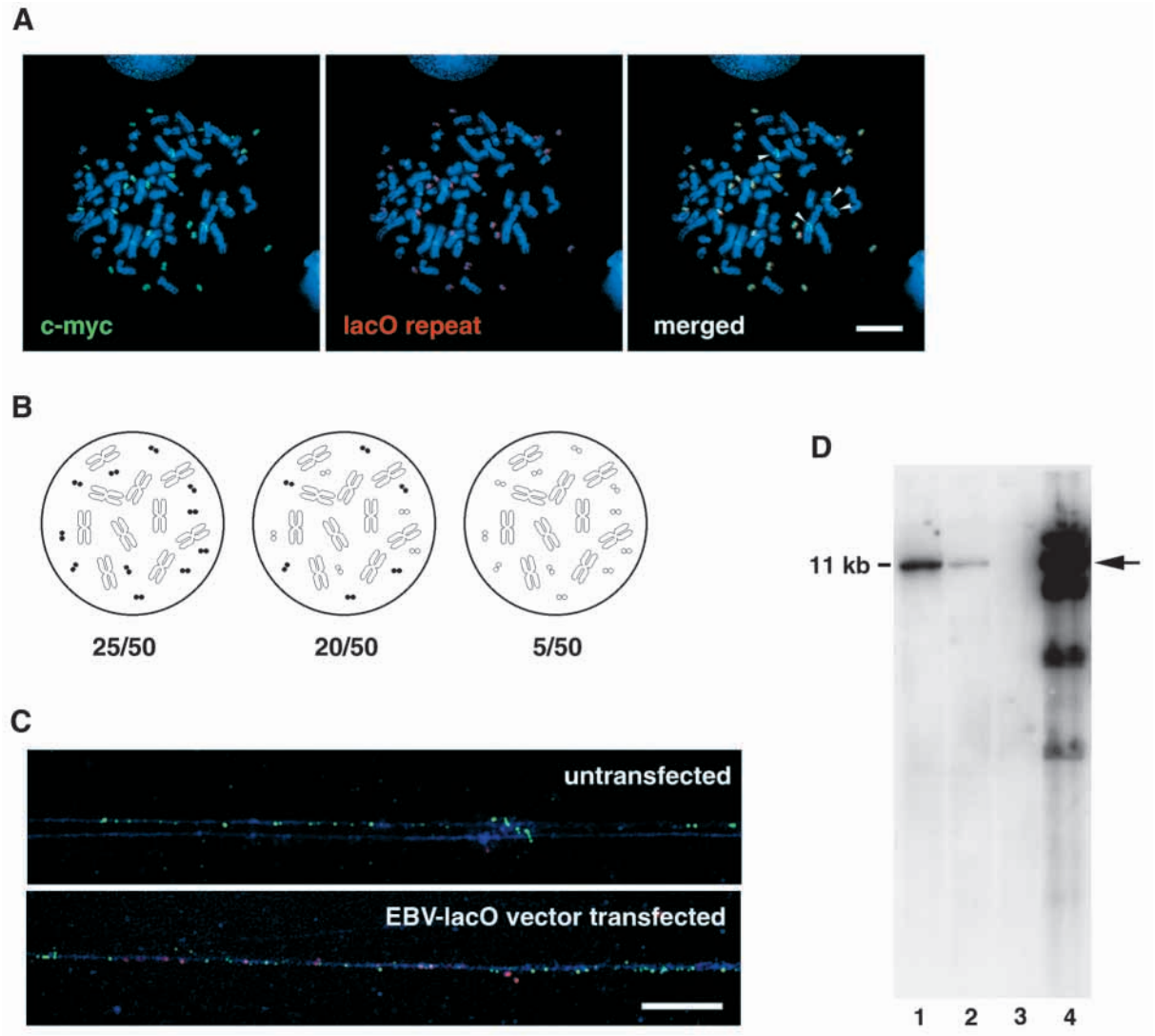

Fig. 2. DMs are preferred targets for EBV vector integration. COLO320DM cells were stably transfected with EBV-lacO vectors, and drug-selected clones were analyzed for the colocalization of EBV vectors and DMs. Representative results obtained by analyzing one of the established clones are shown in this figure. (A) A representative metaphase spread analyzed by dual-color FISH analysis, showing the colocalization of lacO repeats with DMs. DMs are observed as small scattered dots in mechanically spread chromosomes of colcemid-treated cells. Signals detected using c-*myc* cosmid probe (green) and lacO repeat probe (red) are shown. Chromosomes were counterstained with DAPI (blue). Intrachromosomal c-*myc* loci, lacking overlapping red signals, are shown by arrowheads in the merged image. Scale bar, 10 μm. (B) Frequency of colocalization of DMs and lacO repeats in this clone. Paired black dots represent DMs with overlapping lacO signals. 50 metaphase spreads were examined for colocalization, and they were divided into three categories: complete overlap (left), partial overlap (middle), and no overlap (right). The numbers of metaphase spreads in each category are shown. (C) Two-color FISH analyses using chromatin fibers prepared from untransfected COLO320DM cells (top) and cells transfected with the EBV-lacO vector (bottom). Note the lacO signals (red) and c-*myc* signals (green) on the same chromatin fibers (DAPI: blue). Scale bar, 10 μm. (D) Genomic DNAs (10 μg) of parental COLO320DM cells (lanes 1-3, ten copies and one copy equivalent of EBV-lacO plasmids added in lanes 1 and 2, respectively) and the established lacO integrated clone (lane 4) were digested with *Hin*dIII, which clipped out an 11 kb fragment containing the lacO repeats from the EBV-lacO plasmid. Digested DNA samples were analyzed by Southern blotting using ^{32}P-labelled lacO repeat (*Sal*I-*Xho*I fragment of pSV2-dhfr 8.32) (Robinett et al., 1996) as a probe. An arrow indicates the expected size of the fragment containing the lacO repeats.

FISH protocol was not sufficiently sensitive to detect some of the transfected vectors, the combined data strongly suggest that the majority of transfected EBV vectors preferentially colocalize with DMs.

We characterized one of the established clones in detail. First, we examined the frequency of metaphase spreads in which DMs were labelled with lacO repeats. Out of 50 metaphase spreads, 50% (25/50) of the spreads exhibited complete colocalization of lacO/DM signals, and 90% (45/50) of the metaphase spreads contained at least one pair of DMs with overlapping lacO signals (Fig. 2B). On average, each chromosome spread contained 18.3 (±19.2) pairs of DMs, and 12.7 (±13.2) DMs had overlapped lacO signals. Therefore, approximately one third of the DMs in this clone contained lacO repeats, although the number varied from metaphase to metaphase.

The colocalization of DMs and lacO repeats could be due to non-covalent interactions or to integration of the EBV-lacO

vectors into DMs. We applied fiber-FISH analysis to examine the localization of lacO signals and *c-myc* signals on stretched chromatin fibers (Parra and Windle, 1993). The result revealed that arrays of lacO signals were detected on the same DNA fibers exhibiting c-*myc* signals (Fig. 2C). This result indicates that multiple copies of each 19 kb EBV-lacO vector (red dots) integrated into a single DM molecule (which has a size exceeding 1000 kb and is shown as an extended chromatin fiber with green signals). Semi-quantitative Southern blotting revealed that approximately 130 copies of EBV-lacO vectors were present per cell. As there are approximately 13 DMs with integrated lacO repeats per metaphase in this clone (see above), we conclude that each DM contains approximately 10 copies of the EBV-lacO vector (i.e., 130/13). Importantly, Southern blotting analysis revealed one larger fragment and several smaller fragments in addition to the expected-size fragments (Fig. 2D). The complex signal patterns observed by fiber-FISH, together with the many extra bands observed in Southern blotting, indicate that the integration events are likely to be complex, or that further rearrangements occurred subsequent to the initial integration.

EBV-lacO/DM chimeras and free EBV vectors behave differently in prometaphase cells

The established cell lines containing the lacO-integrated DMs were infected with retrovirus expressing the lacR-GFP fusion protein. Retroviral infection resulted in readily detectable lacR-GFP protein expression in approximately 80% of the recipient cells, approximately 20% of which exhibited punctate fluorescent dots representing DMs. The lacR-GFP expressing populations were subcloned to yield clones in which 60% of the cells had punctate fluorescent DMs.

We first focused on the distribution of EBV-lacO/DM chimeras in prometaphase cells, in which chromosomes aggregate briefly into a single, wheel-shaped ring called a chromosome rosette (Nagele et al., 1995). We found that the fluorescent dots always attached to the periphery of the chromosome rosette (Fig. 3A). Immunofluorescence analyses revealed that EBNA-1 protein colocalized with EBV-integrated DMs (Fig. 3B), indicating that EBNA-1 was recruited to the oriP sequences that had been integrated into DMs. We also examined the mitotic distribution of EBV-lacO vectors after transient transfection into COLO320DM cells (see Materials and Methods). We infer that at 3 days post-transfection, most EBV plasmids were not integrated, and one or a few DMs may have contained integrated EBV plasmids. The fluorescent dots representing the transiently transfected EBV-lacO vectors were found to associate randomly with prometaphase chromosomes (Fig. 3C), corresponding well with previous FISH results indicating no preferential peripheral localization (Simpson et al., 1996; Westphal et al., 1998). In this case, EBNA-1 staining was rather diffuse on chromosomes, as observed previously (Grogan et al., 1983; Petti et al., 1990), but still colocalized with the EBV vectors (Fig. 3D). These data are consistent with free EBV-lacO vectors associating randomly with mitotic chromosomes, while DM-integrated EBV vectors localizing at the periphery. Importantly, we found that native DMs and EBV-integrated DMs displayed equivalent mitotic behavior (Fig. 3E,F), although the latter had colocalizing EBNA-1 protein (Fig. 3B). Therefore, it is likely that the mitotic behavior of the chimeric extrachromosomal molecules faithfully represents that of native DMs. These observations justify the use of EBV-lacO/DMs as a tool to analyze DM dynamics.

Mitotic behaviors of DMs, centromeres and microtubules

The fluorescent labeling strategy for DMs described above enables visualization of DMs, centromeres and microtubules simultaneously in various phases of mitosis. Centromeres and spindle microtubules were detected by indirect immunofluorescence while preserving the fluorescence of lacR-GFP. This analysis confirmed that DMs lack centromeric antigens (Barker and Hsu, 1978; Levan and Levan, 1978) (Fig. 4A,C,E,G,I), and that DMs do not associate with kinetochore microtubules (Fig. 4D,F,H). DMs and centromeres exhibited

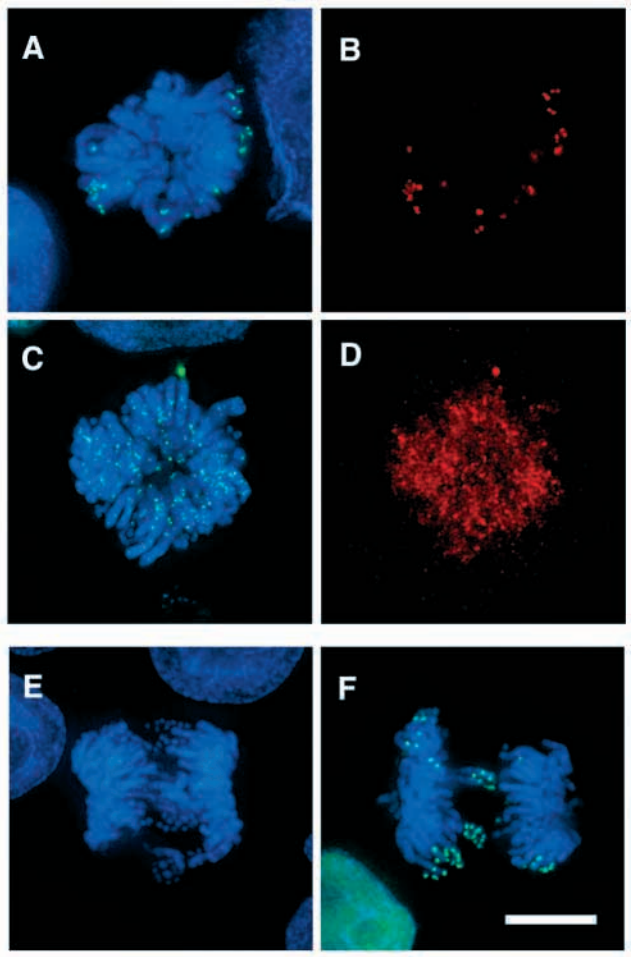

Fig. 3. Localization of DM-integrated and extrachromosomal EBV vectors. (A,C) Prometaphase chromosome rosettes of COLO320DM cells, having either EBV-lacO vectors integrated into DMs after stable transfection (A) or transiently transfected EBV-lacO vectors (B), are shown. Green fluorescent dots represent the lacR-GFP protein recruited to the lacO repeats in the vectors. Chromosomes are counterstained with DAPI (blue). (B,D) The localization of EBNA-1 protein in the same cells as in A and C, shown by indirect immunofluorescence (red). (E) Distribution of native (unlabelled) DMs in an anaphase cell of the parental COLO320DM cell line. Note that DMs make clusters and attach to mitotic chromosomes when cells are not treated with colcemid. (F) Distribution of lacO/lacR-GFP labelled DMs in an anaphase cell of the established cell line. Scale bar, 10 μm.

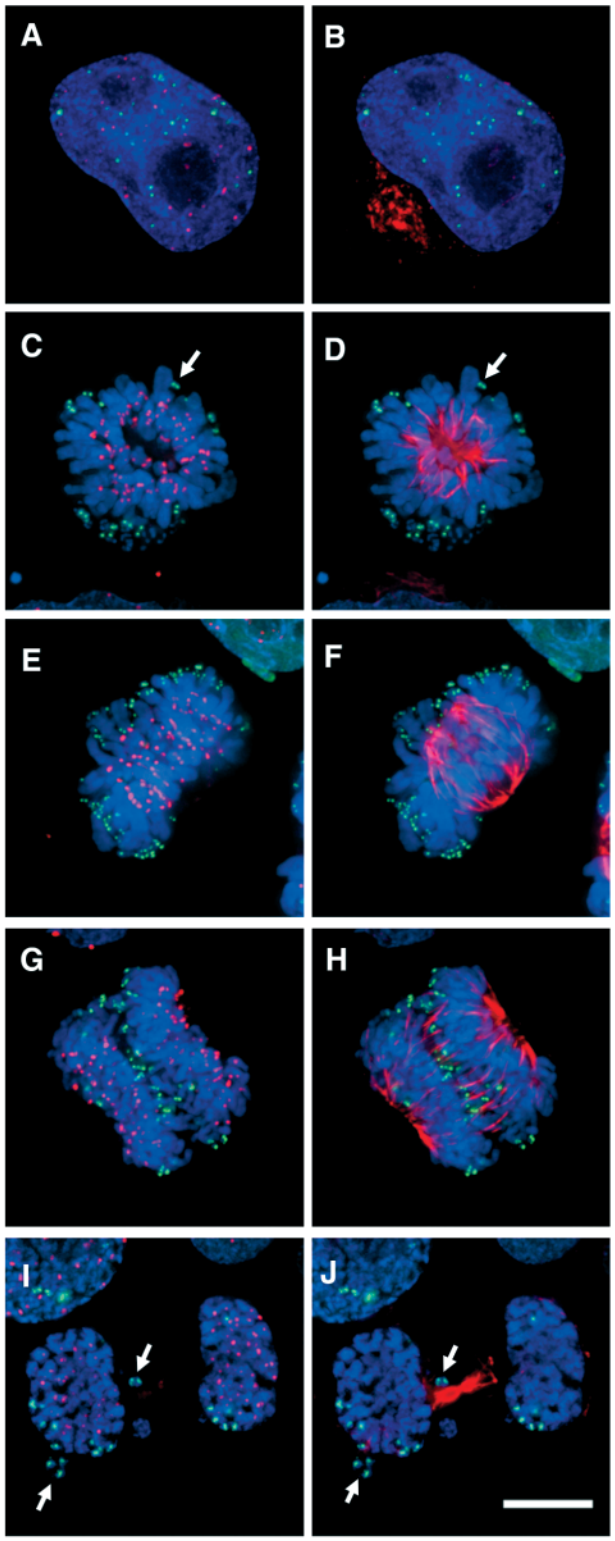

Fig. 4. Mitotic behavior of DMs, centromeres and microtubules. Cells containing labelled DMs (green) were processed for immunofluorescence analyses while preserving the fluorescence of lacR-GFP. Centromeres (red in A,C,E,G,I) and microtubules (red in B,D,F,H,J) in the same cells were detected by indirect immunofluorescence. Chromosomes were counterstained with DAPI (blue). DMs associating with distal chromosomal arms, but not with telomeres, are shown by arrows (C,D). DMs being incorporated into micronuclei are shown by arrows (I,J). Scale bar, 10 μm.

distinctly different behaviors during the phases of mitosis. In interphase cells (Fig. 4A,B), DMs and centromeres were dispersed independently in nuclei. However, in prometaphase cells (Fig. 4C,D), paired fluorescent DM dots attached to the periphery of the chromosome rosettes (Nagele et al., 1995), as described above, while centromeres localized centrally as they were pulled inward by the attached microtubules. In metaphase cells (Fig. 4E,F), DMs did not strictly align on metaphase plates, but rather associated with the periphery of the aligned chromosomes. At the metaphase-anaphase transition (Fig. 4G,H), centromeres of sister chromatids were pulled apart by the attached spindle microtubules. In contrast, some DMs were still clearly observed as double dots in anaphase cells, demonstrating that the connections between sister minute chromosomes are not readily broken at the metaphase-anaphase transition. The majority of DMs, still attached to the distal chromosomal arms, lagged behind centromeric regions that were pulled toward opposing poles in anaphase cells (Fig. 4G,H). In telophase cells (Fig. 4I,J), most DMs were incorporated into daughter nuclei along with the chromosomes, while a minority became entrapped in micronuclei (Shimizu et al., 1996; Tanaka and Shimizu, 2000).

Real time observation of DM behavior using dual-color fluorescent protein labeling

The above results with fixed cells could mask movements occurring in living cells, and do not provide a dynamic view of DM behavior. We addressed these concerns by taking advantage of the in vivo expression of distinguishable fluorescent fusion proteins (Ellenberg et al., 1999) in order to analyze the dynamics of DMs and chromosomes in living mitotic cells. Cells with lacO-labelled DMs were simultaneously infected with two different retroviruses expressing either H2B-CFP or lacR-YFP. H2B-CFP should label chromosomes and DMs, as described previously (Kanda et al., 1998), while lacR-YFP should label only DMs as described above. The feasibility of this dual-color labeling approach was first demonstrated using fixed cells, in which chromosomes and DMs in the same cell were observed with minimal spectral overlap (Fig. 5A). Representative time lapse images demonstrating the behavior of DMs at the metaphase-anaphase transition are shown (Fig. 5B). DMs were found at the tips of chromosome arms in late prometaphase cells (time 00). Subsequently, paired sister chromatids were observed to align on metaphase plates (time 04). When sister chromatids started to separate at the onset of anaphase (time 06), DMs quickly changed their position and lagged behind segregating chromosomes (time 08, 10). In early G_1 phase, significant numbers of DMs were still observed as paired dots (data can be seen in the attached movie), confirming the previous observation obtained by a premature chromosome condensation protocol (Takayama and Uwaike, 1988). These time lapse images correspond very well with the data of fixed cells (Fig. 4). Taken together, the peripheral localization of DMs in prometaphase chromosome rosettes and their lagging behavior in anaphase cells strongly suggest that DMs may be repelled from the spindle poles.

Microtubule inhibitors disrupt DM peripheral localization

The ability to readily visualize DMs without using FISH

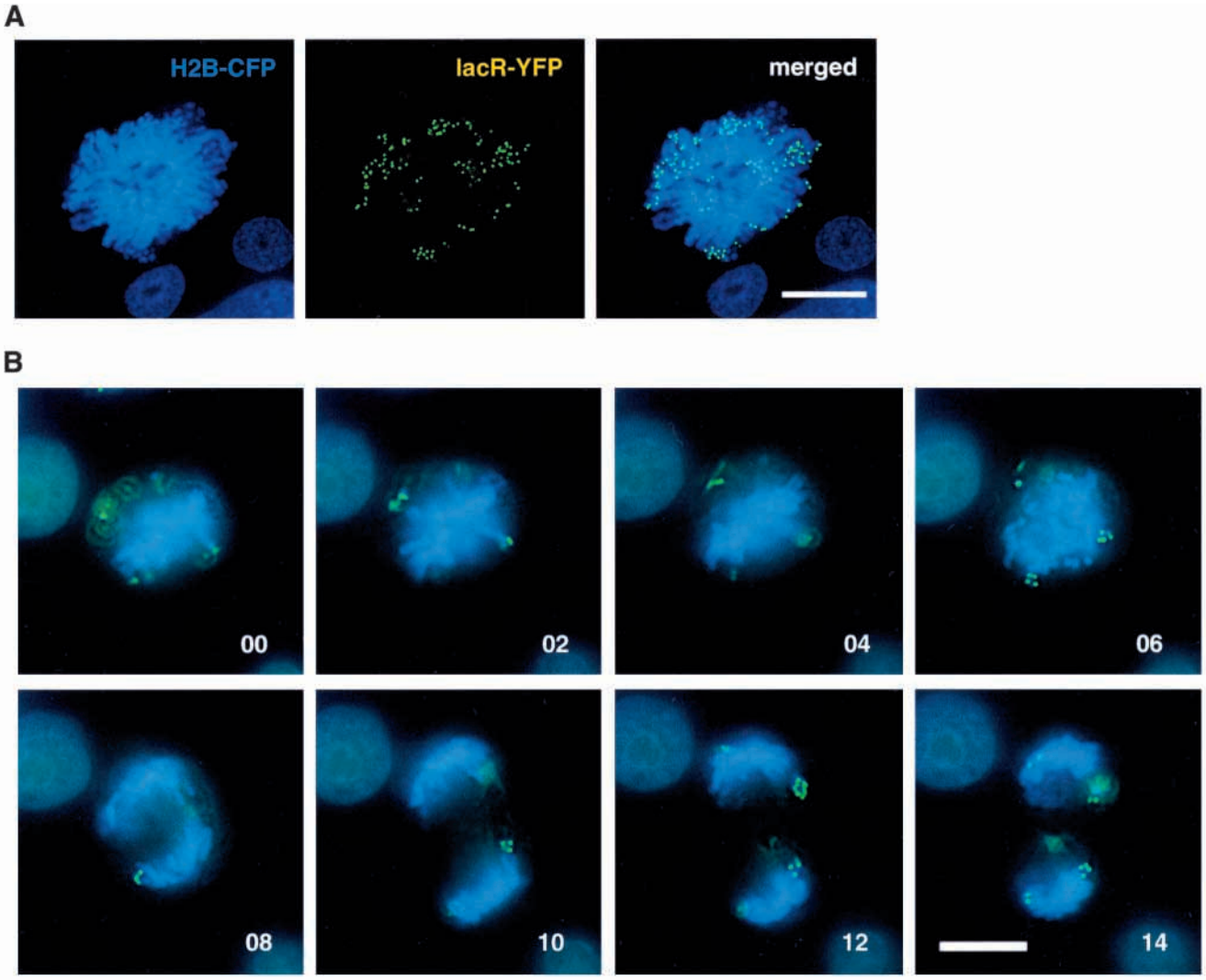

Fig. 5. DM behavior in mitotic cells analyzed by dual-color fluorescent protein labeling. (A) Projection images of a fixed cell, demonstrating the feasibility of dual-color labeling. Entire chromosomes, including DMs, are stained with H2B-CFP (left), while lacO-tagged DMs are specifically stained with lacR-YFP (middle). Merged image is shown in right. Scale bar, 10 μm. (B) Time-lapse images of DM behavior at the metaphase-anaphase transition during mitosis. Single-section, dual-color images were collected at the indicated time points (minutes). Scale bar, 10 μm. The accompanying QuickTime movie shows the DM behavior from late prometaphase to early G_1 phase.

protocol has enabled us to investigate the mechanisms that contribute to their behavior during mitosis. We examined the possibility that DMs are held away from the spindle poles by the microtubule-mediated antipolar force, to which normal chromosome arms are also known to be subjected (Fuller, 1995; Rieder et al., 1986). We treated DM-labelled cells with either a microtubule stabilizer (taxol) or destabilizer (vinblastine). DMs and microtubules were visualized by lacR-GFP and immunofluorescence staining, respectively. Microtubules of taxol-treated cells showed multiple aster-like structures (Fig. 6A), while vinblastine-treated cells exhibited rod-like microtubules (Fig. 6B). In both cases, chromosome organization was completely disrupted and DMs were no longer attached to the periphery of clustered chromosomes. Rather, DMs distributed randomly, although they were still associated closely with chromosomes (Fig. 6). These results support the idea that DMs are repelled from the spindle poles via microtubule-mediated antipolar forces and that microtubules do not mediate the attachment of DMs to mitotic chromosomes.

DISCUSSION

The mechanisms underlying the precision of chromosome segregation are being elucidated with increasing detail. It now appears that some autonomously replicating DNA viruses achieve high efficiency segregation not merely by their high copy number, but rather by having devised strategies to associate with chromosomes (Bastien and McBride, 2000; Ilves et al., 1999; Lehman and Botchan, 1998; Marechal et al., 1999; Skiadopoulos and McBride, 1998). Our data highlight the role of chromosomes as ‘cargo ships’ on which both viral replicons and cellular DMs are loaded to enable their efficient transmission to daughter nuclei.

We found that EBV vectors integrate into DMs at high

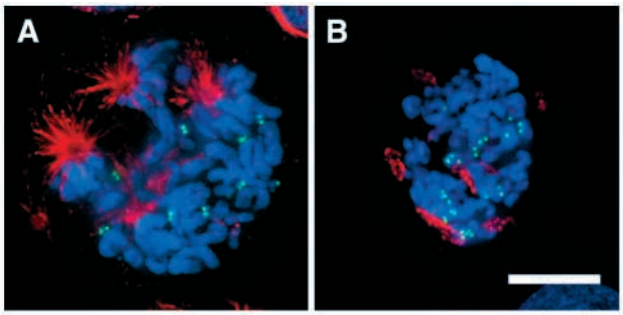

Fig. 6. Microtubule inhibitors disrupt the peripheral localization of DMs. Cells with lacO/lacR-GFP labelled DMs, treated with either taxol (A) or vinblastine (B), were processed for immunofluorescence analysis to detect microtubules (red) while preserving the fluorescence of lacR-GFP (green). Chromosomes were counterstained with DAPI (blue). Note that DMs are no longer at the periphery but still associating with chromosomes. Scale bar, 10 μm.

frequency. This targeted integration of EBV vectors into DMs was totally unexpected since EBV vectors containing oriP and the EBNA-1 gene are usually maintained as extrachromosomal elements without integrating into chromosomes (Yates et al., 1985). We found that EBV vectors randomly associated with mitotic chromosomes as well as DMs after transient transfection (Fig. 3C). This observation corresponds well with the known noncovalent association of EBV vectors with mitotic chromosomes (Harris et al., 1985; Marechal et al., 1999; Simpson et al., 1996; Westphal et al., 1998). However, after stable transfection into DM-harboring cells, we observed that EBV vectors recombined with DMs, and that the chimeric molecules of EBV vectors and DMs were always found at the periphery of mitotic chromosomes (Fig. 3A). We did not detect free extrachromosomal EBV vectors randomly associating with mitotic chromosomes after stable transfection. Interestingly, we found that the same EBV-lacO vector could be maintained extrachromosomally in stably transfected HeLa cells, which do not contain DMs (data not shown). Therefore, the EBV vectors appear to remain as independent extrachromosomal molecules in cells without DMs, while there is a high probability of integration of multiple EBV plasmids into DMs.

The molecular basis of preferential integration into DMs remains a mystery. One may argue that poor EBNA-1 expression under stable conditions in COLO320DM cells results in the highly frequent integration. However, it cannot explain why the EBV vectors integrated into DMs, yet not into normal chromosomes. Furthermore, we observed that the same vector was stably maintained as plasmids in an isogenic cell line lacking DMs (data not shown). This makes it unlikely that insufficient EBNA-1 expression in COLO320DM cells could explain the observed DM integration. Therefore, we infer that it is some property of the DMs that distinguishes them from chromosomes and leads to their being a preferred target for recombination with EBV replicons. One possibility is that DMs and normal chromosomes have different tendencies to undergo recombination. Another possibility is that EBV replicons and DMs share the same subnuclear compartments during S phase of the cell cycle, which increases the probability of recombination between replication intermediates. This would be consistent with the observed requirements of both oriP and EBNA-1 for recombination with DMs. The heterogeneity in the number and fraction of DMs containing integrated EBV-lacO sequences, even in a single clone (Fig. 2B), is most easily explained if one assumes that a single integration event occurred at an early stage of selection shortly after transfection. We infer that replication of the EBV-lacO/DM chimeras, followed by their uneven mitotic segregation, could have generated clones in which the numbers of chimeric extrachromosomal molecules per cell are not uniform even in a single clone. EBV vectors may provide a general strategy for tagging DMs derived from different chromosomal loci, as we succeeded in obtaining EBV-DM chimeras in the CRL2270 neuroblastoma line containing extrachromosomally amplified N-myc amplicons (data not shown).

The chimeric extrachromosomal molecules of DMs and EBV-lacO vectors appear to exhibit the same behavior as native (unlabelled) DMs. The visualization strategy involving lacR-GFP can be used in combination with immunofluorescence, as it does not require harsh denaturation of DNA, and it preserves chromosomal fine structures far better than FISH (Robinett et al., 1996). This sensitive methodology enabled us to visualize DMs together with centromeres and microtubules (Figs 4, 6), and to track the dynamics of DMs and chromosomes in living human cells (Fig. 5). The data confirms and further extends previous analyses using fixed cells (Levan and Levan, 1978)

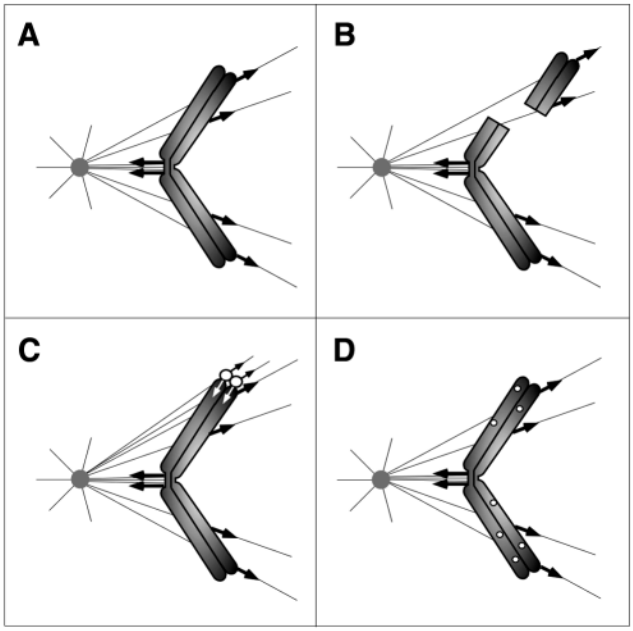

Fig. 7. Model to explain the difference of the dynamics of various acentric DNA molecules. For simplicity, only one pair of mono-oriented prometaphase sister chromatids is drawn in each panel. The four panels show: (A) the behavior of normal mono-oriented sister chromatids; (B) a chromosome arm severed by laser microsurgery (Rieder et al., 1986); (C) a pair of DMs attached to the tip of chromosomes (large white circles); and (D) EBV vectors randomly associating with chromosomes (small white circles). 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 (C). The size of each arrow represents the relative strength of each force.

and our previous findings obtained using H2B-GFP staining (Kanda et al., 1998).

The ability to visualize DMs revealed their frequent association near telomeric regions of chromosomes (Figs 4, 5), raising the possibility that DMs might have an affinity for telomeric regions. This would explain why DMs frequently localize to the periphery of prometaphase chromosome rosettes, and why they lag behind in anaphase cells. The change in DM position at the metaphase-anaphase transition might be explained by the rotation of normal chromosomes. However, two observations argue against the idea of a specific association between DMs and telomeres. First, some DMs were found to associate with chromosomal arms at positions clearly distinct from telomeric regions (Fig. 4C,D). Second, dual-color FISH analyses using a telomeric DNA probe and a c-*myc* cosmid probe (for detecting DMs) revealed that there was no spatial proximity between DMs and telomeric regions in interphase nuclei (data not shown).

Based on these observations, we propose a model in which DMs are subject to two 'forces' in mitotic cells (Fig. 7). The first 'force' appears to push DMs away from the poles. It is well known that, while the kinetochore microtubules pull chromosomes poleward, another force appears to repel chromosomal arms (Fuller, 1995; Heald, 2000) (Fig. 7A). Laser microsurgery experiments demonstrated that severed chromosomal arms immediately moved radially outward to the periphery of the aster (Rieder et al., 1986) (Fig. 7B), indicating the existence of such an astral exclusion force. We made two observations that support the idea that DMs are repelled by microtubule-mediated antipolar force (Fig. 7C). (1) The peripheral localization of DMs becomes apparent only after nuclear membrane breakdown and chromosomes attach to microtubules; (2) disrupting microtubule organization prevents the peripheral localization of DMs. It has been proposed that plus-oriented kinesin-related microtubule motor proteins, distributed along chromosomal arms, mediate this astral exclusion force (Fuller, 1995; Heald, 2000). Human Kid protein (kinesin-like DNA binding protein) (Tokai et al., 1996) is a candidate for generating such antipolar force. Recent studies revealed that Xkid (*Xenopus* homolog of Kid) has an essential role in metaphase chromosome alignment by generating the polar ejection forces (Antonio et al., 2000; Funabiki and Murray, 2000). We recently observed that the DMs that originated from the distal part of chromosome 8 (8q24; c-myc locus) do have Kid protein, just like normal chromosomal arms (data not shown). This is consistent with models suggesting that a 'repelling force' could contribute to DM localization. The antipolar force working on chromosomal fragments is likely to be proportional to their size. This can partly explain the difference between the behavior of severed chromosomal arms and that of DMs, since bigger acentric chromosome fragments should be subject to a stronger antipolar force compared to smaller DMs (Fig. 7B,C). Random chromosomal association of free EBV vectors can be explained by the lack of plus-oriented motor proteins and astral exclusion force on free EBV vectors (Fig. 7D).

The second 'force' acting on DMs keeps them attached to mitotic chromosomes (Fig. 7C). This force is not affected by microtubule disruption. Although there is a study demonstrating that DMs associate with chromosomes via nucleolar material (Levan and Levan, 1978), no further experimental data supporting the idea has been presented. Our finding that DMs are frequent recombinational targets of EBV vectors leads us to propose another model in which DMs may somehow mimic the behavior of viral vectors. It has been suggested that chromosome tethering of EBV vectors is mediated by the cis-acting oriP sequence and trans-acting viral protein EBNA-1 (Krysan et al., 1989; Mackey and Sugden, 1999; Marechal et al., 1999; Middleton and Sugden, 1994; Simpson et al., 1996). We recently found that EBNA-1 appears to serve as a bridge between chromosomes and oriP-containing vectors (Kanda T et al., manuscript in preparation). This observation raises the possibility that DMs may also have cis-acting sequences that recruit cellular transacting factors to them to mediate chromosome association. This possibility is strengthened by the finding that DMs consist of multiple copies of amplicons, each copy of which contains cellular replication origin(s) that are usually associated with scaffold/matrix attachment regions (S/MAR) (Carroll et al., 1991; Pemov et al., 1998). A recent study showed that an episomal vector containing a human S/MAR sequence and SV40 origin is associated with mitotic chromosomes (Baiker et al., 2000). Therefore, it is conceivable that DMs containing multiple S/MARs attach to chromosomal scaffolds, which then gives the appearance that they are associating with mitotic chromosomes, even though there is no direct connection between chromosomes and DMs. The interacting force mediated by S/MAR-bound proteins may be strong enough to compete with the weak antipolar forces working on DMs (Fig. 7C).

The ability of viral replicons and DMs to interact with chromosomes provides a simple solution to the problem of high efficiency segregation of acentric DNA molecules. Interfering with the molecular interactions between viral replicons/DMs and mitotic chromosomes would increase the mitotic loss rate of latently infected viruses, or DMs that are providing survival or selective advantage to cancer cells. Therefore, understanding the molecular interactions that mediate such associations is likely to suggest new molecular targets for anti-viral and anti-cancer therapy.

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