Journal of Cellular Biochemistry 101:552–565 (2007)

Dissection of Mammalian Replicators by a Novel Plasmid Stability Assay

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Abstract A plasmid, bearing a mammalian replication initiation region (IR) and a matrix attachment region (MAR) was previously shown to be efficiently amplified to high copy number in mammalian cells and to generate chromosomal homogeneously staining regions (HSRs). The amplification mechanism was suggested to entail a head-on collision at the MAR between the transcription machinery and the hypothetical replication fork arriving from the IR, leading to double strand breakage (DSB) that triggered HSR formation. The experiments described here show that such plasmids are stabilized if collisions involving not only promoter-driven transcription but also promoter-independent transcription are avoided, and stable plasmids appeared to persist as submicroscopic episomes. These findings suggest that the IR sequence that promotes HSR generation may correspond to the sequence that supports replication initiation (replicator). Thus, we developed a ''plasmid stability assay'' that sensitively detects the activity of HSR generation in a test sequence. The assay was used to dissect two replicator regions, derived from the c-*myc* and *DHFR ori-β* loci. Consequently, minimum sequences that efficiently promoted HSR generation were identified. They included several sequence elements, most of which coincided with reported replicator elements. These data and this assay will benefit studies of replication initiation and applications that depend on plasmid amplification. J. Cell. Biochem. 101: 552–565, 2007.

Key words: gene amplification; homogeneous staining region; replication initiation region; replicator

Gene amplification plays a pivotal role in the malignant transformation of mammalian cells by activating oncogenes or permitting the acquisition of drug resistance (reviewed in [Benner et al., 1991; Schwab, 1999]). Highly amplified genes localize to extrachromosomal double minutes (DMs) or chromosomal homogeneously staining regions (HSRs). Cytogenetically detectable DMs are composed of acentric, atelomeric and autonomously replicating chromatin, composed of circular DNA of a few megabase pairs in size. We previously found that a plasmid with a mammalian replication initiation region (IR) and a matrix attachment region (MAR) was efficiently amplified, to up to 10,000 copies per cell, and that it generated DMs or HSRs composed of plasmid repeats [Shimizu et al., 2001a, 2003]. We observed amplification of plasmids containing IRs from the *DHFR*, c-*myc*, and β-*globin* loci; MARs from the *Ig* κ intron (AR1) and the SV40 early region; and sequences within the *DHFR* and β-*globin* IRs. The c-*myc* IR does not contain a MAR and an exogenous MAR is required for amplification [Shimizu et al., 2001a]. Importantly, the IR is indispensable for efficient amplification of the plasmid, which suggests that it contains necessary sequence information [Shimizu et al., 2001a].

Subsequent studies suggested a mechanism for plasmid amplification [Shimizu et al., 2001a, 2003, 2005], as outlined in Figure 1. A plasmid, bearing an IR and a MAR is multimerized to a large circular molecule in which the plasmid sequences are arranged as tandem repeats (step 1; [Shimizu et al., 2003]). The molecule is

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Japan Society for the Promotion of Science.

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Received 10 September 2006; Accepted 18 October 2006
DOI 10.1002/jcb.21210

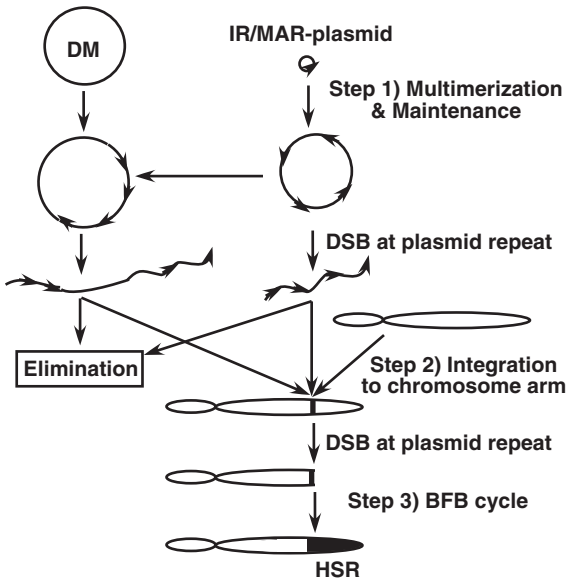


Fig. 1. A model for the generation of HSRs by IR/MAR-containing plasmids. This mechanism was suggested by our previous studies [Shimizu et al., 2001a, 2003, 2005] as well as unpublished observations. In brief, the plasmid bearing a replication initiation region (IR) and a matrix attachment region (MAR) is multimerized to a large circle in which the plasmid sequence (an arrow) is arranged as tandems direct repeat. The large circular molecule may frequently recombine with double minutes (DMs) if they pre-existed in the same cells. If the double strand breakage (DSB) occurred at the plasmid repeat, these circular extrachromosomal DNA may either be eliminated from the cells or be integrated to the chromosome arm. At the chromosome arm, the DSB at the plasmid repeat induces a breakage-fusion-bridge (BFB) cycle that generates a homogeneously staining region (HSR), composed of plasmid repeat. For more detailed explanation, see the introductory section.

replicated autonomously and segregates stably during cell growth, like DMs. If extensive multimerization occurs, the plasmid may be cytogenetically recognized as a DM [Shimizu et al., 2005]. Alternatively, the large circle may frequently recombine with DMs, if they are present. If the plasmid-derived tandem repeats are integrated into the chromosome (step 2), they efficiently initiate a breakage-fusion-bridge (BFB) cycle (step 3), which generates an HSR composed of plasmid repeats [Shimizu et al., 2005]. This amplification system was very useful in basic studies of chromosomal structure and function [Shimizu and Shingaki, 2004; Shimizu et al., 2005; Bosisio et al., 2006], and it has been adapted for the efficient production of recombinant proteins (N. Shimizu et al., manuscript in preparation).

The BFB cycle model has been frequently invoked for the genomic instability seen in human cancer cells [reviewed in Fenech, 2002; Masuda and Takahashi, 2002]. In this model, chromosomal breakage, followed by replication and the end-to-end fusion of sister chromatids, generates a mitotically unstable dicentric chromosome, which undergoes subsequent breakage near the first break site. Multiple cycles of BFB lead to the amplification of genes near the break site. Indeed, it has been reported that double strand breaks (DSBs) introduced under different conditions can trigger a BFB cycle [Pipiras et al., 1998; Ciullo et al., 2002; Zhu et al., 2002 and references therein]. In the case of HSRs derived from IR/MAR-bearing plasmids, the BFB cycle can be initiated by DSBs in the chromosomally integrated plasmid repeats [Fig. 1, step 3; Shimizu et al., 2005].

In a previous study [Shimizu et al., 2003], we showed that HSRs were efficiently generated from plasmid constructs (pSFVdhfr, pΔB, or pΔBN) in which transcription of the blasticidin resistance (*BSR*) gene was not terminated, thus permitting the transcriptional machinery to collide with the presumed replication fork, derived from the IR. In contrast, if transcription was terminated by the addition of a 3′ poly-A addition and processing sequence (pΔB.poly A or pΔBN.poly A), these plasmids did not generate HSRs. Furthermore, if the hypothetical replication fork arriving from the IR was stopped by an orientation-dependent replication fork barrier [RFB; Putter and Grummt, 2002], HSRs were not generated (pΔB.RFB Dir), whereas they were efficiently produced if the RFB was in an inverted orientation (pΔB.RFB Rev). On the other hand, if sequences having MAR activity (AR1) were inserted at the collision point (pΔB.AR1 or pΔBN.AR1), the generation of HSRs was greatly elevated. Therefore, we suggested that collision between transcription and replication complexes in MAR sequences destabilizes the plasmid, probably by inducing DSBs that trigger the BFB cycle and generate HSRs. If the multimeric plasmid is in the form of extrachromosomal circular DNA, DSBs will cause it to be eliminated from the cell (depicted in Fig. 1). We have shown that DSBs lead to the detachment of DMs from mitotic chromosomes, and DMs remain in the cytoplasm after mitosis and are subsequently eliminated [Shimizu et al., 2000; Tanaka and Shimizu, 2000]. Alternatively, DSBs linearize circular plasmid multimers and might facilitate their integration into the chromosome (Fig. 1,

step 2), because extrachromosomal molecules are frequently integrated into broken chromosomes [Lin and Waldman, 2001; Miller et al., 2003; Porteus et al., 2003].

A replicon model for the initiation of DNA synthesis postulated that a trans-acting regulatory factor (initiator) binds and stimulates a cis-acting specific sequence (replicator), leading to the initiation of replication [reviewed in Gilbert, 2001; Aladjem and Fanning, 2004]. There is evidence for this paradigm in bacterial and yeast cells; however, there has been a long-term debate concerning its relevance to mammalian cells. One of the major obstacles was the lack of a plasmid with a mammalian replicator that shows episomal maintenance. It has been shown that IRs from several loci can act as replicators, because these sequences support replication initiation at ectopic chromosomal sites [reviewed in Aladjem and Fanning, 2004]. However, the failure to construct a stable episome with a mammalian replicator might come from our ignorance about the rule that governs the stability of those plasmids. Our previous results, as described above, suggested that the orientation of specific modules may destabilize IR/MAR-containing plasmids. Thus, our initial aim was to address the question whether we could actually stabilize the IR/MAR-containing plasmid by the avoidance of such orientation.

In this report, we demonstrate that IR/MAR-containing plasmids can be stabilized or destabilized, if we consider the collision between the replication from IR and not only the promoter-driven transcription, but also the promoter-independent transcription. These achievements suggested that the tendency of IR/MAR-containing plasmids to generate HSRs appears to reflect the replicator activity of the IR, which suggested our second aim of this study—the dissection of mammalian replicators. Thus, we developed a novel plasmid stability assay and dissected two replicator sequences from the human c-*myc* and the hamster *DHFR* loci.

MATERIALS AND METHODS

Plasmid Construction

The construction of pΔBN.AR1 (Fig. 2A) and pSFV-V was previously described [Shimizu et al., 2003]. The plasmid pV was derived from pSFV-V by removing a *Not*I-*Nru*I fragment, containing the entire hygromycin resistance expression cassette and inserting at the same site, a synthetic oligonucleotide containing a multiple cloning site (introducing the *Kpn*I-*Not*I-*Asc*I-*Nru*I polylinker downstream of the *BSR* gene). This modification regenerated the *Nru*I but not the *Not*I site of pSFV-V, so that the resulting plasmid had a single *Not*I site. The HSV poly-A addition sequence (1,357 bp) was amplified by PCR, using the HSVpAKpnIR and HSVpAKpnIL primer set to create a *Kpn*I-tailed product that was inserted into the *Kpn*I site of pV (pVA; Fig. 2C). The sequences of all PCR primers used in this study are shown in Supplementary Table S1. All amplifications were performed using KOD polymerase under standard conditions. The 4.6 kb *ori-β* region of *DHFR* IR was excised from pSFVdhfr [Shimizu et al., 2001a] by *Not*I digestion. The 2.4 kbp c-*myc* IR was excised from pNeo.Myc-2.4 [McWhinney and Leffak, 1990] by *Hin*dIII/*Not*I double digestion. These IR fragments were blunt ended and ligated to an *Asc*I adaptor oligonucleotide. The *DHFR* IR fragment was then inserted into the *Asc*I site of pVA in the orientation where the internal MAR sequence [the *Hin*fI fragment in Shimizu et al., 2001a] was distal to the *BSR* transcript (pVA.D; Fig. 2D). pVAx2.D (Fig. 2F) was constructed by inserting the HSV poly-A addition sequence (see above) into the *Nru*I site of pVA.D by blunt end ligation.

The series of plasmids used in the experiment shown in Figure 4 (pT.IR.MAR; Fig. 2B) was constructed as follows. The AR1 MAR (375 bp) was excised from pAR1 [Shimizu et al., 2001a] by *Hin*dIII/*Bam*HI double digestion and both ends were blunted. The SV40 early region MAR (900 bp) was amplified by PCR [Shimizu et al., 2001a], using the SV40L and SV40R primer set and pNeo.Myc-2.4 as a template. The RFB sequence (118 bp) was amplified by PCR as previously described [Shimizu et al., 2003], using the RFB Not IL and RFB Not IR primer set and pSV2.SB2 as a template, and the product was tailed at both ends with *Not*I sequences. The RFB fragment was inserted into the *Not*I site of pVA in an orientation that blocked the replication fork from the IR. The AR1 and SV40 MAR fragments (see above) were inserted at the *Nru*I site of the resulting plasmid by blunt end ligation. This plasmid was then digested with *Asc*I and the 2.4 kbp c-*myc* IR, 4.6 kbp *DHFR* IR, or 4,361 bp *Hin*d III fragment of phage lambda was inserted at that site.

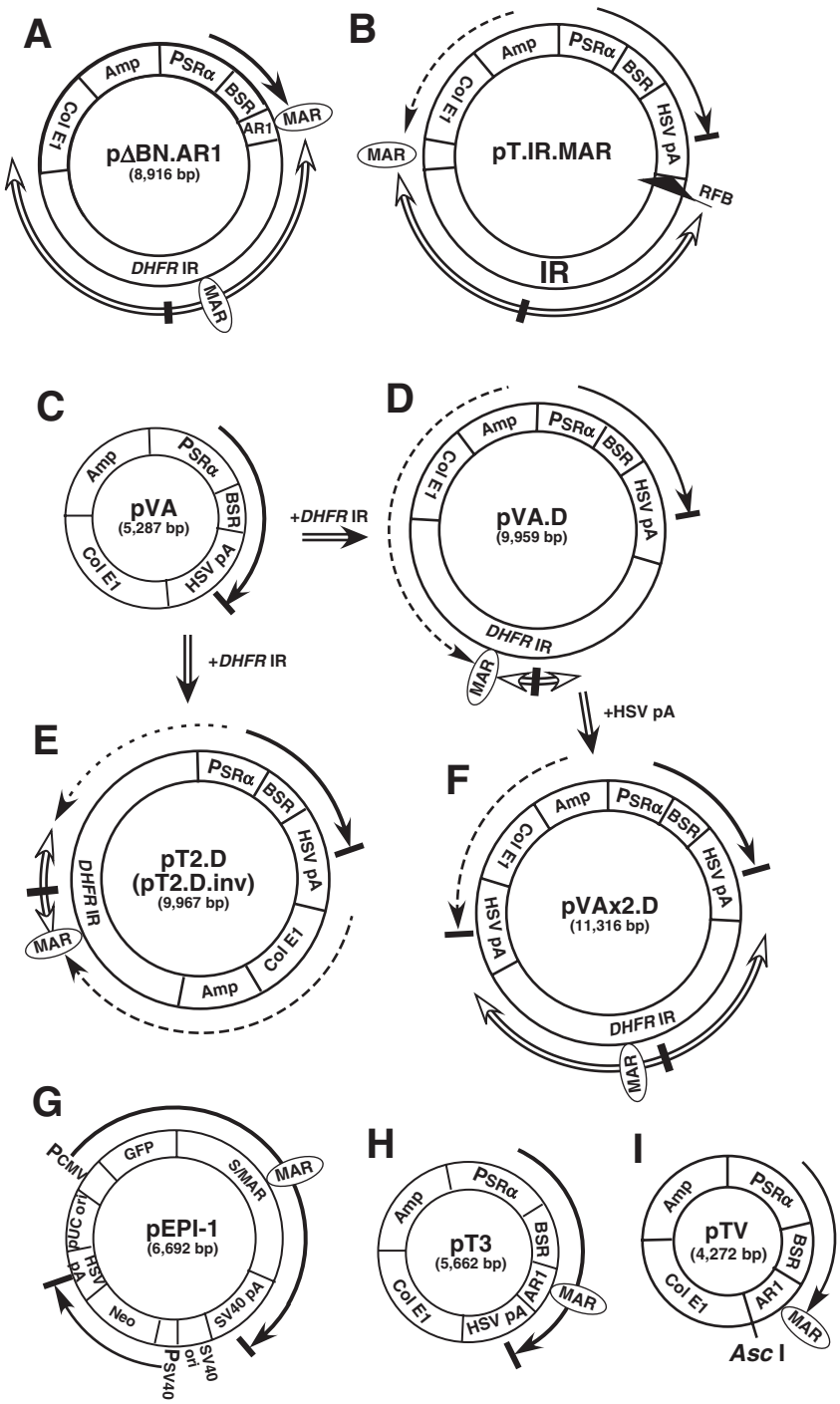


Fig. 2. Thes structures of plasmids used in this study: The construction of these plasmids is described in Materials and Methods. For pT.IR.MAR (**B**), several kinds of IR and MAR in various combinations were inserted at the positions indicated in the plasmid map. These plasmids were used in Figure 4. pT2.D.inv had identical construct with pT2.D (**E**), except for that the direction of DHFR IR was inverted. The black arrows indicate the direction of transcription, which is in some cases terminated by poly-A addition sequence (pA). The dashed black arrows indicate the direction of hypothetical non-coding transcription. The white arrows indicate the presumed bidirectional replication forks that start in IRs. Regions showing MAR activity are noted.

pT2.D (Fig. 2E) or pT2.D.inv was obtained by inserting the 4.6 kbp *DHFR* IR (see above) at the *Eco*RI site of pVA by blunt end ligation so that it had the same or opposite orientation, respectively, relative to *BSR* transcription as that of pΔBN.AR1. pEPI-I [Jenke et al., 2004; Schaarschmidt et al., 2004] was a generous gift from Dr. Daniel Schaarschmidt (Department of

Biology, Universität Konstanz). pTV (Fig. 2I) was derived by inserting the AR1 MAR into the *Kpn*I site of pV by blunt end ligation. pT3 (Fig. 2H) was then derived from pTV by inserting the HSV poly-A addition sequence at the *Not*I site by blunt end ligation. The subregion of the 2.4 kbp c-*myc* IR (C0–16) or 4.6 kbp *DHFR* IR (D1–11) was amplified by PCR using pNeo.Myc 2.4 or pSFVdhfr DNA as a template and the primer sets indicated in Supplementary Table S1. These primers included the *Asc*I site and products were digested with *Asc*I and ligated to *Asc*I-digested pTV.

Other Methods

Human colorectal carcinoma COLO 320DM, COLO 320HSR tumor cell lines, and the human HeLa cell line were obtained and cultured as described [Shimizu et al., 2001a]. COLO 320DM cells had many endogenous DMs harboring amplified c-*myc* genes, and isogenic COLO 320HSR cells had the same amplicon at an HSR rather than in DMs. All plasmids were purified using the Qiagen plasmid purification kit (Qiagen, Inc., Valencia, CA). Residual trace amounts of endotoxin were removed with the MiraCLEAN endotoxin removal kit (Mirus, Madison, WI), and plasmids were transfected into cells, using the GenePorter 2 lipofection kit (Gene Therapy Systems, San Diego, CA) according to the manufacturer's recommended protocol. Blasticidin (5 μg/ml; Funakoshi, Tokyo) was added to the culture 2 days after transfection. Thereafter, half of the culture medium was replaced with fresh medium, containing the drug every 3–5 days. After 4–8 weeks as indicated in each figure, a portion of the culture was harvested to examine HSR generation. Metaphase spreading, probe preparation, and FISH were performed, according to a previously published protocol [Shimizu et al., 2001b]. The slides were viewed under an inverted fluorescence microscope (ECLIPSE TE2000-U, Nikon) with a 100× objective (Nikon Plan Fluor, NA 1.30 oil) and an appropriate filter set specific for each fluorochrome. Frequency of HSR was scored by viewing more than 60 metaphase cells for each polyclonal transformants. In the data appearing in this study, we incorporated small chromosomal dots of FISH signal, which was not counted in the previous study [Shimizu et al., 2003], into the HSR count. Digital images were acquired with a Fuji FinePix S1Pro digital camera (Fuji Film Co., Tokyo) and merged with Adobe Photoshop (R) version 4.0J.

RESULTS

Is the IR/MAR-Containing Plasmid Stable, If There Is No Apparent Collision Between the Replication and Transcription Machinery?

The IR/MAR plasmid always efficiently generated HSRs or DMs in transfected cells. A representative image of HSR or DMs is shown in Figure 3A,B, respectively. We had suggested that a head-on collision between the replication and transcription machinery in the MAR results in the generation of HSRs. Therefore, the avoidance of such structures might stabilize the plasmid. One such construct is illustrated as pT.IR.MAR in Figure 2B. To avoid collision, the transcription of *BSR* is terminated by poly-A addition 3′ processing signal, and the presumed replication fork from the IR was stopped by an orientation-dependent RFB. We had shown that a MAR, in addition to an IR, is necessary for plasmid amplification [Shimizu et al., 2001a]. Furthermore, many reports suggested that attachment to the nuclear matrix is required for replication initiation [reviewed in Hyrien et al., 1997]. Therefore, we included a MAR in the plasmid but carefully placed it at a site where collision between the replication and transcription complexes was unlikely. In all, we prepared 11 new plasmids with different IR (*DHFR* or c-*myc*) and MAR (*Ig* κ AR1 or SV40) configurations, as shown in Figure 4, and transfected them into COLO 320DM cells. We selected stable transformants, prepared metaphase chromosome spreads, and detected plasmid sequences by FISH. The frequency of cells, bearing HSRs among all transformants was determined by examining metaphase cells, and the results are summarized in Figure 4. Unexpectedly, we found that every plasmid, bearing both an IR and a MAR, generated HSRs at significant frequencies, irrespective of the source and orientation of the IR and irrespective of the source of the MAR. However, HSR generation was seen only in cells transfected with plasmids bearing an IR but not with plasmids without an IR, even if it contained a MAR. Furthermore, if we used plasmids bearing unrelated sequences of similar length (fragments from phage λ DNA) at the site of the IR, HSRs were never generated. Therefore, these results provide additional evidence that the

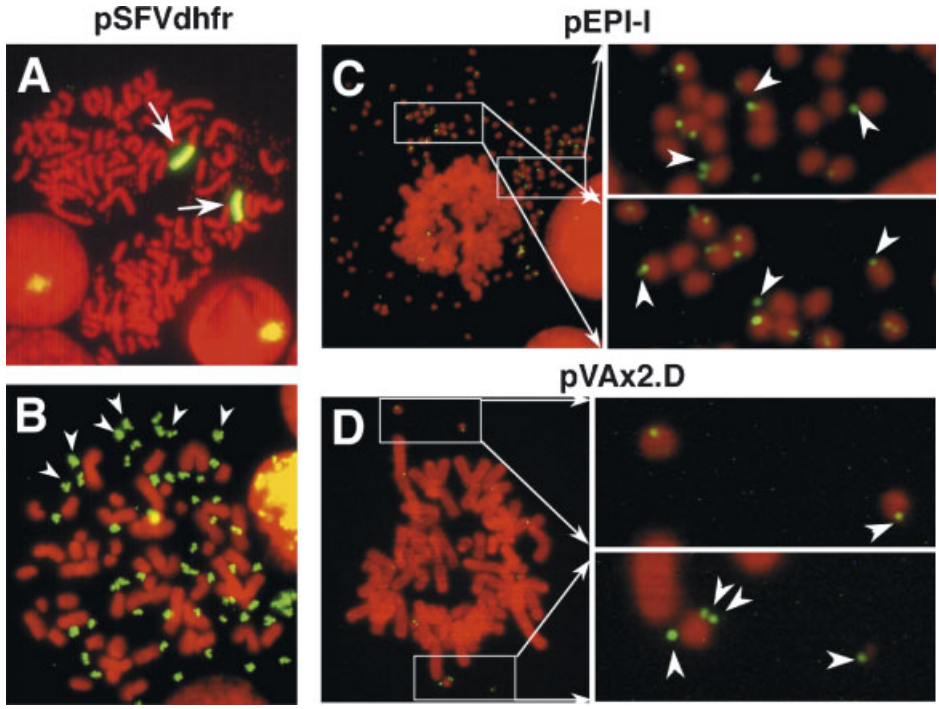
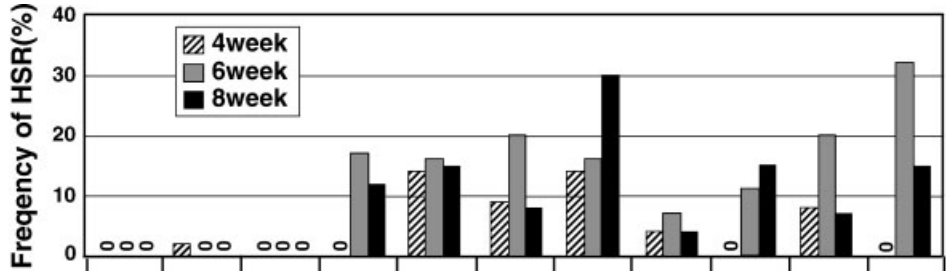


Fig. 3. Localization of plasmid sequences in stable transformants. Plasmid pSFVdhfr (**A**, **B**), pEPI-I (**C**), or pVAx2.D (**D**) was transfected into COLO 320DM cells. Metaphase chromosome spreads were prepared from polyclonal transformants 8 weeks (A, B) or 6 weeks (C, D) after transfection and blasticidin selection. The spreads were hybridized with a biotinylated probe prepared from each plasmid, and the probes were detected by FITC-streptavidin (green) and the DNA was counterstained with PI (red). Representative images of HSRs (A; arrows) and DMs (B; arrowheads) are shown. The enlarged images of the boxed areas in C and D show small submicroscopic DNA with hybridizing signals (arrowheads). The signals are specific because they do not appear when the same probe is hybridized to chromosome spread from parental COLO 320DM cells that do not contain plasmid sequences. Note that most of the signals adhered to DMs that were present in these cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sequence information of IR is absolutely required for HSR generation.

IR/MAR-Containing Plasmids Are Stable, if Collision With Promoter-Independent Transcription Is Avoided

In contrast to initial expectations, the above experiment revealed that IR/MAR-containing plasmids were unstable and generated HSRs, even if an apparent collision at the MAR between replication and transcription complexes was avoided. However, if we hypothesize there is a promoter-independent transcription, coming from the opposite direction (dashed line in Fig. 2B), it should collide at the MAR with the replication fork coming from the IR. The idea was examined by constructing a series of plasmids (Fig. 2C–F). We transfected these plasmids into three human tumor cell lines (COLO 320DM, COLO 320HSR, and HeLa) and determined whether they generated HSR in these cells, as summarized in Figure 5. The average size of HSRs formed in HeLa cells was much less than that formed in COLO 320 lines for unknown reasons (data not shown). pΔBN.AR1 (Fig. 2A), in which the apparent collision occurs at the MAR, is a positive control plasmid and it always generated HSRs at high frequency in all cell lines examined. pVA is a vector without IR/MAR elements and it never generated HSRs in either cell line. These observations reinforce the requirement of IR for HSR generation. In contrast, the insertion of *DHFR* IR into this plasmid (pVA.D) converted it to an amplification-prone plasmid, which can be explained by collision between the replication machinery and the hypothetical promoter-independent transcription, as shown in Figure 2D. This argument is supported by the finding that blocking this hypothetical transcription by inserting a poly-A sequence (Fig. 2F; pVAx2.D) led to stabilization of the plasmid, and it never generated HSRs in either cell line. On the other hand, pT2.D (Fig. 2E) and pT2.D.inv, which differ in the orientation of *DHFR* IR, were also constructed so as to avoid collision. However, in



IR		none	none	λ–DNA fragment	*DHFR* (+)	*DHFR* (-)	*DHFR* (+)	*DHFR* (-)	*c-myc* (+)	*c-myc* (-)	*c-myc* (+)	*c-myc* (-)
MAR		AR1	SV40	SV40	AR1	AR1	SV40	SV40	AR1	AR1	SV40	SV40
Frequency of HSR	4 w	0% (0/49)	2% (1/50)	0% (0/49)	0% (0/50)	14% (6/43)	9% (4/45)	14% (7/49)	4% (2/46)	0% (0/49)	8% (4/50)	0% (0/48)
	6 w	0% (0/51)	0% (0/51)	0% (0/50)	17% (7/44)	16% (7/45)	20% (10/51)	16% (8/50)	7% (3/45)	11% (5/47)	20% (10/50)	32% (17/53)
	8 w	0% (0/49)	0% (0/49)	0% (0/52)	12% (6/49)	15% (7/46)	8% (4/50)	30% (16/53)	4% (2/47)	15% (7/46)	7% (4/55)	15% (8/53)

Fig. 4. IR/MAR-containing plasmids generate HSRs even if there is no apparent collision between the replication and transcription machineries. A series of pT.IR.MAR plasmids (Fig. 2B) was constructed using different IR/MAR combinations. The IR position contained the *DHFR* IR or c-*myc* IR in either orientation (− or +), a 4,361 bp *Hin*d III fragment of lambda-phage DNA or no insert (none). The MAR position contained a fragment from the *Ig k* locus (AR1) or SV40 early region that showed MAR activity. These plasmids were transfected into COLO 320DM cells and stable transformants were selected on blasticidin. After 4, 6, or 8 weeks, a portion of polyclonal transformants was harvested. Plasmid sequences were detected on chromosomal spreads and the frequency of metaphase cells that contained plasmid sequences in an HSR was determined. The results are summarized in both table and graph formats.

this case, HSRs were generated at the frequency comparable to pVA.D (Fig. 5). This might be explained by the possibility that non-coding transcription that collides with replication from IR might still be present, and poly-A sequences that flank both sides of the IR may be necessary to completely prevent collision.

It was reported that a MAR module, placed inside a transcription unit, is sufficient and that an IR is not required for replication and maintenance of a mammalian episome [Jenke et al., 2004; Schaarschmidt et al., 2004]). Therefore, we transfected pEPI-I (Fig. 2G) and a similar plasmid that was constructed by us (pT3; Fig. 2H) into three human tumor lines. Like pEPI-I, pT3 had a MAR module (AR1) placed inside the BSR transcription unit. We obtained polyclonal stable transformants with these plasmids and analyzed them by FISH. Consequently, we found that these plasmids never generated HSRs (Fig. 5) or cytogenetically detectable DMs (data not shown) in any of the three cell lines tested. Instead, we frequently observed tiny FISH-specific signals (Fig. 3C). These signals were specific because the same procedure did not generate such signal in the slide from parental COLO 320DM. Since PI, which stains DNA, only faintly stained these elements, they were not classified as DMs but as submicroscopic episomes. Such episomal signals frequently stuck to DMs, which pre-existed in COLO 320DM cells. Importantly, we found that pVAx2.D also showed identical episomal maintenance in the majority of stable transformants (Fig. 3D). These observations are discussed below.

Dissection of the c-*myc* Replicator With the Plasmid Stability Assay

The above results, together with our previous works, suggested that the HSR-generation activity inside IR might correspond to replicator. Thus, we tried to identify the minimum sequences required for the generation of HSRs. We showed that the arrangement promoter-gene-MAR-IR generated HSR at high frequency, and we explained this as the collision between the replication and the transcription machinery at the MAR. Therefore, we developed a vector plasmid, pTV (Fig. 2I), into which we inserted the IR fragment at the *Asc*I cloning site. If the fragment contained HSR-generation activity, the plasmid will efficiently generate an HSR after transfection to the cells. We refer to

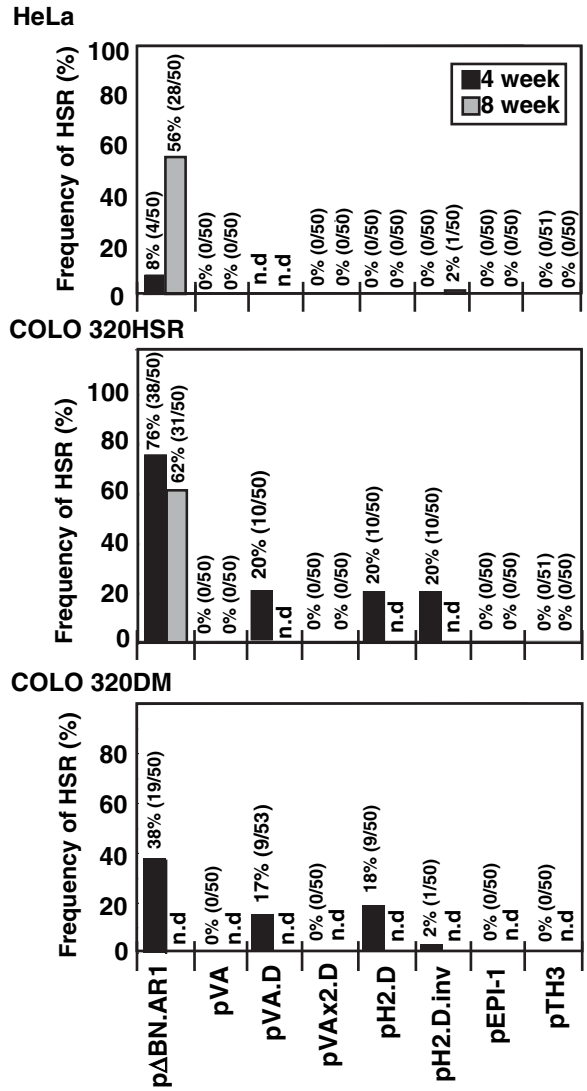


Fig. 5. IR/MAR-containing plasmids do not generate HSR if collision between replication forks and non-coding transcripts is avoided. The indicated plasmids were transfected into HeLa, COLO 320HSR, or COLO 320DM cells. After culturing 4 or 8 weeks in the presence of blasticidine, metaphase chromosome spreads were prepared from polyclonal stable transformants and plasmid sequences were detected by FISH. The frequency of cells with plasmid-derived HSRs was scored as in Figure 4. nd; not done.

this assay as "the plasmid stability assay." The results shown above (Figs. 4 and 5) suggested that the assay should detect the HSR-generation activity with high sensitivity and low background.

Using this assay, we first dissected the c-*myc* IR, which localizes at the promoter region of the gene (Fig. 6A). We inserted the entire 2.4 kbp c-*myc* IR (pCf.l) or nine subfragments of varying length and position (pC0–9) into the *Asc*I site of pTV. We transfected these plasmids into COLO 320DM cells and analyzed stable transformants by FISH. The results (Fig. 6) showed that the vector pTV did not generate HSRs, which confirmed the low background of this assay. Furthermore, there were fragments showing high HSR-generation activity, whereas there were also fragments that did not show any activity, indicating that the assay was suitable for dissecting the IR. Among the fragments tested, the fragment in pC3 showed the highest HSR-generation activity and it was the shortest; therefore, we subdivided this region and generated pC10–16. Among these plasmids, only pC12 generated HSRs at high frequency (Fig. 6C) and other plasmids had a negligible effect. Therefore, we interpret these results to mean that cooperation between regions cI (core I) and cII (core II), which are indicated in Figure 6B,C, is required for HSR generation. Region cI contains a duplex unwinding element (DUE), as predicted by the "WEBTHERMODYN" program (http://www.gsa.buffalo.edu/dna/dk/WEBTHERMODYN/), and region cII contains a potential topoisomerase II-binding site that was predicted by the "MAR-Wiz" program (http://www.futuresoft.org/MAR-Wiz/). Consistent with this interpretation, pC3, 1, 2, and 7, which bear both cI and cII regions, generated HSRs at high frequency. The frequencies for pC3 and pC1 were even higher than that for pCf.l, which suggests the presence of sequences that suppress HSR-generation activity in the full length c-*myc* IR. pC9 does not include the cII region although it generated HSRs. This may suggest that the cII' region depicted in Figure 6B may substitute for the cII region, although cII' does not contain a potential topoisomerase II-binding site. The presence of both cII and cII' in a single construct may suppress HSR generation because the effect of pC6 was less than that of pC3, 1, 2, or 7. Furthermore, the region N (negative), indicated in the figure might suppress the HSR generation, because pC0 generated HSRs at much lower frequency than did pC9 or 6. The region N contains a poly-purine sequence that tends to form a triplex/non-B structure. Such sequences have been postulated to slow or arrest replication fork progression [Rao et al., 1988; Rao, 1994]. pC8 and pC5 did not generated HSRs, because they contain only cI and cII', respectively.

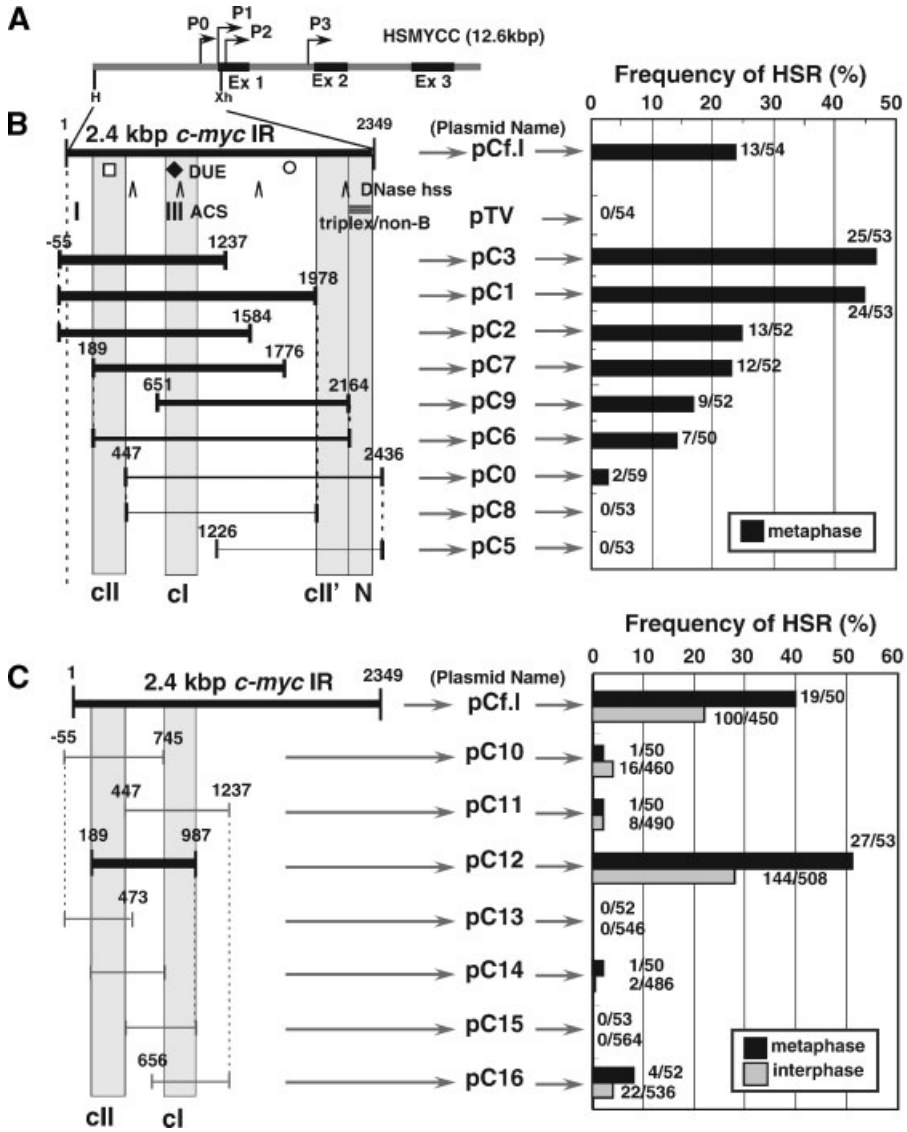


Fig. 6. Dissection of the c-*myc* replicator by the plasmid stability assay. The c-*myc* locus (Genbank HSMYCC; accession number X00364) is outlined in (**A**). The 2.4 k bp c-*myc* IR corresponds to the *Hin*dIII (H)-*Xho*I (Xh) fragment in the promoter region. In **panel B**, duplex unwinding element (DUE), ACS, DNase hypersensitive site (hss), and triplex/non-B DNA sequences are indicated according to Liu et al. [2003]. A white square indicates a potential topoisomerase II-binding site, and a white circle indicates sequences that match the core 20 bp of a 36-bp human consensus sequence that was reported to support autonomous plasmid replication [Price et al., 2003]. The entire 2.4 kbp c-*myc* IR (pCf.I.) or nine fragments of varying length and position (pC0–9) were inserted into the *Asc*I site of pTV (Fig. 2I). Plasmid DNA was transfected into COLO 320DM cells and polyclonal stable transformants, obtained 6 weeks after transfection, were analyzed by FISH. The bar graphs show the frequency of cells bearing HSRs. The plasmids are ordered according to the HSR frequency. The widths of the lines showing the fragment position (**left panel**) roughly represent the HSR frequency. The fragment that showed the highest activity of HSR generation (pC3) was subdivided and examined in **panel C**. These data suggest that the cI (core I) and cII (core II) regions are required for HSR generation. These results were used to evaluate the data in B and the region cII′ (cII-like) and the region N (negative) were suggested.

Dissection of the *DHFR* Replicator by the Plasmid Stability Assay

The *DHFR* origin of replication consists of many inefficient initiation sites scattered throughout a 55-kbp intergenic spacer, with *ori-β*, *ori-β′*, and *ori-γ* subregions being preferred [depicted in Fig. 7; Kobayashi et al., 1998; Dijkwel et al., 2002; Lin et al., 2005 and references therein]. We have used the 4.6 kbp *Bam*HI/*Hin*dIII fragment containing *ori-β* in our preceding experiments and we now

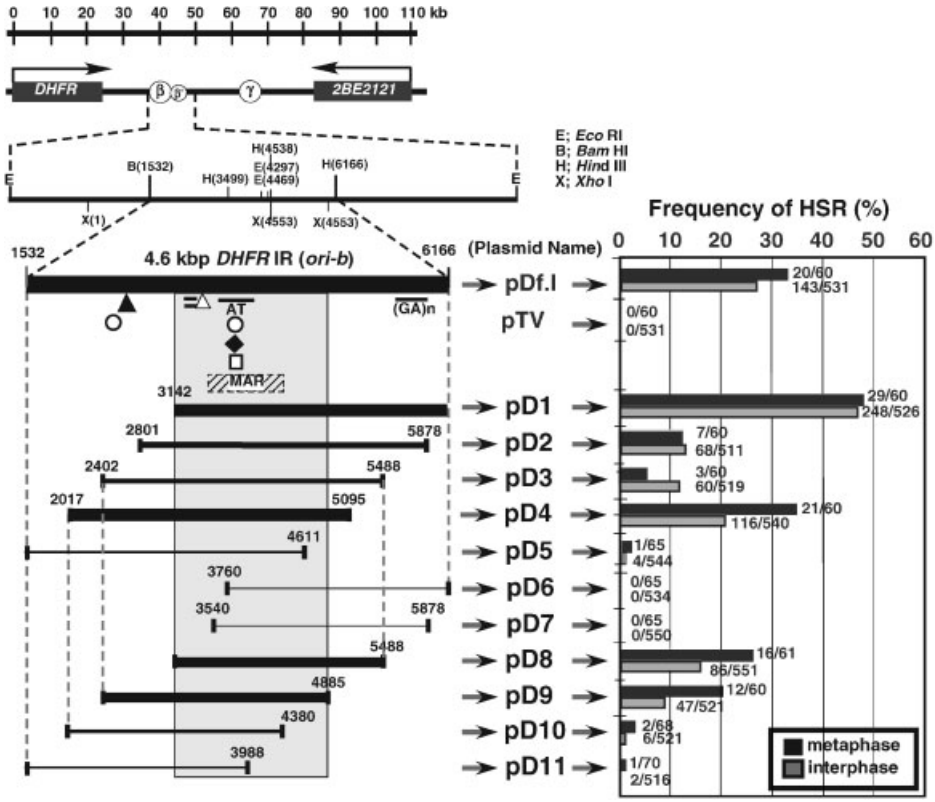


Fig. 7. Dissection of the *DHFR* replicator by the plasmid stability assay. The position of *ori*-β in the *DHFR* locus and its physical map is illustrated in the **top panel**. Base positions were adapted to Genbank CFORIDHFR (accession number X94372). The 4.6 kbp *Bam*HI-*Hin*dIII fragment was dissected. This region contains several elements, as follows. The *Hin*fI fragment (3,520–4,322; a hatched box) shows in vitro MAR activity [Shimizu et al., 2001a]. A 4-bp sequence located within the replication initiation site (black triangle; 2,659), a region of intrinsically bent DNA (parallel horizontal lines; 3,426–3,474) and a RIP60 protein-binding site (white triangle; 3,474–3,485), and the AT-rich element (3,695–4,039) are four regions reported as essential for replicator activity [Altman and Fanning, 2004]. Two white circles (2,466–2,483 and 3,809–3,826) indicate sequences that match (18 bp) the core 20 bp of a 36-bp human consensus sequence that was reported to support autonomous plasmid replication [Price et al., 2003]. The white square and black diamond indicate the potential topoisomerase II-binding site (3,770–3,850) and the potential DUE (3,801–3,851), respectively. A GA dinucleotide repeat (5,751–5,986) is indicated. The full length fragment (pDf.l.) or the region indicated in the figure (pD1–11) was inserted at the *Asc*I site of pTV vector, transfected into COLO 320DM cells, and analyzed as in the case of the c-*myc* replicator (Fig. 6). The HSR frequencies among metaphase or interphase cells were determined and are shown in the graph. The widths of the lines indicating the area of fragment (**left panel**) roughly represent the HSR frequency. These data suggest that the region from 3,142 to 4,885 (gray box) is essential for HSR generation.

dissected the region. Namely, fragments that were generated by PCR were cloned in the vector pTV and the resulting constructs were tested by the plasmid stability assay as before. The results are summarized in Figure 7. We interpret the data to mean that a region encompassing base positions 3,142–4,885 is required for HSR generation. This region contains a *Hin*fI fragment, for which we previously found in vitro MAR activity [Shimizu et al., 2001a]. On the other hand, Altman and Fanning [2004] reported that at least four defined sequence elements are specifically required for the replicator activity of the *ori-β* region. These include an AT-rich element, a 4-bp sequence located within the initiation site, a region of intrinsically bent DNA located between these two elements, and a RIP60 protein-binding site, adjacent to the bent region. Our data show that if the bent DNA and RIP 60-binding site (pD7) or, in addition to these, a part of AT-rich (pD6) were deleted, the HSR-generation activity was completely diminished, whereas full HSR-generation activity was seen if these elements were present (pD1). Therefore, at least either bent DNA or RIP 60-binding site was essential for HSR generation. Furthermore, the minimum region required for HSR generation also

includes the potential topoisomerase-II binding site that was predicted by the "MAR-Wiz" program as well as the DUE that was predicted by the "WEBTHERMODYN" program. There is also a core human consensus sequence that was reported to support autonomous plasmid replication [Price et al., 2003]. Because pD10 or 5 exhibits a less efficient HSR-generation activity than pD9, we conclude that the region from 4,380 to 4,885 is necessary. However, we have not deduced any sequence features for this region.

DISCUSSION

Relationship Between the Replicator and HSR-Generation Activities of the IR

We have studied a 2.4 kbp c-*myc* IR [Trivedi et al., 1998; Malott and Leffak, 1999; Liu et al., 2003] and a 4.6 kbp *DHFR ori-β* [Altman and Fanning, 2001, 2004] that was previously shown to act as a replicator at an ectopic chromosomal site. Furthermore, it was reported that the 2.4 kbp c-*myc* IR supported autonomous plasmid replication [McWhinney and Leffak, 1990; McWhinney et al., 1995], however, *DHFR ori-β* IR did not [Burhans et al., 1990]. On the other hand, as has been repeatedly reinforced in our study, the sequence information inside the IR is strictly required for the generation of HSRs from IR/MAR-containing plasmids (Fig. 4). This suggests that the HSR-generation activity of IR likely represents the replicator activity of the same sequence. The following observations also support this argument. First, we showed previously that the c-*myc* IR lacks a MAR and an exogenous MAR was required for HSR generation [Shimizu et al., 2001a]. The indispensable role of MAR in replication was frequently suggested [for *DHFR ori-β*, see Pemov et al., 1998; for review, see Hyrien et al., 1997]. However, the initiation sites in mammalian chromosomes are not defined by attachments to the nucleoskeleton [Ortega and DePamphilis, 1998] and the removal of the MAR has no significant effect either on the frequency or timing of initiation in *DHFR* locus, instead, it may be required for local chromatid separation [Mesner et al., 2003]. Therefore, the precise role of MAR in both the replication and the HSR generation should be clarified in the future study. Second, if we assume the replication fork arrives from the IR, a collision with the transcription apparatus was always associated with HSR generation. Namely, both the collision and the HSR generation could be prevented by stopping either transcription or replication by the addition of a poly-A signal or the inclusion of an orientation-specific RFB, respectively [Shimizu et al., 2003 and this study]. Replication may be required during all steps of HSR generation (Fig. 1). The initial plasmid multimerization to tandem repeated circles might be mediated by recombination at the time of replication [Shimizu et al., 2003]. The persistence of the plasmid or plasmid multimers also requires autonomous replication. Furthermore, the DSBs required at steps 2 and 3 of Figure 1 may arise from collision between the replication and transcription complexes. DSB, caused by the collision between replication and transcription had been suggested in bacteria or yeast cells, but it may also play a pivotal role in genome instability during malignant transformation of animal cells.

Stabilization of IR/MAR-Containing Plasmids

The collision between replication and transcription at the MAR was suggested to destabilize IR/MAR-containing plasmids and generated HSR [Shimizu et al., 2003]. Therefore, we examined whether avoidance of such collision might stabilize the plasmid and prevent HSR generation. Consequently, the plasmid was stabilized if we placed two poly-A addition sequences at both ends of IR (pVAx2.D). It might be possible that the poly-A sequences had some other effect than blocking a transcript. However, our previous work prefers the interpretation that there should be the hypothetical promoter-independent transcription, in addition to promoter-driven transcription, and two poly-A sequences blocked both transcription and prevented collision. We tried to detect the former transcription, but it was not successful, probably because scarce amount of transcription might influence the stability.

Recently, it was reported that episomal plasmids might be constructed without a replicator, if they contained specific consensus sequences [Trivedi et al., 1998] or there was a MAR linked to an upstream active transcription unit [Stehle et al., 2003; Jenke et al., 2004; Schaarschmidt et al., 2004]. The latter plasmid pEPI-I (Fig. 2G) had a structure with the order, promoter/gene/MAR/poly-A, and our pT3 (Fig. 2H) or pTV (Fig. 2I) had the similar structure, albeit the latter plasmid did not

contain poly-A. All these three plasmids never generated HSR (Figs. 5, 6), however, it did if we inserted IR or its fragments at the downstream of MAR in pTV (Figs. 6, 7). It was reported that the origin recognition complex (ORC) bound to pEPI-I at many sites scattered throughout the plasmid [Schaarschmidt et al., 2004]. The insertion of IR at the downstream of MAR might lead to the preferential binding of ORC to the IR. Such hypothesis should be examined by the future experiments.

The pVAx2.D never generated HSR or any detectable plasmid sequences in the chromosome; instead, we frequently detected these sequences in submicroscopic episomes (Fig. 3D). The similar signals were also observed for pEPI-I (Fig. 3C). The presence of such episomes suggests that these molecules are stably maintained during cell division. However, when we examined extrachromosomal DNA by Southern blot hybridization, we could not detect the band corresponding to a plasmid of the same shape and size (data not shown). A similar result was obtained for DNA, prepared from polyclonal transformants or isolated clones (about 10 clones for each plasmid) that were derived from cells transfected with our pVAx2.D as well as with pEPI-I. pEPI-I was reported to be maintained as a stable episome [Jenke et al., 2004]. We have currently no idea why we could not reproduce the published result. However, it might be explained by the difference of HeLa sublines or the method to isolate transformants, and our condition might favor the multimerization of the plasmid that make the molecular detection more difficult.

The episomal plasmid signal was frequently associated with pre-existing DMs (Fig. 3C,D). This observation might reflect the frequent recombination of autonomously replicating extrachromosomal molecules, which was first demonstrated for an episomal plasmid bearing the Epstein Barr virus *EBNA-1* gene and a latent origin of replication [*OriP*I; Kanda et al., 2001].

Dissection of a Replicator by a Novel Plasmid Stability Assay

As discussed above, the HSR-generation activity likely represents a replicator. Therefore, we developed a plasmid stability assay that detects HSR-generation activity in a defined sequence. Dissection of the c-*myc* replicator suggested that both DUE and the potential topoisomerase-binding site were indispensable for the HSR generation. These two elements were also included in the minimum region required for HSR generation inside *DHFR* IR. An important role for DUE and topoisomerase in replication initiation has already been suggested. Namely, DUE plays a pivotal role in replication initiation from the c-*myc* and/or *DHFR* IR [Ishimi et al., 1994; Liu et al., 2003; Casper et al., 2005]. It has been suggested that DUE modulates the assembly of DNA replication complexes [Casper et al., 2005]. On the other hand, a sequence that closely resembles the *Drosophila* topoisomerase II consensus was reported around yeast ARS [Matsuoka et al., 1993] and in a human inactive X-specific replication origin [Koina and Piper, 2005]. Furthermore, topoisomerases are involved in the initiation of simian virus 40 minichromosome replication [Halmer et al., 1998].

In addition to these two elements, the minimum region required for HSR generation in the *DHFR* IR (3,142–4,885) contains sequences showing MAR activity and many reported elements that were involved in replication initiation. On the other hand, Altman and Fanning [2004] reported that four elements are essential for the replicator activity of this region, and at least the bent DNA or the RIP60-binding site was indispensable for our plasmid stability assay. They reported that a 4-bp sequence in the initiation site was required for replicator activity, whereas our data showed the sequence was dispensable. As they stated, the sequence was very short and the effect on replicator activity was modest, therefore, any other sequence in our plasmid construct might substitute for the 4-bp sequence. This sequence is inside the mapped initiation site [Pelizon et al., 1996], but replication initiation might arise elsewhere if the original site was removed. In the case of the c-*myc* replicator, many initiation sites are scattered along the 2.4 kbp IR [Liu et al., 2003]. Altman and Fanning [2001] also noted that a GA dinucleotide repeat (starting from 5,751 in our numbering) is required for the replicator. However, our data showed that this region is dispensable for amplficator activity. As Altman and Fanning [2001] did not mention this region in their more recent study, its effect might be modest.

Taken together, the dissection of the c-*myc* and *DHFR* replicator suggest that many sequence elements positively or negatively

affect the HSR generation. Because the HSR-generation activity of IR likely represents the replicator activity of the region, our dissection of replicators, as well as our novel plasmid stability assay, will have benefits for studies of DNA replication initiation. Furthermore, the minimum region required for HSR generation revealed here would be useful for basic biological studies or for applications in protein production by improving IR/MAR-plasmid-based gene amplification technology.

ACKNOWLEDGMENTS

This work is supported by a Grant-in-Aid for Scientific Research (B) with a Grant no. 17370002. We thank Dr. Daniel Schaarschmidt (Department of Biology, Universität Konstanz) for kindly providing the plasmid pEPI-I.

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