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Extrachromosomal double minutes and chromosomal homogeneously staining regions as probes for chromosome research

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Abstract

Gene amplification in human cancer cells generates two cytogenetically identifiable structures: extrachromosomal double minutes (DMs) and the chromosomal homogeneously staining region (HSR). DMs are composed of autonomously replicating circular DNA of genomic origin, and they tell us about how the extrachromosomal elements may behave in the cells, how they were entrapped by the micronuclei and how they were eliminated from the cells. On the other hand, the episome model predicts that extrachromosomal elements excised from the chromosome arm might generate DMs, and the breakage-fusion-bridge (BFB) cycle model explains the generation of the HSR. In accordance with this, a plasmid bearing a mammalian replication initiation region (IR) and a matrix attachment region (MAR) mimics gene amplification and generates DMs and HSRs *de novo*. The IR/MAR gene amplification system extends our understanding on the mechanism of gene amplification and the behavior of amplified genes. Furthermore, the system may suggest the way how the extrachromosomal elements in general may alter the chromosome architecture and function.

1. Gene amplification and Cancer

Genomic instability is one of the fundamental features of human cancer cells. Consequently, gene amplification is a frequent abnormality restricted to these cells (for recent reviews for gene amplification, see (Murnane and Sabatier 2004; Debatisse and Malfoy 2005; Myllykangas and Knuutila 2006; Albertson 2006; Murnane 2006). In single cell organisms, worms, insects and amphibians, regulated amplification of certain genes is employed for normal development (reviewed in Claycomb and Orr-Weaver 2005); however, gene amplification is strictly prohibited in normal mammalian cells in order to keep the genome integrity. In contrast, amplification of various oncogenes was frequently associated with certain type of cancer. Therefore, gene amplification is considered to be one of the major mechanisms of oncogene activation, and cells with amplified oncogenes may gain a growth advantage through the overproduction of protein products. For example, a direct correlation between amplification and protein over-expression was reported for HER-2/neu proto-oncogene in human breast and ovarian cancer (Slamon et al. 1989). Furthermore, c-*myc* gene number was almost proportional to its mRNA level in human colorectal cancer cell line (Shimizu et al. 2007a), and comparative genomic hybridization on cDNA microarrays revealed hundreds of novel genes whose overexpression is attributable to gene amplification in breast cancer (Hyman et al. 2002). Similarly, amplification of drug-resistance genes may allow cancer cell growth in the presence of cytotoxic drugs, and it was associated with the overproduction of corresponding protein (Wahl et al. 1979; and reviewed in Albertson et al. 2003). However the proportionality between DNA copy number and the amount of protein was not established in the strict sense.

Highly amplified genes manifest themselves as either of two cytogenetically identifiable structures; *i.e.,* double minutes (DMs; named after the paired minute chromatin body that was detected among the metaphase chromosome spread) and the chromosomal homogeneously staining region (HSR; see Figure 1A to C). Amplified genes most frequently localize at DMs rather than the HSR in primary tumor cells (reviewed in Benner et al. 1991). The DMs are autonomously replicating extrachromosomal genetic elements of genomic origin. They are composed of circular DNA (Hamkalo et al. 1985; VanDevanter et

al. 1990) that does not require telomeric ends, and usually they do not have active centromeres (Rattner and Lin 1984; Haaf and Schmid 1988). Despite their acentric nature, they are delivered to the daughter cell nucleus by sticking to the mitotic chromosomes (Levan and Levan 1978; Kanda et al. 2001; Kanda and Wahl 2000; see Figure 1F).

2. DMs tell us about the intracellular behavior and elimination through micronuclear entrapment of extrachromosomal elements.

2.1 Loss of amplified genes from cancer cells results in reversion of the tumor cell phenotype. The copy number of amplified genes may increase or decrease during the growth of cells (Kaufman et al. 1981). Because gene amplification is responsible for the malignant transformation of certain cancer cells, a decrease in the copy number of amplified genes results in the reversion of tumor cell phenotypes (Von Hoff et al. 1992), the arrest of cell growth (Shimizu et al. 1994), the expression of differentiation markers (Shimizu et al. 1994; Eckhardt et al. 1994) or apoptotic cell death (Von Hoff et al. 1992). This argument was made using human colorectal carcinoma COLO 320DM cells with amplified c-*myc* (Von Hoff et al. 1992), human promyelocytic leukemia HL-60 cells with amplified c-*myc* (Shimizu et al. 1994; Eckhardt et al. 1994), neuroblastoma cells with amplified N-*myc* (Ambros et al. 1997) and glioblastoma cells with amplified epidermal growth factor receptor genes (*EGFR*; Canute et al. 1998). Therefore, if we may artificially reduce the number of amplified genes, we may cure many cancers (Snapka 1992; Van and Von 1995; Stewart et al. 1996).

2.2 An active mechanism may eliminate amplified genes from the cells. DMs are acentric extrachromosomal chromatin that are distributed unevenly to the daughter cells through mitosis (Levan and Levan 1978), and cells with a lower number of DMs may appear. In addition, several pieces of evidence have suggested the presence of an active elimination mechanism. For example, the spontaneous appearance of cells that had lost most of their amplified genes was significantly more frequent than the level anticipated by unequal distribution only (Shimizu et al. 1994). Furthermore, a decrease in the amplified genes on DMs was accelerated by treatment of the cells with certain drugs or radiation. Such

drug-induced loss of amplified genes was most frequently reported for the DNA replication inhibitors such as hydroxyurea (HU) at lower concentrations (50 to 150 μM) than those that completely stopped DNA replication (1 to 2 mM). This HU-treatment resulted in a copy number decrease of the amplified *DHFR* in hamster CHO cells (Snapka and Varshavsky 1983; Wani and Snapka 1990; Nevaldine et al. 1999), amplified c-*myc* in human colorectal carcinoma COLO 320DM cells (Von Hoff et al. 1990; Von Hoff et al. 1992) or human promyelocytic leukemia HL-60 cells (Eckhardt et al. 1994) and amplified *EGFR* in human glioblastoma cells (Canute et al. 1996). The HU treatment reduced the number of DMs in advanced ovarian carcinoma *in vivo* (Raymond et al. 2001). The inhibitor of poly (ADP-ribose) polymerase or dimethyl sulfoxide also reduced the copy number of amplified c-*myc* in HL-60 cells (Shima et al. 1989). Furthermore, ionizing radiation accelerated the loss of amplified *MDR1* on DMs in multi-drug resistant KB cells (Sanchez et al. 1998; Schoenlein et al. 2003) or amplified c-*myc* in COLO 320DM cells (Schoenlein et al. 2003). It was suggested that low levels of cytotoxic drug may selectively kill cells with a high copy number amplification (Wani and Snapka 1990; Wani et al. 1990). However, copy numbers only decreased when the amplified genes resided on the DMs, and a low dose of HU had no effect on the cells with an identical level of gene amplification if the amplified genes resided on the HSR (Snapka and Varshavsky 1983; Von Hoff et al. 1992). Therefore, these treatments appeared to accelerate the active elimination process operating for DMs.

2.3 DMs are selectively incorporated into the micronuclei which may explain their elimination. Treatment with a low dose of HU (Von Hoff et al. 1992; Shimizu et al. 1996) or ionizing radiation (Sanchez et al. 1998) increased the frequency of DM-enriched micronuclei as well as inducing a loss of amplified genes on DMs. The selective entrapment of DMs with amplified N-*myc* into the micronuclei was also observed *in vivo* neuroblastoma tumor cells (Valent et al. 2001). In general, it was frequently reported that the content of micronuclei was eliminated from the cells; despite the fact that it is not known how elimination proceeds. For example, hypoploidy of the X-chromosome in peripheral leukocytes of an aged woman was related to the selective entrapment of the inactivated

X-chromosome to the micronuclei (Tucker et al. 1996; Dyer et al. 1989; Hando et al. 1997). Furthermore, many studies assume that the chromatin left behind the separating anaphase chromosome would diminish from the cells through the formation of micronuclei. The micronuclear entrapment of DMs may therefore explain their elimination.

2.4 How the content of micronuclei is eliminated from cells. It is possible that a replication defect in the micronuclei, if it exists, may explain the apparent elimination of the micronuclear content. However, BrdU-labeling showed that DNA replication takes place inside most micronuclei in S-phase cells (Tanaka and Shimizu 2000; Utani et al., unpublished). Therefore, the above possibility appears to be unlikely, but more extensive studies are needed to reach a conclusion because a minor portion of the micronuclei may not be replicated. It is also possible that micronuclei are degraded *in situ* in the cytoplasm; however, evidence for this is scarce. On the other hand, particles close to the micronuclei were found in the culture fluid of COLO 320DM cells (Shimizu et al. 2000). Such extracellular micronuclei were highly enriched in DMs, were wrapped with both the nuclear lamina and the cytoplasmic membrane, and the DNA inside them did not suffer any extensive degradation. These particles were not the apoptotic bodies, because lamin and DNA are degraded during apoptosis. Furthermore, a partially purified fraction of extracellular micronuclei had scarcely been contaminated by the apoptotic bodies (Shimizu et al. 2000). Therefore, at least a portion of the DM-enriched micronuclei appeared to be extruded from the cells as extracellular micronuclei.

The extracellular extrusion of micronuclei was reported in the course of erythrocyte maturation in a developmentally regulated manner (Parton et al. 1991; Schriever et al. 1997), and the process was further enhanced by cytochalasin B both *in vivo* and *in vitro* (Nito et al. 1988). The presence of extracellular DNA was also reported in the culture supernatant (Anker et al. 1975) or frequently in the serum (for a recent review, see Vlassov et al. 2007), and it may have originated from DNA in the extruded micronuclei because origination from the apoptotic bodies was suggested to be unlikely. The extrusion of micronuclei may be mediated by cytoplasmic membrane blebbing that is active during mitosis (Lee et al. 2004) or

a pre-apoptotic state (Takashina and Nakayama 2007) that may not result in apoptosis. Extracellular micronuclei allow us to speculate that they may be the origin of some kind of enveloped virus that has the cytoplasmic membrane as an envelope and the extrachromosomal DNA as a genome.

In order to understand the fundamental mechanism operating for elimination of the extrachromosomal genetic elements, an alternative approach was the tracking of microinjected DNA in live cells. Such experiments showed the presence of an apparently active mechanism that rapidly aggregated the DNA microinjected at the nucleus. After mitosis, the aggregate was left at the cytoplasm like the micronuclei, and it persisted long-term or eventually disappeared from the cytoplasmic rim (Shimizu et al. 2005a).

To understand the elimination of DMs or injected DNA, time-lapse observation of living cells is necessary. However, such time-lapse observation may not be an easy task, because our unpublished study suggested that the elimination event was infrequent and, once initiated, it proceeded rapidly. Thus, high-resolution, three-dimensional, short-interval and long-term time-lapse experiments should be repeated to clearly show the elimination process.

2.5 Why and how DMs are entrapped by the micronuclei. The entrapment of DMs by the micronuclei was highly selective, as revealed by fluorescence in situ hybridization (FISH) that detects the sequences on DMs (Von Hoff et al. 1992; Shimizu et al. 1996; Shimizu et al. 1998; Tanaka and Shimizu 2000). Furthermore, DNA in the purified micronuclei was highly enriched in the sequences on DMs (Shimizu et al. 1996), therefore the DNA could be used as a DM-paint FISH probe (see Figure 1A to C) or a probe that identified the chromosomal locus that was amplified in the cancer cells. Such selective incorporation of DMs into the micronuclei was a result of the behavior of DMs during cell cycle progression (summarized in Figure 2).

The acentric DMs were segregated to the daughter cell nucleus by sticking to mitotic chromosomes; this was called “hitchhiking” (Levan and Levan 1978; Kanda et al. 2001; Kanda and Wahl 2000). As a result of hitchhiking, DMs localize preferentially at the nuclear periphery during G1 phase (Itoh and Shimizu 1998). DMs are euchromatin because the

genes on them are actively transcribed (Utani et al. 2007; Shimizu et al. 2007a) and they are replicated at early S phase (Shimizu et al. 2001b). Thus, location of DMs at the nuclear periphery is unusual, because the periphery is usually enriched in heterochromatin. From such a position, DMs re-located to the nuclear interior during early S phase (Itoh and Shimizu 1998) when DMs themselves were replicated (Shimizu et al. 2001b). At that time, early S phase, treatment of the cells with a low concentration of HU induced the detachment of DMs from the chromosomes at the next mitosis (Tanaka and Shimizu 2000). Detached DMs were left in the cytoplasm at G1, and generated micronuclei.

Shimizu et al examined the effect of HU by simultaneously detecting DMs and DNA damage by visualizing phosphorylated histone H2AX (γH2AX). They showed that low concentrations of HU induced replication-associated DNA damage throughout the nucleus, which scarcely overlapped with DMs in early S phase cells (Shimizu et al. 2007b). However, if the cells were passaged into fresh medium, chromosomal γH2AX signals were gradually diminished reflecting their repair, whereas the remaining signals were frequently associated with the DMs. In metaphase to anaphase cells, the γH2AX signal was scarcely detected at the chromosome, whereas it was almost predominantly detected at the DMs. These DMs were usually aggregated and lagged behind the segregating chromosomes, which resulted in the formation of micronuclei specifically enriched in DMs. The precise molecular mechanism remains to be established, however, it suggests that the consequence of DNA damage on DMs appears different from the one on the chromosome arms. It was a long-term mystery why two types of micronuclei were simultaneously present in the same cell, *i.e.* the micronuclei specifically enriched in DMs and the ones with only chromosomal material. The above result clearly explains it.

2.6 What the heterogeneity of micronuclei means. There are many types of micronuclei among the cell population in a culture. They are heterogeneous in respect to their size, chromatin density as revealed by DNA-staining, or the presence of nuclear lamina (Willingale-Theune et al. 1989; Paulin et al. 1996). Tanaka and Shimizu reported that most of the DM-enriched micronuclei were not wrapped with lamin B during G1 phase, and they

were associated with lamin B at the early S phase when nuclear lamina is synthesized and dynamically rearranged (Tanaka and Shimizu 2000). Interestingly, they suggested that the lamin-entrapment of DMs at the outside nuclear rim is accompanied by a nuclear bud-shaped appearance. It corresponded with the proposal that DM-enriched micronuclei might be generated through nuclear budding (Shimizu et al. 1998). The involvement of nuclear budding in micronuclei formation was frequently suggested (for a review, see Zaharopoulos et al. 1998). In addition, Haaf et al showed that the Rad 51 foci were eliminated from the interphase nucleus and they formed micronuclei (Haaf et al. 1999). Furthermore, DNA microinjected in the nucleus was aggregated, it moved across the nuclear membrane to the cytoplasm and it formed a micronucleus-like structure (Shimizu et al. 2005a). Thus, all of these studies suggested that the interphase generation of micronuclei through nuclear budding is possible. It is also known that micronuclei are formed by the lagging chromatid or by the chromatin bridge between the daughter nuclei (Hoffelder et al. 2004; Fenech 2007). Therefore, the heterogeneity of micronuclei may reflect the differences in their formation mechanism; alternatively the heterogeneity may reflect their change during cell cycle progression. In order to clarify this point, further study on the life cycle of micronuclei is needed.

Whether DMs in the micronuclei are transcribed or not is an important question because the amplified genes that are critical for cancer development reside on the DMs. Untani et al (Utani et al. 2007) showed that the DMs were actively transcribed in the micronuclei and the transcript moved to the cytoplasm. Furthermore, transcription was strictly restricted to the micronucleus that was associated with the nuclear lamina. Because the fraction of the micronuclei with lamina changed during cell cycle progression (Tanaka and Shimizu 2000), the micronuclear entrapment of DMs could change the transcriptional regulation of amplified genes on DMs, therefore, it could profoundly influence the cell phenotype (Utani et al. 2007).

2.7 The intracellular behavior and the elimination of DMs may be common among autonomously replicating extrachromosomal elements. The hitchhiking manner of

segregation appears to be utililzed by a broad spectrum of the autonomously replicating exrtachromosomal elements, because several viral nuclear plasmids including papilloma virus (Lehman and Botchan 1998), Epstein-Barr virus (EBV; Marechal et al. 1999), Kaposi's sarcoma associated herpes virus (KSHV; Ballestas et al. 1999), and simian virus 40 (SV40; Baiker et al. 2000) also use a similar mechanism during segregation (reviewed in Calos 1998; Botchan 2004). Tethering was usually mediated by binding of the viral-encoded protein to the cellular chromosomal protein; *e.g.* the EBV-encoded EBNA-1 protein binds both the viral latent origin of replication (*Ori* P) and chromosomal EBP2 protein (Kapoor et al. 2005), the papilloma virus-encoded E2 protein binds to the C-terminal domain (CTD) of the chromosomal Brd4 protein (You et al. 2005), and the KSHV-encoded LANA protein binds to nucleosomal histone H2A-H2B (Barbera et al. 2006). Thus, overexpression of the CTD of Brd4 in papilloma-virus transformed cells released the viral DNA from the mitotic chromosome, and it resulted in the complete elimination of viral DNA from the cells and the reversion of the tumor phenotype (You et al. 2005). This consequence suggested a close similarity to the case of DMs. Interestingly, human *EBP2* was essential for cell proliferation, and the yeast *EBP2* homologue played a role in ribosome biogenesis (Tsujii et al. 2000) and localized to the nucleolus during interphase. Many nucleolar proteins localized to the perichromosomal layer of the mitotic chromosome (reviewed in Van Hooser et al. 2005), and it was suggested previously that the nucleolar-derived materials tethered DMs to the chromosomes (Levan and Levan 1978).

The intracellular behavior of DMs and their entrapment into the micronuclei may be common among a broad spectrum of extrachromosomal elements in general, because such behavior originated from the hitchhiking manner of segregation during mitosis and because hitchhiking is the only known manner by which extrachromosomal elements may be transmitted to the daughter cells.

3. Generation of DMs and HSR from the IR/MAR plasmid tells us how the extrachromosomal elements alter the chromosome architecture.

3.1 The IR/MAR plasmid efficiently initiates gene amplification. Shimizu et al found that a plasmid bearing a mammalian replication initiation region (IR) and a nuclear matrix attachment region (MAR) efficiently initiates gene amplification in mammalian cells, and it *de novo* generated structures indistinguishable from DMs or HSR in primary cancer cells; alternatively it was amplified at the pre-existing DMs, if the cells already had DMs that were generated during cancer development (Shimizu et al. 2001a; Shimizu et al. 2003). The IR is the DNA region where DNA replication initiates; it is scattered throughout the entire human genome (for reviews, see Gilbert 2001; Biamontim et al. 2003; Aladjem and Fanning 2004)). The MAR (also known as SAR; scaffold attachment region) is a sequence that is defined by its ability to bind to the isolated nuclear matrix/scaffold. There is no consensus sequence for the MAR, but it is usually an extremely AT-rich sequence. It was suggested that DNA replication requires anchorage to the nuclear matrix/scaffold, thus it requires the MAR (reviewed in Anachkova et al. 2005). Therefore, the sequence showing MAR activity is frequently found in the IR (*e.g.* IR from *DHFR* or β-*globin* locus); while some IR (*e.g.* IR from c-*myc* locus) do not contain the sequence showing MAR activity and such IR probably depends on the distantly located MAR. Sequences showing strong MAR activity may also be found at regions unrelated to the IR (*e.g.* AR1 sequence from *Ig k* intron; Tsutsui et al. 1993).

The mammalian IR was indispensable for the amplification of the IR/MAR plasmid, because the vector sequence alone or unrelated sequences of similar length never supported the amplification (Shimizu et al. 2003; Hashizume and Shimizu 2007). Therefore, sequence information inside the IR was necessary for the plasmid amplification. Furthermore, the MAR was also required for the efficient amplification of the plasmid (Shimizu et al. 2003; Hashizume and Shimizu 2007). As described above, an indispensable role for the MAR in DNA replication was frequently suggested. Thus, autonomous extrachromosomal replication of the plasmid may be required for efficient plasmid amplification. On the other hand, the plasmid bearing the EB virus *Ori P* and *EBNA-1* gene, which was autonomously replicated and stably segregated in mammalian cells, showed amplification at DMs (Kanda et al. 2001; Shimizu et al. 2001a); however, it never generated HSR (Shimizu et al. 2001a).

Therefore, amplification at DMs may reflect the autonomous replication of the introduced plasmid, whereas amplification at the HSR requires another additional feature of the plasmid. I hereafter call the amplification method the “IR/MAR gene amplification method”.

3.2 The IR/MAR plasmid provides a useful *in vitro* model for gene amplification. There have been many hypotheses for the mechanism of gene amplification in human cancer cells. Among these, the “episome model” proposed a central role for the extrachromosomal circular episome (reviewed in Wahl 1989). This episome can be excised from the chromosome arm (Carroll et al. 1988), it was replicated autonomously (Carroll et al. 1987), and it generated DMs where the amplicon sequences were arranged as a tandem repeats (Wahl et al. 1984). Another hypothesis to explain how genes may be amplified is the “Breakage-Fusion-Bridge (BFB) cycle model”, which was originally proposed by B. McClintock more than 50 years ago (McClintock 1951). This model has been used to explain the generation of a variety of gene amplifications or genomic instabilities in human cancer cells (reviewed in Murnane and Sabatier 2004; Debatisse and Malfoy 2005; Murnane 2006; Haber and Debatisse 2006; Bailey and Murnane 2006)), including the amplification of the *DHFR* or *AMPD2* genes in CHO cells (Kaufman et al. 1983; Trask and Hamlin 1989; Ma et al. 1993; Toledo et al. 1992) and the *CAD* gene in Syrian hamster cells (Smith et al. 1990; Smith et al. 1992). The BFB cycle has also been reported to be responsible for genetic intratumor heterogeneity (Gisselsson et al. 2000). In this model, a chromosomal breakage followed by replication and end-to-end fusion of sister chromatids generates a mitotically unstable dicentric chromosome, which leads to another breakage close to the first one. Multiple cycles of BFB lead to the multiplication of the genes near the breakage. Thus, the model required a double strand breakage for initiation. This break can be produced by a recombination-activating gene product in the case of *IgH*/c-*myc* co-amplification (Difilippantonio et al. 2002; Zhu et al. 2002), by the expression of fragile sites (Coquelle et al. 1997; Ciullo et al. 2002), by the *HIV Vpr* gene product (Shimura et al. 1999), by the expression of the I-*Sce* I mega-endonuclease (Pipiras et al. 1998), or by a folate deficiency which is a common feature of human tumor cells *in vivo* (Crott et al. 2001; Kimura et al. 2004). That the BFB cycle involves multiple

breakages both at initiation and at later stages may be related to the observation of gene amplification in cells with disrupted cell cycle checkpoint machinery (Yin et al. 1992; Livingstone et al. 1992) (Paulson et al. 1998), or in cells with a defective DNA damage repair system that was mediated by either DNA mismatch repair (Lin et al. 2001; Chen et al. 2001) or nonhomologous end joining (Mondello et al. 2001; El-Hizawi et al. 2002).

In order to understand the molecular mechanism of gene amplification, an *in vitro* model system that mimics gene amplification was definitively required. Therefore, a cosmid bearing a genomic copy of the PALA-resistant CAD gene (Wahl et al. 1984) or a plasmid bearing the methotrexate-resistant *DHFR* gene (reviewed in Omasa 2002) was transfected into Chinese hamster ovary (CHO) cells. The latter system was frequently used for the study of gene amplification and for recombinant protein production. However, such experimental systems utilized only drug-resistant genes; therefore, the question of why amplification occurred was hard to answer. Furthermore, the efficiency of gene amplification was usually low; and it took quite a long period of time, usually a few months, before the target genes were amplified. On the other hand, the IR/MAR gene amplification method utilized the introduction of the plasmid that had a defined sequence for replication and transcription, and it utilized only one-step selection based on drug resistance. As a result, up to 80 % of the transformed cells had highly amplified copies of the plasmid sequences as DMs or the HSR, which are cytogenetically indistinguishable from the ones generated during cancer development (Shimizu et al. 2001a; Shimizu et al. 2003). Thus, the method appears to be a useful model to uncover the mechanism of gene amplification in human cancer cells. Thus, a model that explains how the IR/MAR plasmid generates DMs and HSR was proposed (Shimizu et al. 2005b), and it was shown in Figure 3 with some modifications. In below, I will explain the mechanism according to this model.

3.3 How DMs are generated from the IR/MAR plasmid. DMs that were generated by the IR/MAR gene amplification method had the plasmid sequence as an orderly tandem direct repeat (Shimizu et al. 2003). The HSR also had the plasmid as the direct repeat but its orderly arrangement was somewhat disrupted. The chromatin fiber FISH applied to the

cloned cells revealed that a few tens of the plasmid sequence continued in DMs, and the sequences continued as a long array without any interruption in the HSR (Shimizu et al. 2003). The copy numbers of the plasmid sequences were from a few hundreds to a few thousands per cell (Shimizu et al. 2003; Shimizu et al. 2007a). A novel and a convenient feature of this gene amplification system was that any DNA could be co-amplified by co-transfection with the IR/MAR plasmid (Shimizu et al. 2003; Shimizu et al. 2007a); thus, even lambda phage DNA could be easily amplified. Fiber FISH analysis on such co-amplified structures suggested that recombination between extrachromosomal elements appeared to be very frequent, and that the co-transfected DNA was ligated to the IR/MAR plasmid after the early stages of transfection (Shimizu et al. 2003). Such frequent recombination was also seen between the plasmid repeat and the DMs if they pre-existed in the transfected cells (Shimizu et al. 2005b). Namely, dual-color FISH analysis revealed that there were either DMs composed with only the plasmid repeat or DMs intermixed with the plasmid repeat and the pre-existing DMs. By summarizing these results, a model was suggested for the generation of DMs. Namely, the IR/MAR plasmids are initially replicated as extrachromosomal circles, and during this stage, they are multimerized to a large circular molecule with a plasmid direct repeat. If the multimerization proceeds extensively, such large circles may be detected as cytogenetically detectable DMs. If the cells already have DMs, the amplified plasmid repeat frequently recombines with pre-existing DMs.

It was previously reported that extrachromosomal closed circular (ecc) DNA is present in normal cells from almost all tissues (Gaubatz 1990), and the content increased during the malignant transformation. It is highly conceivable that a portion of the excised molecules had the IR/MAR sequence because the sequence frequently exists in the genome, as described above; therefore, such molecules may generate DMs.

3.4 How the HSR is generated from the IR/MAR plasmid. The IR/MAR plasmid-derived HSR was frequently located at the end of the metaphase chromosome (Shimizu et al. 2003; Shimizu et al. 2005b). Furthermore, the HSR frequently formed the anaphase bridge (Shimizu et al. 2003; Shimizu et al. 2005b), and the plasmid sequences were located in the

middle of the bridge. These observations were consistent with the fact that the HSR was generated by the BFB cycle. The anaphase bridge was severed in the middle, where the plasmid repeat was located. It broke before nuclear membrane reformation or cytokinesis (Shimizu et al. 2005b); thus, the bridge appeared to be severed by the mechanical tension applied from the spindle, as is usually supposed in the BFB cycle model. Importantly, the bridge was always severed almost in the middle irrespective of the length of the plasmid repeat (Shimizu et al. 2005b). Severing the middle of the anaphase bridge was reported in another instance where the BFB cycle was progressing (Lo et al. 2002). However, it is curious that the long and homogeneous plasmid repeat was always severed in the middle because mechanical tension should sever such a structure at random. This suggested the presence of a specific point in the middle of the anaphase bridge; this point was most plausibly explained by the formation of large palindrome that generates a fragile cruciform structure (Shimizu et al. 2005b). The cruciform may be resolved by enzyme activity similar to the Holliday junction resolvase, as suggested in yeast cells (Narayanan et al. 2006; Lobachev et al. 2007). However, such a reaction does not explain why HSR elongates, despite the fact that it may explain how the BFB cycle initiates. On the other hand, single strand nucleases such as the Mre11 complex may attack the hairpin loop of the cruciform structure; however, this mechanism also cannot explain why the HSR elongates. The classical BFB cycle model predicts that the fluctuation of the breakage point around the exact middle of the anaphase bridge would result in unequal segregation of the HSR sequence to the daughter cells, which would contribute to the lengthening of the HSR. Precise molecular understanding of this mechanism should be an important future task.

The above results suggest that DMs or the submicroscopic episome that is composed of tandem IR/MAR-plasmid repeats, were integrated to the chromosome arm where the repeat initiated the BFB cycle that generated the HSR. Chromosomal integration may be mediated by breakage at the plasmid repeat followed by its re-ligation to the chromosomal breakage site. The capture of extrachromosomal DNA to the chromosomal breakage site was reported in mammalian cells (Lin and Waldman 2001). If the breakage was not ligated to the chromosome, the plasmid repeat would be eliminated through

entrapment into the micronuclei, as described in the earlier section. At the chromosome arm, a break at the plasmid repeat would initiate the BFB cycle as described, and the frequency of the break would relate to the frequency of the HSR generation. This will be the focus of the next section.

3.5 Head-on collision between replication and transcription may induce strand breaks that either initiate HSR generation or fragment HSR. The break at the plasmid repeat, which initiates the BFB cycle and generates the HSR, may arise from head-on collision between the replication fork from the IR and the transcription machinery from the promoter (Shimizu et al. 2003). This was suggested by constructing several plasmids where the replication fork or the transcription machinery were stopped by the presence of the orientation-specific replication fork barrier (RFB) sequence or the poly A addition 3'-processing sequence, respectively. It was reported that the collision between transcription and replication resulted in DNA breakage in bacterial and yeast cells (for a review, see Rothstein et al. 2000). IR/MAR plasmid amplification in animal cells further suggested the involvement of the MAR, because insertion of the sequence showing strong MAR activity at the collision point resulted in elevated HSR generation (Shimizu et al. 2003). It was suggested that the MAR is anchored to the nuclear matrix/scaffold by topoisomerase II, because the protein was a major constituent of the matrix/scaffold (Berrios et al. 1985; Earnshaw et al. 1985), its cleavage consensus sequence is frequently clustered in the MAR (Cockerill and Garrard 1986), and the purified enzyme directly interact with the MAR (Adachi et al. 1989). Therefore, a DNA that anchored to the nuclear matrix might become a recombination hot spot (Svetlova et al. 2001).

On the other hand, continuous strong activation of the tetracycline-inducible promoter in the IR/MAR plasmid during plasmid amplification prevented the generation of a long HSR (Shimizu et al. 2007a). Under such conditions, a short HSR or DMs were preferentially generated. Furthermore, if transcription was activated in cloned cells that already had a long HSR, the HSR was fragmented, converted to a short HSR and many DMs were generated (Shimizu et al. 2007a). The process was greatly enhanced by the presence of

5-azacytidine that decreased the DNA methylation level and enhanced transcription. It was suggested that many breaks were produced under strong transcriptional activation, thus leading to fragmentation of the long HSR. On the other hand, DMs rather than the HSR were preferentially found in tumor cells grown *in vivo*, whereas cells with an HSR predominated during long-term culture (reviewed in Benner et al. 1991). The most important difference between conditions *in vivo* and *in vitro* was the oxygen concentration, which might alter the promoter strength (Pouyssegur et al. 2006; Rocha 2007) that could in turn change the level of conflict between replication and transcription. Therefore, IR/MAR plasmid amplification also provides a unique model for the analysis of inter-conversion between the HSR and DMs.

3.6 The rule that destabilizes the IR/MAR-bearing plasmid may be applied to the construction of episome vectors. Many episome vectors were developed utilizing the viral sequences for replication and maintenance. Among these, it was reported that the combination of the SV40 virus origin of replication and the MAR sequence rendered the plasmid the ability of replication and maintenance (Piechaczek et al. 1999; Baiker et al. 2000). The same group then found that the replication origin was dispensable, and showed that the plasmid bearing a MAR between the promoter and the poly A addition sequence was stably maintained in animal cells (Jenke et al. 2004; Schaarschmidt et al. 2004). On the other hand, it is a long-lasting but as-yet unattained task to establish a stable episome vector using a mammalian replication IR. Such a vector will benefit the study of how DNA replication initiates from a defined sequence, and it may be used as a therapeutic vector that does not involve viral genes or as a vector for protein overproduction. Remarkably, DMs are autonomously replicating stable extrachromosomal molecules of genomic origin; thus, the presence of DMs suggests that artificial generation of an episomal vector using genomic sequences is possible.

One of the reasons why construction of an episome vector is difficult might be our ignorance about the conditions that destabilize such a vector. For example, the plasmid may be stable if collision between transcription and replication at the MAR is avoided. In actual

fact, the plasmid still generated an HSR even if the collision between promoter-driven transcription and the hypothetical replication fork from the IR was avoided by the insertion of a poly A addition sequence or an RFB sequence. However, the plasmid never generated an HSR if transcription of the promoter-independent orientation was also blocked (Hashizume and Shimizu 2007). Such a plasmid appeared to be stably maintained as an extrachromosomal element, as FISH detected tiny signals among metaphase chromosomes (Hashizume and Shimizu 2007). Promoter-independent transcription may represent the mechanism that surveys and maintains the genome of yeast or mammalian cells (Lu and Gilbert 2007; Vasiljeva et al. 2008). Therefore, the avoidance of such collisions appears to be one of the critical points for constructing episomal vectors.

3.7 The "plasmid stability assay" provides a novel method to dissect replicator sequences.

Sequence information for the IR was necessary to generate the HSR. Thus, Hashizume et al (Hashizume and Shimizu 2007) developed "the plasmid stability assay", which measured the HSR generation activity of the test sequence. In that assay, if replication initiated from the test sequence, it efficiently collided with the promoter-driven transcription at the MAR, and the plasmid generated an HSR. Using this assay, the IR from the human c-*myc* locus (2.4 kbp) and the IR from the hamster *DHFR* locus (4.6 kbp) were dissected. The assay reproducibly measured the HSR generation activity with high sensitivity and quite a low-background; the assay revealed that only about an 800 bp fragment (c-*myc* IR) or about a 1,700 bp fragment (*DHFR* IR) was necessary and enough for efficient HSR generation. These fragments contained several elements that were suggested to be required for DNA replication initiation: *e.g.* the duplex unwinding region, potential topoisomerase II binding sequence, autonomous replicating consensus sequence (ACS), DNase hypersensitive site, triplex/non-B DNA sequence, RIP60 protein-binding site, or a sequence that matches the core 20 bp of a 36-bp human consensus sequence that was reported to support autonomous replication (Price et al. 2003). The sequence that supports autonomous replication in mammalian cells (the "replicator sequence") has suffered from much debate (reviewed in Aladjem and Fanning 2004; Gilbert 2001)). The above result suggests that the plasmid

stability assay provides a novel, convenient and sensitive method to determine the replicator sequence.

4. The HSR tells us about the essence of chromosome structure in the nucleus.

The IR/MAR gene amplification method can easily and efficiently amplify any desired sequence as the extrachromosomal DMs or the chromosomal HSR. Thus, by tagging DMs or the HSR with the lactose operator (Lac O) sequence, the DMs or the HSR could be visualized by the expression of the lactose operator protein fused to green (or cyan) fluorescence protein. Using this method, the mitotic behavior of the HSR that was crucial for the BFB cycle (Shimizu et al. 2005b) or the intranuclear behavior of DMs in relation to the γH2AX signal (Shimizu et al. 2007b) was examined. In addition, the induced transcript from the genes on DMs was simultaneously visualized in different colors in live cells to see how transcription proceeds from the DMs (Utani et al. 2007). Furthermore, the chromosomal tandem plasmid array generated by the IR/MAR gene amplification method was used for the analysis of the binding reaction between a sequence-specific DNA binding protein and its target sequence (Bosisio et al. 2006).

On the other hand, Shimizu et al (Shimizu et al. 2001b) found that the natural HSR, which was generated during cancer development, was replicated during late S phase and that the pulse-labeled site of replication appeared as multiple bands at the following metaphase of mitosis. Because such replication bands were usually associated with the chromosomal G/R-band, its appearance inside the homogenous HSR without a G/R-band was unexpected. More intriguingly, the replication bands also appeared inside the HSR of long and homogeneous plasmid arrays (Shimizu and Shingaki 2004). Analysis of S phase nuclei showed that the compact HSR subchromosomal domain was replicated from the outside to the inside. Such analysis was possible because the HSR was replicated at the last stage of S phase when very few other chromosomal regions were replicated. To explain this series of results, a model was proposed in which the HSR was folded as a giant coiled-coil structure in the nucleus at late S phase, and it replicated from the outside to the inside (Shimizu and Shingaki 2004). The model was the first one to suggest how the few-tens of megabase pairs

of chromatin folded and replicated inside the nucleus. It also suggested that the chromosomal bands might originally arise from the way how the huge chromatin was folded and replicated inside the nucleus.

5. Conclusion

I have reviewed how the number of genes in a cell might decrease or increase. The mechanism leading to gene-number decrease was uncovered by examining the intracellular behavior, micronuclear entrapment and elimination of the DMs, which were genome-derived autonomously replicating extrachromosomal elements. Whereas, the gene-number increase was efficiently mimicked by the IR/MAR plasmid, which may be maintained in the transfected cells as an extrachromosomal element during at least limited term. Therefore, autonomously replicating extrachromosomal elements appear to play a central role for both gene-number decrease and increase. On the other hand, in the living mammalian cells, there are many other types of extrachromosomal elements that include several kinds of viruses, transposons, or artificially introduced DNA. There is also eccDNA that was excised from the chromosome arm, and I have wrote that eccDNA may be amplified and generate DMs, because significant portion of eccDNA may have IR and MAR sequence. I also mentioned that some viral nuclear plasmid may be segregated to daughter cells by a similar manner as the one utilized by DMs. Furthermore, the microinjected DNA appears to mimic the behavior of DMs at least in part. Therefore, the model explaining gene-number decrease or increase, which was obtained by examining DMs or the IR/MAR plasmid, may have more general implications. If the model may be expanded to a broad spectrum of extrachromosomal elements, if the additional mechanism may be uncovered in another situations, and if the mechanism may be further clarified in molecular detail, the total set of rules would be highly valuable because it will explain, and even predict, the behavior, amplification and elimination of any extrachromosomal element that have important biological, therapeutical or industrial implications.

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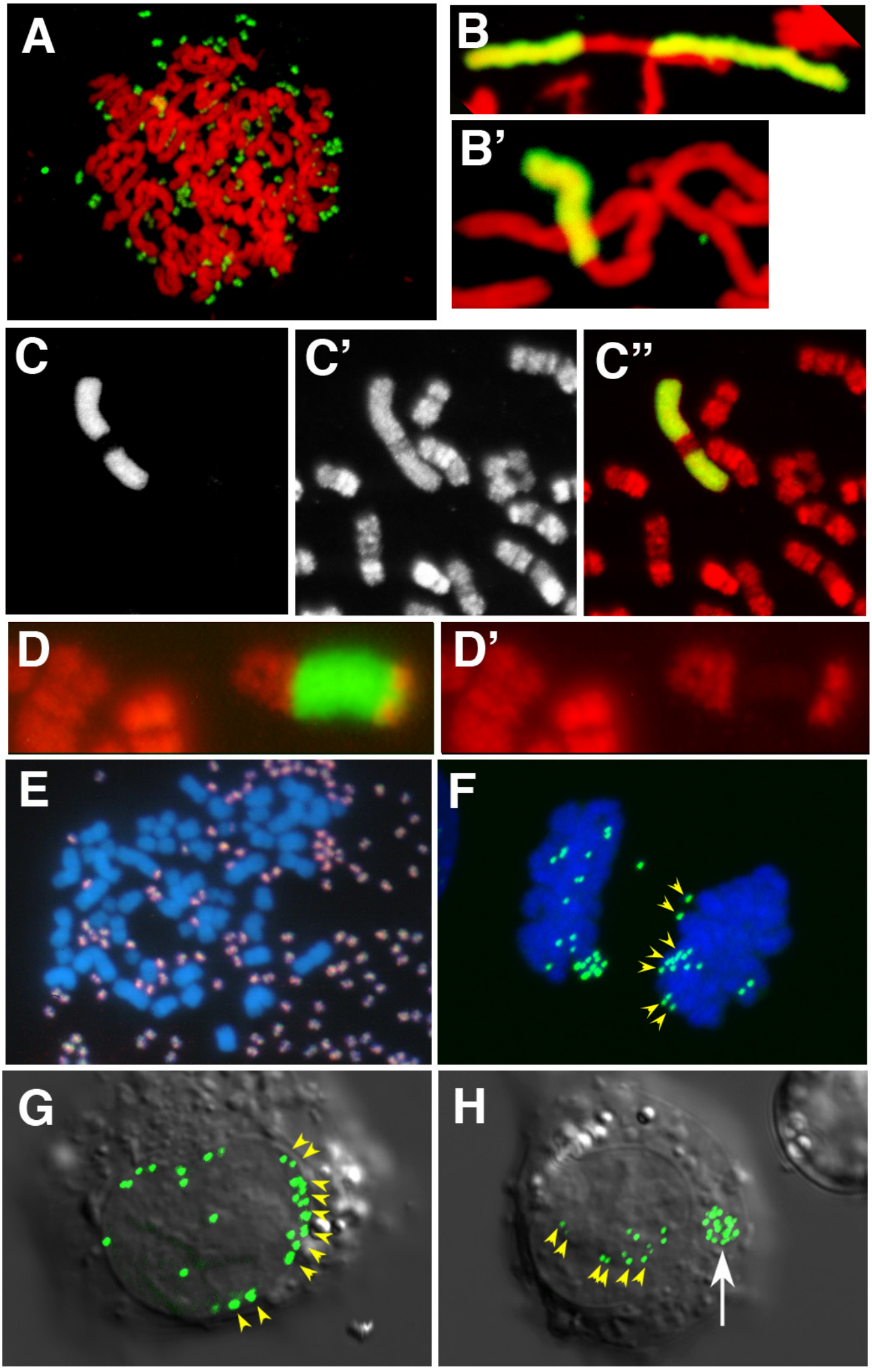
(Legends to Figures)

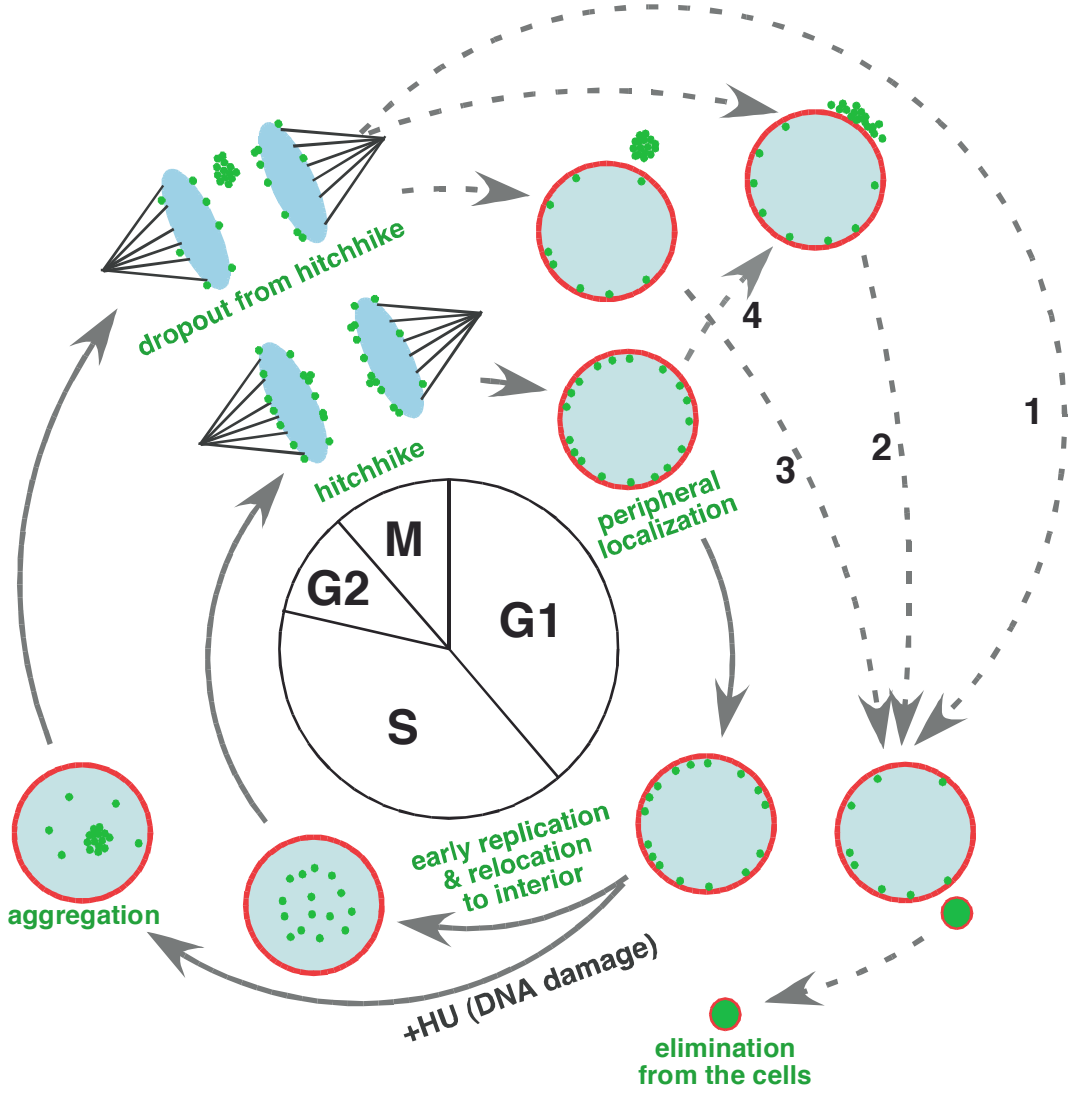
Figure 1. DMs and HSR that was formed during oncogenesis (A to C) or that was formed by introduction of IR/MAR plasmid (D to H). DMs (A; green) or HSR (B; green) was detected by FISH using DM-paint probe among the prometaphase chromosome (stained by propidium iodided in red) prepared from human COLO 320DM or COLO 320HSR cells. HSR is a homogeneous region composed of DM-derived sequences. The HSR in COLO 320HSR (C) or the artificial HSR generated by IR/MAR plasmid in COLO 320DM cells (D) was simultaneously hybridized with DM-paint probe (C and C" green) or IR/MAR plasmid probe (D green) and the Alu probe (C', C" red, D red and D'). The highly repetitive Alu sequence was homogeneously distributed in the HSR formed during oncogenesis (C) whereas it was absent in artificially generated HSR (D), suggesting that the latter HSR, in this case, was composed solely of the plasmid-derived sequences. By co-transfecting the IR/MAR plasmid (red) and the plasmid bearing Lactose operator repeat (LacO, green), both sequence was amplified at multiple DMs in COLO 320 cells (E). The LacO repeat was visualized by the binding of Lactose repressor-GFP fusion protein in live cells (F to H, yellow arrowheads). The DMs thus visualized was segregated by sticking to the anaphase chromsome (F), localize at the nuclear periphery in G1 phase (G) and the interior at S phase (H). In H, a portion of DMs was selectively incorporated into the micronucleus (arrow). A to C are from (Shimizu et al. 2001b), D from (Shimizu et al. 2005b), F to H from (Shimizu et al. 2007b), and E is an original.

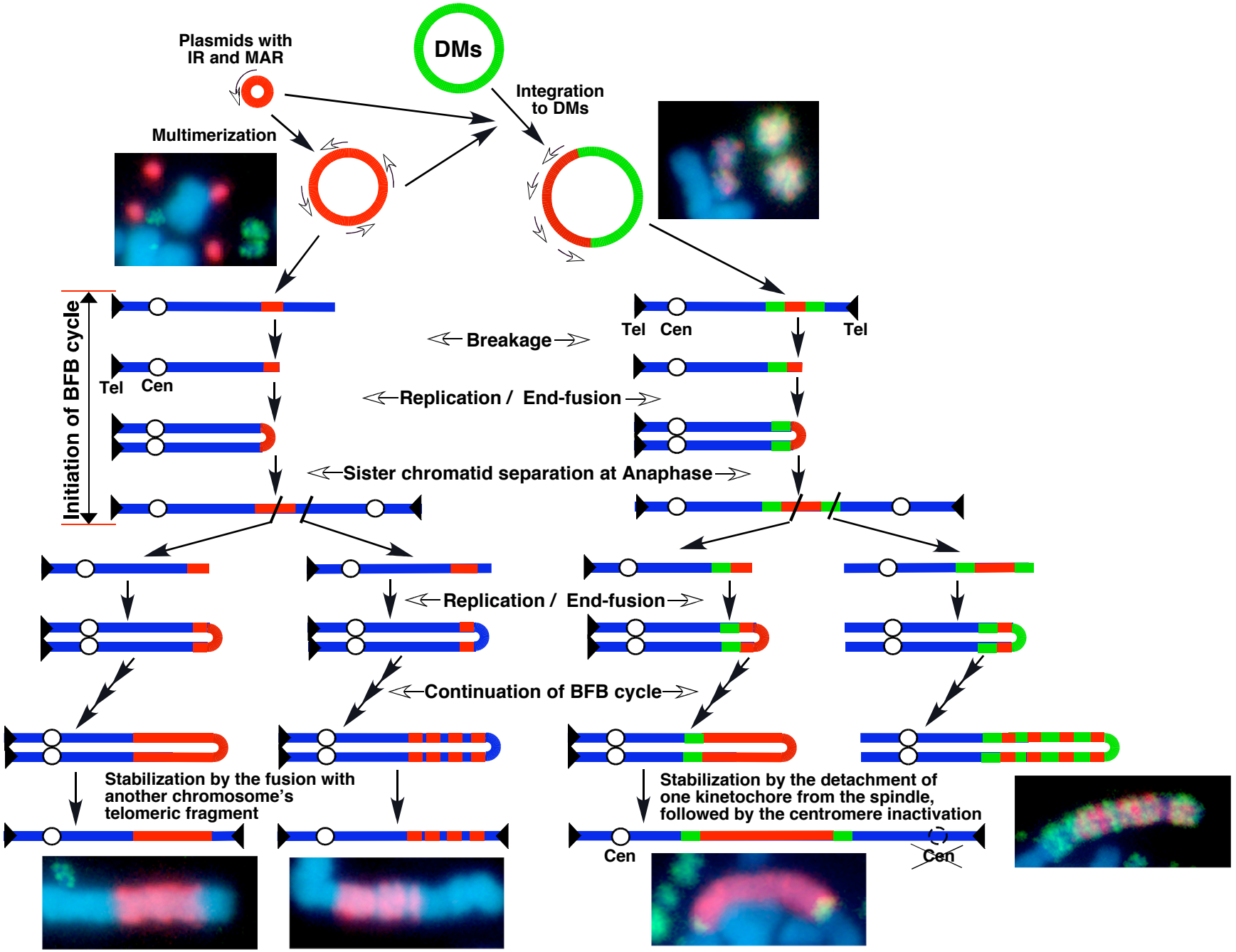
Figure 2. Intracellular behavior and elimination of DMs. DMs are depicted in green, nuclear lamina is red, and chromatin is blue. Acentric DMs are segregated during M phase by sticking to the chromosome (hitchhike). Consequently, they localize at the nuclear periphery and moved to interior at the early S phase while DMs themselves are replicated. DNA damage at that time induced aggregation of DMs, drop-out from the hitchhike at the next M phase, and generation of the micronuclei (1 to 3). The aggregated DMs may be wrapped by lamina just after the mitosis (1) or they may be left naked during G1 phase (2, 3).

DMs may also move from the nucleus to the cytoplasm during interphase (4). The micronuclei may be eliminated from the cells. For detailed explanation, see the text.

Figure 3. The plasmid sequences, the DM-derived sequences, and the chromosome sequences are denoted as red, green and blue bold lines, respectively. The open circles denoted as Cen represent centromeres. The black triangles denoted as Tel represent telomeres. The actual FISH images representatives for each structure are shown in this figure. It is from (Shimizu et al. 2005b) with some modifications.


A
B
B'
C
C'
C''
D
D'
E
F
G
H


dropout from hitchhike
hitchhike
peripheral localization
1
2
3
4
M
G2
G1
S
aggregation
early replication & relocation to interior
+HU (DNA damage)
elimination from the cells


Plasmids with
IR and MAR
DMs
Integration
to DMs
Multimerization
Initiation of BFB cycle
Tel
Cen
Tel
Cen
Tel
Breakage
Replication / End-fusion
Sister chromatid separation at Anaphase
Replication / End-fusion
Continuation of BFB cycle
Stabilization by the fusion with
another chromosome's
telomeric fragment
Stabilization by the detachment of
one kinetochore from the spindle,
followed by the centromere inactivation
Cen
Cen