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Interconversion of Intra- and Extra-Chromosomal Sites of Gene Amplification by Modulation of Gene Expression and DNA Methylation

Noriaki Shimizu,* Naoyuki Hanada, Kohichi Utani, and Naoki Sekiguchi

Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima, Hiroshima 739-8521, Japan

Abstract We previously showed that plasmids containing a mammalian replication initiation region and a matrix attachment region were efficiently amplified to few thousand copies per cell, and that they formed extrachromosomal double minutes (DMs) or chromosomal homogeneously staining regions (HSRs). In these structures, the plasmid sequence was arranged as a tandem repeats, and we suggested a mechanism of plasmid amplification. Since amplification was very efficient, easy, and convenient, it might be adapted to a novel method for protein production. In the current study, we found that gene expression from the tandem plasmid repeat was suppressed. We identified several strategies to overcome this suppression, including: (1) use of higher concentrations of antibiotic during cell selection; (2) treatment of cells with agents that influence DNA methylation (5-azacytidine) or histone acetylation (butyrate); (3) co-amplification of an insulator sequence; and (4) co-amplification of sequences that encode a transcriptional activator. Expression from the plasmid repeat was always higher at DMs compared to HSRs. We found that continuous activation of a plasmid-encoded inducible promoter prevented the generation of long HSRs, and favored amplification at DMs. Consistent with this finding, there was a synergistic effect of transcriptional activation and inhibition of DNA methylation on the fragmentation of long HSRs and the generation of DMs and short HSRs. Our results indicate that both transcriptional activation and DNA methylation regulate the interconversion between extra- and intra-chromosomal gene amplification. These results have important implications for both protein production technology, and the generation of chromosomal abnormalities found in human cancer cells. J. Cell. Biochem. 102: 515–529, 2007. © 2007 Wiley-Liss, Inc.

Key words: gene amplification; recombinant protein production; genome instability; double minutes; homogeneously staining region

Gene amplification plays a pivotal role in the malignant transformation of mammalian cells, leading to the activation of oncogenes or the development of drug-resistance (for recent reviews, see [Albertson, 2006; Myllykangas and Knuutila, 2006]). Highly amplified genes usually localize to either of two cytogenetic loci, double minutes (DMs), or homogeneously staining regions (HSRs). DMs are multiple sites of extrachromosomal paired chromatin observed in metaphase chromosomes. Cytogenetically, DMs appear as circular DNA of few Mbp in size, and they are devoid of centromeres and telomeres. The amplification unit (amplicon) usually ranges from a 100 kilobases to 1 megabase. An HSR, on the other hand, is a chromosomal region containing a long stretch of amplified sequences. In cells derived from the same tumor, DMs, and HSRs usually have identical amplicons, suggesting that there is an interconversion between these two structures. In fact, growth in vivo favors cells bearing DMs, whereas long term passage in vitro usually gives rise to cells bearing HSRs (for a review, see [Benner et al., 1991]).

We and others previously found that the elimination of DMs from tumor cells leads to reversion of tumor phenotype and cellular differentiation [Eckhardt et al., 1994; Shimizu et al., 1994]. These findings not only reinforced

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*Correspondence to: Noriaki Shimizu, Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima, Hiroshima 739-8521, Japan.
E-mail: shimizu@hiroshima-u.ac.jp

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the importance of gene amplification in tumorigenesis, but also suggested potential therapeutic applications. The elimination of DMs was mediated by the selective entrapment of DMs into cytoplasmic micronuclei [Von Hoff et al., 1992; Shimizu et al., 1996]. Such entrapment was the result of a novel intracellular behavior of DMs [Tanaka and Shimizu, 2000], which might be common among a broad spectrum of autonomously replicating, extrachromosomal genetic entities.

When a plasmid bearing a mammalian replication initiation region (IR) and a matrix attachment region (MAR) was transfected into human tumor cells, it was efficiently amplified, to nearly ten-thousand-copies per cell, and formed structures indistinguishable from DMs and/or HSRs [Shimizu et al., 2001a, 2003]. Subsequent studies suggested that the plasmid was multimerized to a large circular molecule in which the plasmid sequence was arranged in a tandem repeat [Shimizu et al., 2003]. If multimerization was extensive, the circular molecule appeared cytogenetically as DMs. Alternatively, if the large circular molecule integrated into the chromosome arm, the plasmid repeat appeared to efficiently initiate the Breakage-Fusion-Bridge (BFB) cycle, and formed a chromosomal HSR [Shimizu et al., 2005]. An HSR was most frequently generated using a plasmid in which the putative replication fork arising from the IR would meet at the MAR with the transcription machinery coming from the promoter region [Shimizu et al., 2003]. These data indicated that the interaction between the transcriptional and replication machinery results in a double strand break, triggering the BFB-cycle and generating an HSR. Gene amplification mediated by an IR/MAR-bearing plasmid was very useful not only for studying the mechanism of gene amplification, but also for the basic biology that utilizes the prominent feature of the amplified structure [Shimizu and Shingaki, 2004; Shimizu et al., 2005; Bosisio et al., 2006].

The aim of the current study was to adapt the IR/MAR amplification methodology to protein over-production. Gene amplification has been widely used for the production of recombinant protein (for a review, see [Wurm, 2004]). The most common method used is to select cells carrying an amplified dihydrofolate reductase (*DHFR*) gene using the inhibitor methotrexate (MTx). However, this is a highly time-consuming and labor-intensive method. In comparison, IR/MAR-mediated gene amplification is simple and highly efficient; that is, one-step selection resulted in a cell population in which up to 70% of the cells had a few-thousand copies of an amplified gene [Shimizu et al., 2003]. Furthermore, this method has the advantage that any DNA sequence could be co-amplified, if it was co-transfected with the IR/MAR-plasmid [Shimizu et al., 2003]. In the current study, we demonstrated that expression of amplified genes from the tandem plasmid repeat was usually silenced to a low level of expression, consistent with the phenomenon known as repeat-induced gene silencing (RIGS) [Garrick et al., 1998; Henikoff, 1998; McBurney et al., 2002]. We demonstrated several strategies for overcoming this phenomenon, and increasing gene expression using the IR/MAR gene amplification system. We also present the serendipitous finding that gene expression and the macroscopic structure of the amplified gene have a reciprocal regulatory relationship.

MATERIALS AND METHODS

Plasmids and DNA

pTet-ON and pTRE-d2EGFP were purchased from Clontech (Valencia, CA). The plasmid bearing an insulator sequence derived from the chicken β-globin locus (pHS4; [Yusufzai and Felsenfeld, 2004]) was a kind gift from Dr. Gary Felsenfeld (NIDDK, NIH, Bethsda, MD). The structures of pSFVdhfr and pSFV-V have been previously described [Shimizu et al., 2003]. Briefly, pSFVdhfr (Fig. 1A) contained an IR (4.6 kbp), termed *Ori β* from the Chinese hamster *DHFR* genomic region, which included a sequence with MAR activity [Shimizu et al., 2001a]. The plasmid also encoded the blasticidine (*BS*) and hygromycin (*Hyg*) resistance genes. The former gene was driven by the SRα promoter and did not contain a poly A termination sequence. We deleted the IR/MAR sequences from pSFVdhfr to generate pSFV-V (Fig. 1B). Plasmid pDHFR.TRE-d2EGFP (Fig. 1C) bear an IR/MAR sequence from non-coding downstream of *DHFR* locus and a d2EGFP gene driven by TRE-promoter. It was derived from pSFVdhfr by removing the *Hyg* expression cassette, using *Bam* HI and *Nru* I, and replacing it with the *Xho* I/*Bgl* I fragment of pTRE-d2EGFP using blunt-end ligation. The latter fragment contained sequences encoding the TRE-promoter, d2EGFP, and SV40 poly A, and

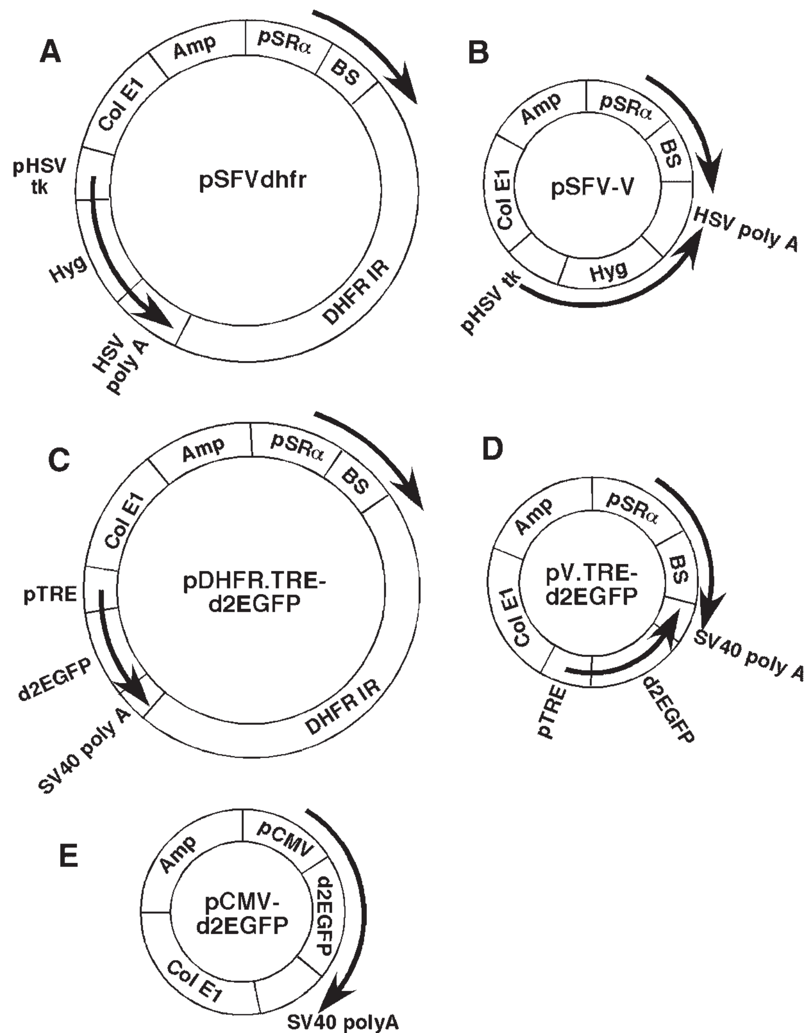


Fig. 1. Plasmid used in this study. The abbreviations used in this figure are; pSRα, SRα promoter; BS, blasticidine resistance; DHFR IR, replication initiation region from downstream non-coding region of hamster DHFR gene loci (ori β); HSV poly A, herpes simplex virus (HSV) poly A addition sequence; Hyg, hygromycin resistence; pHSVtk, HSV thymidine kinase promoter; Col E1, colicin E1; Amp, ampicilin resistence; pTRE, TRE promoter; d2EGFP, d2EGFP coding; SV40 poly A, SV40 poly A addition sequence; pCMV, CMV promoter. The arrows indicate the direction of transcription.

the direction of transcription was towards the *DHFR* IR/MAR sequence. A variant of enhanced green fluorescence protein (d2EGFP), which has a very short half-life (ca. 2 h) in live cells, was used throughout this study; thus, fluorescence should reflect real time gene expression. The TRE promoter was activated using the doxycyclin (Dox)/rtTA complex, with rtTA supplied via the expression plasmid pTet-ON. The control plasmid, pV.TRE-d2EGFP (Fig. 1D) had the same structure as pDHFR.TRE-d2EGFP, but lacked the IR/MAR sequences. pCMV-d2EGFP (Fig. 1E) was constructed by replacing the *Xho* I/*Sac* II fragment of pTRE-d2EGFP, encompassing the TRE promoter, with the *Xho* I/*Sac* II fragment of pTet-ON, encompassing the CMV promoter.

Cell Culture, Transformation, and Analysis of Protein Expression

The human colorectal carcinoma cell lines COLO 320DM (CCL 220) and COLO 320HSR (CCL 220.1) were obtained and maintained as described previously [Shimizu et al., 1996]. The chinese hamster cell line CHO-K1 (CCL 61) was obtained from Cell Research Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University, Japan.

WI-38 (CCL 75) normal human fibroblasts were obtained from American Type Culture Collection. All plasmids were purified from bacteria using a Qiagen plasmid purification kit (Qiagen, Inc., Valencia, CA), and cells were transfected using a GenePorter 2 lipofection kit (Gene Therapy Systems, San Diego, CA) according to the manufacture's instructions. A clone of COLO 320DM stably expressing rtTA, which we termed COLO 320DM (pTet-ON), was developed by transfecting COLO 320DM cells with pTet-ON, and culturing the cells in the presence of 400 μg/ml neomycin (Life Technologies, Inc., Rockville, MD) for 4 weeks. Ten independent clones were obtained by limiting dilution cloning. Clones were transfected with pTRE-d2EGFP, and Dox (Clontech) was added 2 days post-transfection. We selected the clone that emitted the lowest level of green fluorescence before transfection, and the highest level of green fluorescence 2 days after the addition of Dox. Sodium butyrate and 5-aza 2′-deoxycytidine (5-aza) were purchased from Sigma (St. Louis, MO).

Unless otherwise noted, the expression of d2EGFP was analyzed 2 days after the addition of 1 μg/ml Dox to the cells. Cells were then resuspended in phosphate buffered saline (PBS) and either examined directly using fluorescence microscopy, or using fluorescence activated cell sorting (FACS, Beckton Dickinson Co., Franklin Lakes, NJ). The results of the cell sorting analysis are expressed as "relative d2EGFP expression", which was calculated by multiplying the percentage of cells emitting more than two fluorescence unit (horizontal axis of each graph) among all viable cells by the mean fluorescence value of that population of cells. The "relative d2EGFP expression" values, and the value of each of the two factors in the calculation, are presented in each FACS graph.

Other Method

Total genomic DNA and total RNA were simultaneously isolated using TRI-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacture's instructions. RNA was converted to cDNA using reverse transcriptase. All the primer sequences used in this study are available upon request. Competitive PCR was performed according to a previously published protocol [Shimizu et al., 1996].

Metaphase spreading, probe preparation, and fluorescence in situ hybridization (FISH) were performed according to previously published protocols [Shimizu et al., 2001b]. Slides were viewed using an inverted fluorescence microscope (ECLIPSE TE2000-U, Nikon) equipped with a 100× objective lens (Nikon Plan Fluor, NA 1.30 oil) and an appropriate filter set specific for each fluorochrome. Digital images were acquired with a Fuji FinePix S1Pro digital camera (Fuji Film Co., Tokyo) and merged with Adobe Photoshop CS (Adobe Systems, Inc.).

RESULTS

Gene Expression Driven From Tandem Plasmid Repeats was Silenced, Whereas Expression due to Oncogenesis-Associated Amplification was not

Human COLO 320HSR (Fig. 2A) and COLO 320DM (Fig. 2B) cells have an identical c-*myc* amplicon in their HSRs and DMs, respectively [Shimizu et al., 2001b]. In the HSRs of COLO320HSR cells, the *Alu* repeat was distributed homogeneously (Fig. 2A′). Competitive PCR analysis of genomic DNA showed that c-*myc* was amplified 60- and 30-fold in COLO 320DM and COLO 320HSR cells, respectively, compared with human normal diploid WI-38 cells (Fig. 2E). These values were in good agreement with previously reported values [Shimizu et al., 1996, 2001b]. Reverse transcriptase-mediated competitive PCR analysis revealed that the amount of c-*myc* RNA was approximately proportional to the c-*myc* DNA copy number (Fig. 2E), which was also consistent with previously reported data [Shimizu et al., 2001b].

On the other hand, we previously transfected IR/MAR-bearing pSFVdhfr into COLO 320DM cells [Shimizu et al., 2003], and obtained stable clone 12 and 22 in which the plasmid was amplified at multiple DMs (Fig. 2C) or HSR (Fig. 2D), respectively. The HSRs of clone 22 cells were devoid of human *Alu* sequences, suggesting the absence of genomic sequence (Fig. 2D′). By using these cells, we had shown that the plasmid was arranged as a head to tail tandem array in the amplified region [Shimizu et al., 2003]. In the current study, we were interested in quantifying the copy number of a plasmid-encoded gene, the *BS* gene, and the level of *BS* gene transcripts. As a control, we examined polyclonal stable transformants of COLO 320DM cells transfected with pSFV-V, which lacked IR/MAR sequences. As seen in

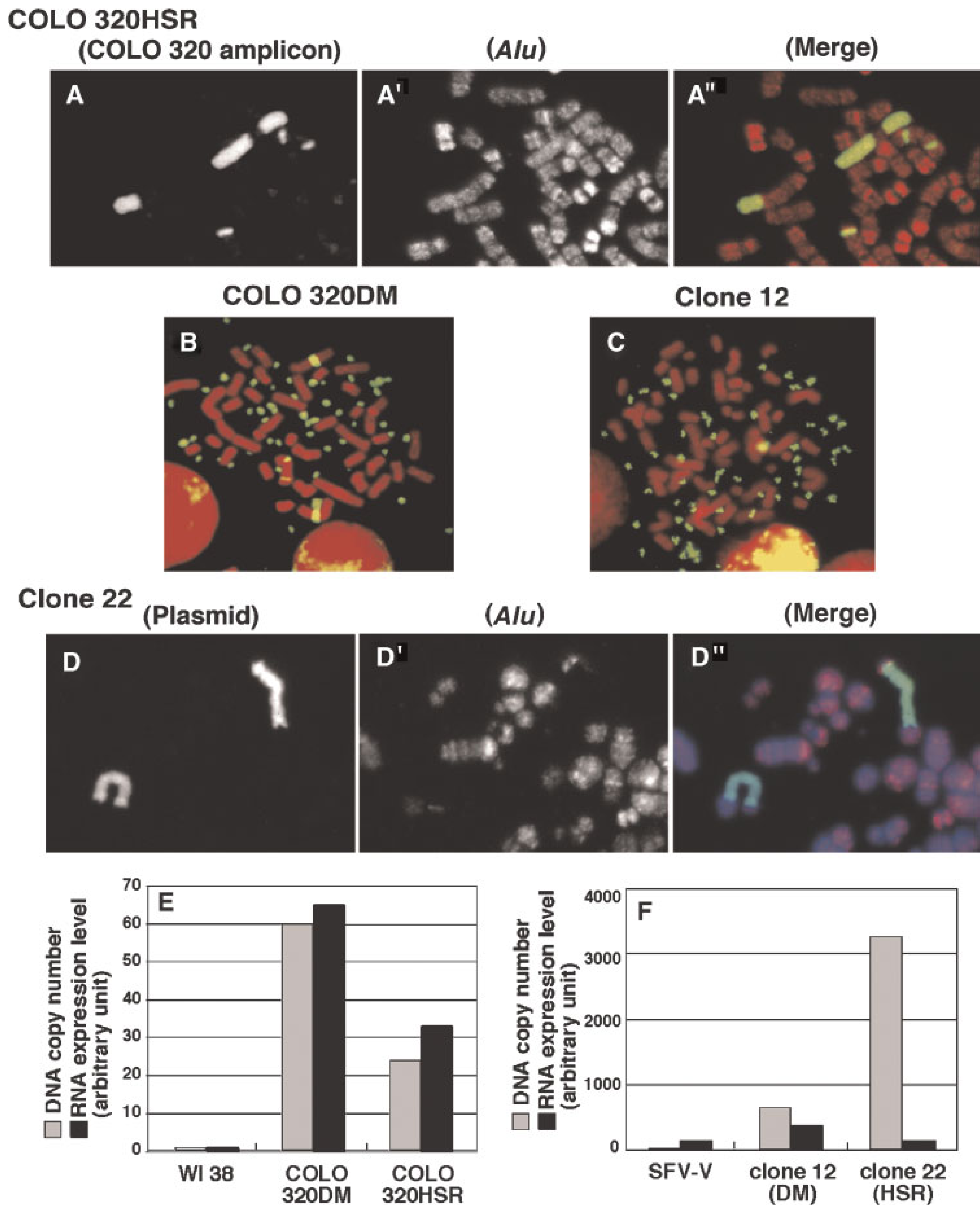


Fig. 2. Suppression of gene expression from a tandem plasmid repeat, but not from oncogenesis-associated amplification. **A**, **B**: Metaphase spreads prepared from COLO 320HSR (A) or COLO 320DM (B) cells were hybridized with a micronuclei-derived probe prepared from COLO 320DM cells, which painted amplified sequences on DMs [Shimizu et al., 1996]. The hybridized probe appears as green in the images. Clone 12 (**C**) and clone 22 (**D**) were obtained as previously described[Shimizu et al., 2003]. Metaphase spreads prepared from these cells were hybridized with a probe derived from the plasmid used in the original transfection (pSFVdhfr), whch was detected in green. For A and D, human *Alu* sequences were simultaneously detected with an *Alu*-specific probe, in red [Shimizu et al., 2001b]. For B and C, chromosomes were counterstained with propidium iodide (PI), in red. For D, chromosomes were counterstained with DAPI, in blue. **E** and **F**: DNA and RNA were isolated from the indicated cell lines, and RNA was converted to DNA by reverse transcriptase. The copy number and level of RNA for c-*myc* (E) or *BS* (F) was measured using competitive PCR [Shimizu et al., 1996]. As a control, we used the human normal diploid cell line WI-38 (E), and stable transformants of COLO 320DM cells carrying the IR/MAR-lacking plasmid pSFV-V (F). DNA copy number and RNA levels are expressed relative to the values obtained for the control cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2F, the *BS* DNA copy number in clones 12 and 22 was several hundred- or thousand-fold higher than in pSFV-V transformants. However, the amount of *BS* RNA was not proportional to the DNA copy number. These results suggested that gene expression from the plasmid repeat was profoundly suppressed. The amount of *BS* RNA relative to *BS* DNA was higher in DM-bearing cells (clone 12) compared to HSR-bearing cells (clone 22), suggesting that gene expression driven by the plasmid repeat was higher when the plasmid was in an extra-chromosomal setting, compared to a chromosomal environment. The silencing of chromosomal HSRs was consistent with the observation that the HSRs appeared as condensed heterochromatin by DAPI staining (Fig. 2D″), and with our previous finding that the timing of replication of the HSRs was very late [Shimizu and Shingaki, 2004].

Increasing the Concentration of Antibiotic During Cell Selection Yielded Cells With a Higher Level of Gene Expression

To assess gene expression in the IR/MAR plasmid-based system, we used the reporter gene *d2EGFP*, which encodes a GFP variant with a very short half-life in cells. We constructed a plasmid pDHFR.TRE-d2EGFP (Fig. 1) that has an IR/MAR sequence from the non-coding downstream of *DHFR* locus and a d2EGFP gene driven by TRE-promoter, as described in the Method section. A plasmid pV.TRE-d2EGFP (Fig. 1) was a control plasmid that lacked IR/MAR sequences. Expression of *d2EGFP* was under the control of the inducible TRE-promoter in both plasmids. COLO 320DM (pTet-ON) cells were transfected with either plasmid, and transformants were selected in the presence of 5 μg/ml of BS for approximately 4 weeks. We confirmed by FISH analysis that approximately 50% of the pDHFR.TRE-d2EGFP (which contains IR/MAR sequences)-transformants had highly amplified plasmid sequences at HSRs or DMs, while pV.TRE-d2EGFP (which does not contain the IR/MAR sequences) transformants were negative. We then added Dox to the cells to induce TRE-promoter activity, and analyzed the expression of d2EGFP using FACS analysis 2 days later. As seen in Figure 3, d2EGFP expression was much higher in the IR/MAR-bearing pDHFR.TRE-d2EGFP-transformants (Fig. 3B) compared to the negative control pV.TRE-d2EGFP-transformants (Fig. 3A). We continued to culture the pDHFR.TRE-d2EGFP-transformants in the presence of increasing concentrations of BS, and after approximately 3 weeks, obtained cells that grew in 320 μg/ml BS. When we examined d2EGFP expression in cells growing in each concentration of BS, we found that it increased in proportion to the concentration of BS in the cell culture medium (Fig. 3B–D and the black circles in Fig. 3G).

Co-Amplification With an Insulator Sequence Yielded Cells With a Higher Level of Gene Expression

While gene expression from the plasmid tandem repeat in the IR/MAR system was silenced, there was an apparent absence of such silencing associated with oncogenesis-related gene amplification (Fig. 2E). This could be due to the structure of the latter, which was much more complex than the former. Previous results in our lab indicated that any sequences might be co-amplified by co-transfection with an IR/MAR-plasmid: when we co-transfected cells with lambda phage DNA and an IR/MAR-plasmid to increase the complexity of the amplified structure, we found that d2EGFP expression was increased (data not shown). It was previously reported that the insulator sequence protected the spreading of heterochromatin and increased transgene expression [Recillas-Targa et al., 2002; Tajima et al., 2006]. Therefore, we co-transfected a plasmid bearing the HS4 insulator [Yusufzai and Felsenfeld, 2004] and an IR/MAR-bearing plasmid to COLO 320 (pTet-ON) cells, and found that the level of d2EGFP was higher in the co-transfected transformants compared to the single transformants (compare Fig. 3B,E, or C,F). Furthermore, the effect of co-transfection with the HS4 insulator and increasing the concentration of BS was additive (Fig. 3G, compare white and black circles).

Co-Amplification of the Gene for the Transcriptional Activator Protein Yielded Cells With a Higher Level of Expression

The above results were generated using COLO 320DM cells stably expressing the transactivator protein rtTA. However, when we transfected these cells with pTet-ON to transiently overexpress rtTA, the TRE promoter-driven expression of *d2EGFP* was significantly increased in a portion of the cells (data not shown). These results suggested that the

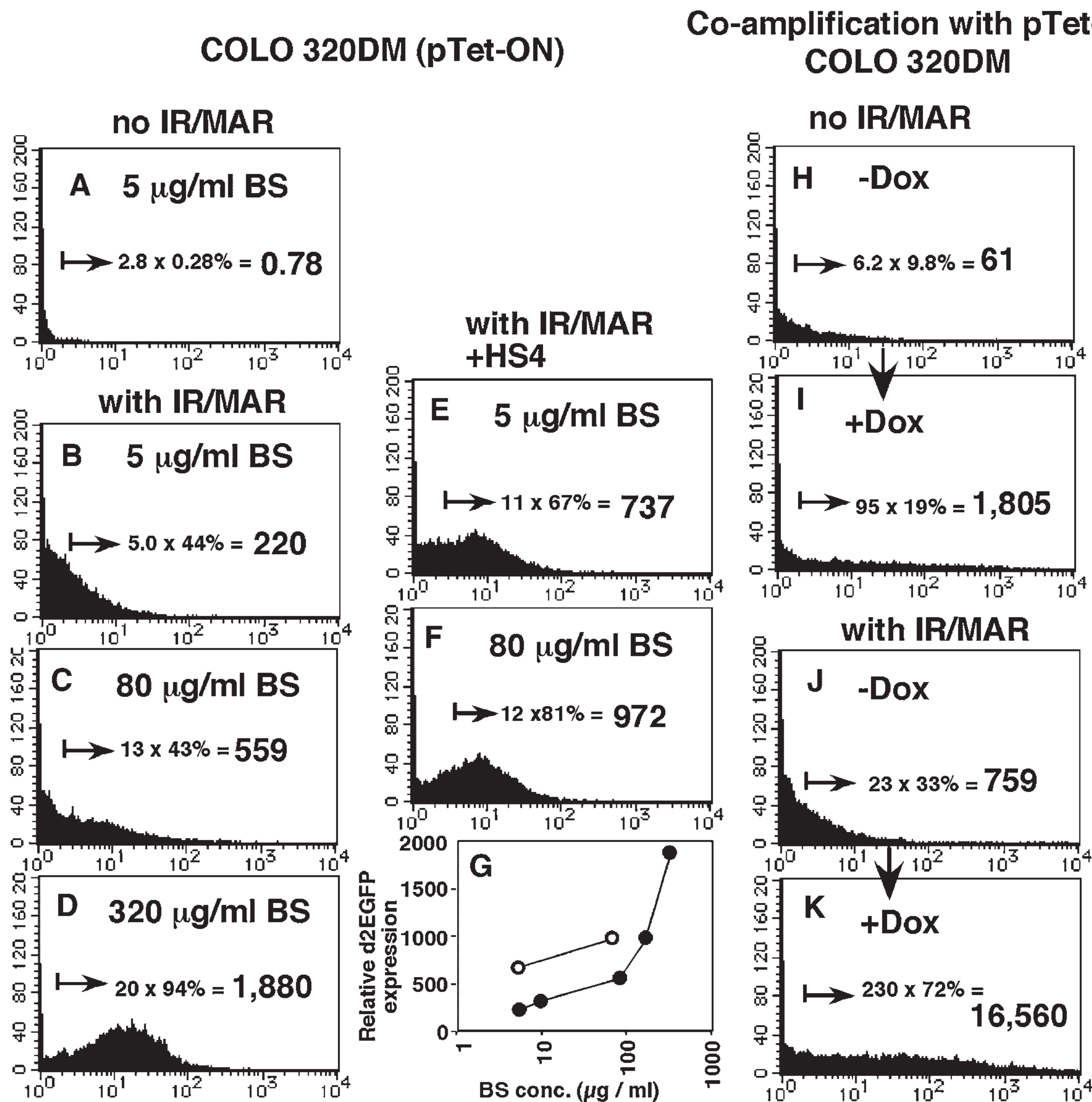


Fig. 3. Several methods elevated the protein expression from the amplified IR/MAR-plasmid in COLO 320DM cells. COLO 320DM (pTet-ON) cells were transfected with either pV.TRE-d2EGFP lacking IR/MAR sequences (**A**), pDHFR.TRE-d2EGFP, which contained IR/MAR sequences (**B**–**D**), or a mixture of pDHFR.TRE-d2EGFP and pHS4 (**E**, **F**). Stable transformants were selected in the presence of 5 μg/ml BS for 4 weeks. Subsequently, the concentration of BS in the medium was doubled each time the cells were sub-cultured (3–5 days interval). The expression of d2EGFP in these polyclonal populations at various concentrations of BS was measured by FACS analysis. ''Relative d2EGFP expression'' was calculated as described in Methods by multiplying ''mean'' and ''percent of expressing cells'', and is noted in each panel. G: ''Relative d2EGFP expression'' was plotted against BS concentration for pDHFR.TRE-d2EGFP-transformants (black circles), or pHS4/pDHFR.TRE-d2EGFP-transformants (white circles). **H**–**K**: COLO 320DM cells were co-transfected with a mixture of pTet-ON and either pV.TRE-d2EGFP (H, I) or pDHFR.TRE-d2EGFP (J, K) Stable transformants were selected in the presence of 5 μg/ml BS for 4 weeks, and the expression of d2EGFP was analyzed before (H, J) and after (I, K) the addition of 1 μg/ml Dox for 2 days.

amount of rtTA was a limiting factor in expression driven by the TRE-promoter. Therefore, we co-transfected the wild type COLO 320DM cells with pTet-ON, and either pV.TRE-d2EGFP (which does not contain the IR/MAR sequences) or pDHFR.TRE-d2EGFP (which contains IR/MAR sequences), to co-amplify the two plasmids. In polyclonal transformants, d2EGFP expression was observed in the absence of Dox, indicating that the promoter was somewhat "leaky" (Fig. 3H or J). However, addition of Dox to these cells greatly increased the level of

d2EGFP (Fig. 3I,K). Importantly, the relative level of d2EGFP expression increased several ten-fold using this co-amplification strategy (compare Fig. 3B,K), and expression was nearly ten-fold higher in the IR/MAR-bearing transformants compared to the non-IR/MAR-bearing transformants (Fig. 3I,K).

The Gene Expression was Higher From DMs Than HSRs

We isolated several clones of the COLO 320DM(pTet-ON) transformants from pDHFR.TRE-d2EGFP (Figs. 3E) or the COLO 320DM transformants from pDHFR.TRE-d2EGFP and pTet-ON (Fig. 3J), and examined the location of the plasmid sequence and d2EGFP expression in these clones using FISH and FACS analysis, respectively. The results from the clones derived from the transformant depicted in Figure 3E are shown in Table I, and data from two representative clones derived from the transformant depicted in Figure 3J are shown in Figure 4. The results indicated that d2EGFP expression was consistently higher in cells in which the plasmid repeat was localized to DMs (Table I, Fig. 4A,B), compared to HSRs (Table I, Fig. 4J,D,E).

Inhibition of DNA Methylation Elevated Gene Expression From the Tandem Plasmid Repeat

Monoclonal, stable pDHFR.TRE-d2EGFP-transformants from Colo 320DM (Table I, Fig. 4) were exposed to 5-aza for 5 days, and Dox for 2 days prior to harvest. Under these conditions, in every sample analyzed, the expression of d2EGFP was enhanced by the addition of 5-aza. Furthermore, the effect of 5-aza was always higher in HSR-bearing cells compared to DM-bearing cells (Table I, Fig. 4).

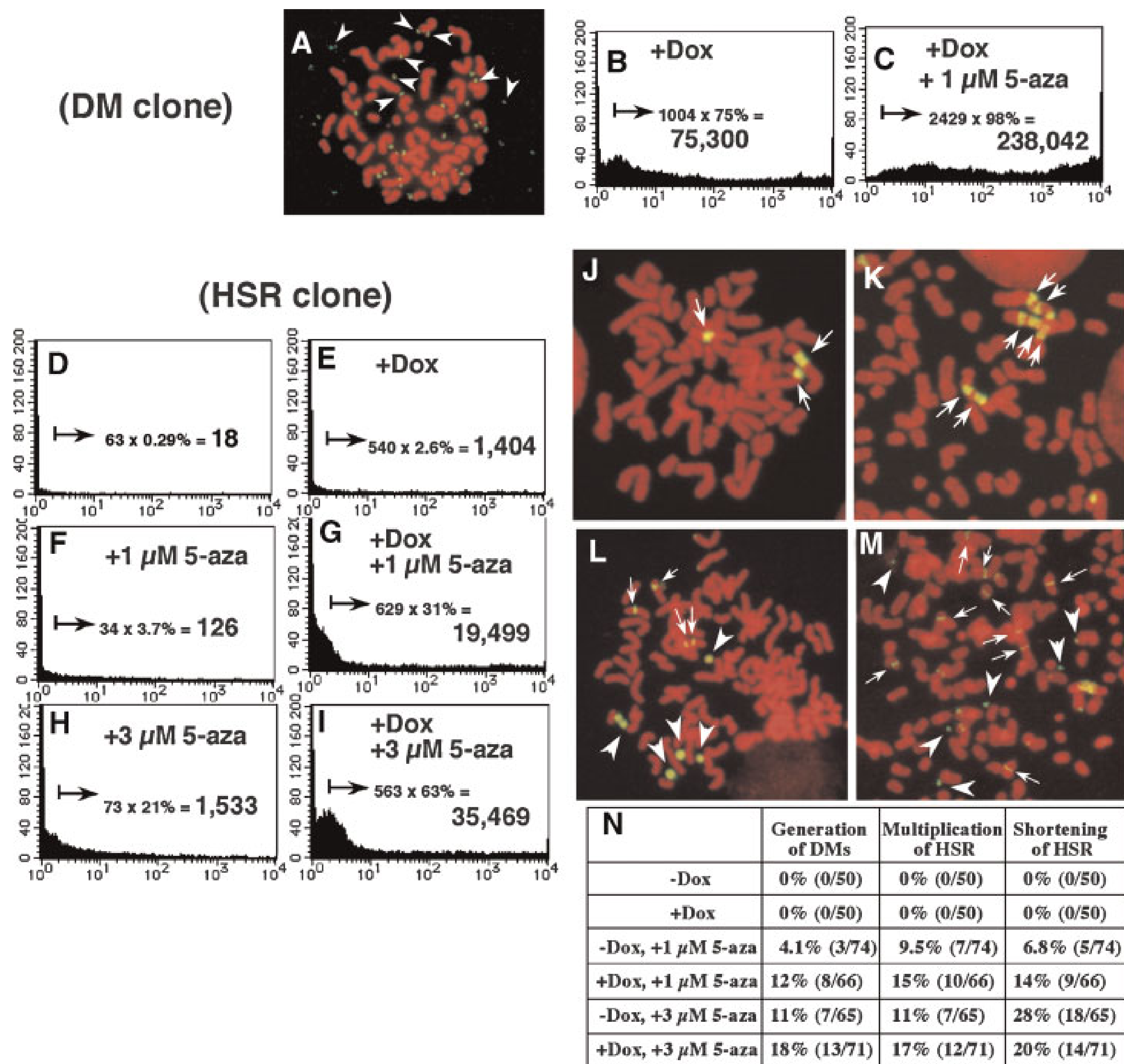
Inhibition of DNA Methylation Together With Transcriptional Activation Resulted in Fragmentation of Large HSRs

We were interested in whether the macroscopic structure of the tandem plasmid repeat changed in response to transcriptional activation or inhibition of DNA methylation. Plasmid sequences were analyzed by FISH before and after addition of 5-aza and/or Dox. In a primary, HSR-bearing clone, the plasmid sequence was present solely at three chromosomal HSRs in all 50 metaphase cells examined (Fig. 4J,N). The addition of Dox alone did not appear to change

TABLE I. d2EGFP Expression in the Clones of COLO 320DM Transformants From pDHFR.TRE-d2EGFP and pTet-ON

				Relative
Clone no.[a]	Treatment[b]	Mean[c]	Percent[c]	d2EGFP expression[c]
DM clone 1	None	71	90	6,390
	+1 μM 5-Aza	150	88	13,200
	+3 μM 5-Aza	268	93	24,924
DM clone 2	None	58	72	4,176
	+1 μM 5-Aza	35	72	2,520
	+3 μM 5-Aza	69	81	5,589
HSR clone 1	None	53	1.7	90
	+1 μM 5-Aza	37	13	481
	+3 μM 5-Aza	71	33	2,343
HSR clone 2	None	3.5	0.32	1.1
	+1 μM 5-Aza	18	7	126
	+3 μM 5-Aza	38	25	950
HSR clone 3	None	34	0.46	16
	+1 μM 5-Aza	18	6.1	110
	+3 μM 5-Aza	41	24	984
HSR clone 4	None	3.3	0.23	0.76
	+1 μM 5-Aza	15	6.5	98
	+3 μM 5-Aza	28	22	616
HSR clone 5	None	4.1	0.17	0.70
	+1 μM 5-Aza	26	5.4	140
	+3 μM 5-Aza	33	17	561
HSR clone 6	None	8.1	45	365
	+1 μM 5-Aza	16	57	912
	+3 μM 5-Aza	19	56	1,064

[a]Each clones were classified into either DM clone or HSR clone by FISH applied to the metaphase spread.
[b]Indicated concentration of 5-aza was added to the culture on 5 days before the cell sorter analysis. Dox was added to every culture 2 days before the analysis.
[c]The 'Relative d2EGFP expression' was calculated by multiplying the 'mean' and the 'percent' of expressing cells. See the Methods for detail.



	Generation of DMs	Multiplication of HSR	Shortening of HSR
-Dox	0% (0/50)	0% (0/50)	0% (0/50)
+Dox	0% (0/50)	0% (0/50)	0% (0/50)
-Dox, +1 μM 5-aza	4.1% (3/74)	9.5% (7/74)	6.8% (5/74)
+Dox, +1 μM 5-aza	12% (8/66)	15% (10/66)	14% (9/66)
-Dox, +3 μM 5-aza	11% (7/65)	11% (7/65)	28% (18/65)
+Dox, +3 μM 5-aza	18% (13/71)	17% (12/71)	20% (14/71)

Fig. 4. Interconversion of intra and extrachromosomal amplification by transcriptional activation and inhibition of DNA methylation. We obtained clones of the transformants shown in Figure 3J using limiting dilution, and analyzed them using FISH and FACS to determine plasmid localization and d2EGFP expression, respectively. Two representative clones in which the amplified plasmid sequences were localized at either DMs (arrowheads in **A**) or HSRs (arrows in **J**) are shown. Expression was always higher in the DM-containing clone (**B**, **C**) compared to the HSR-containing clone (**D**, **E**). Gene expression in the HSR-containing clone was elevated by pre-treatment with 1 μM or 3 μM 5-aza for 5 days, or a combination of 5-aza for 5 days and Dox for the last two days of incubation (**F–I**). Metaphase FISH analysis revealed several novel structures of the plasmid sequence following treatment that were not seen in the original clone: multiple HSRs in a chromosome (arrows in **K**), large extrachromosomal DMs (arrowheads in **L**), small extrachromosomal DMs (arrowheads in **M**), or very short HSRs (small arrows in L and M). The frequencies of these structures after the various treatments are summarized in **N**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the structure of the HSRs; however the addition of 5-aza resulted in the appearance of several novel structures, in addition to the original HSRs: extrachromosomal structures that closely resembled DMs (arrowheads in Fig. 4L,M); multiple HSRs in a chromosome (arrows in Fig. 4K); and very short HSRs (small arrows in Fig. 4L,M). These novel structures were induced by either 1 or 3 μM 5-aza alone, and the addition of Dox further increased their frequency (Fig. 4N). Because these novel structures were never seen in non-treated cells, these results suggested that the original HSRs were partially excised, generating extrachromosomal elements, leaving short chromosomal HSRs. The generation of multiple HSRs in a

chromosome could be due to initiation of the BFB cycle by DSBs near the HSR.

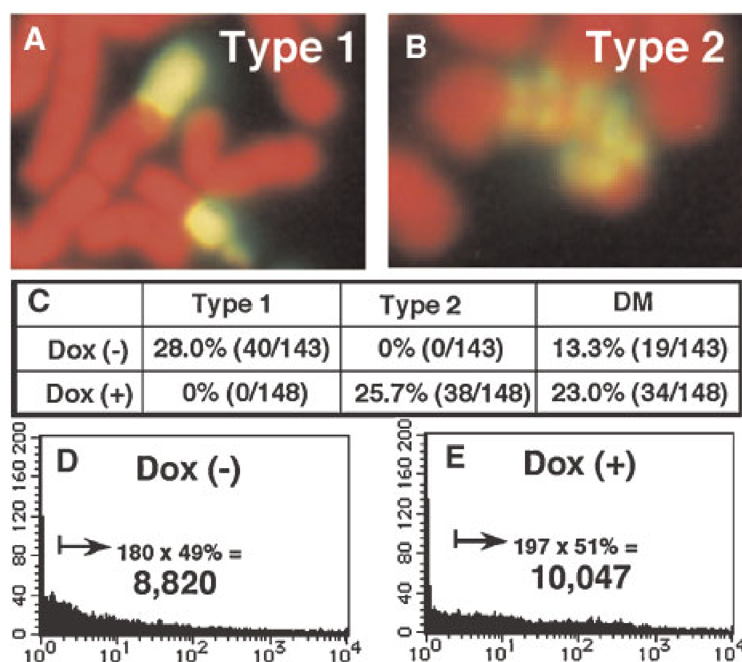
Active Gene Expression Prevents the Generation of Large Amplified Structures

We were interested in whether activation of the TRE-promoter during cell selection had any effect on the process of gene amplification. COLO 320DM were co-transfected with pDHFR.TRE-d2EGFP and pTet-ON, and stable transformants were selected in the presence of BS, with or without Dox. Plasmid sequences were then analyzed by FISH. As a result, selection in the absence of Dox led to the appearance of large homogeneous HSRs (Type 1; Fig. 5A,C); in contrast, cell selection in the presence of Dox yielded transformants that were totally devoid of Type 1 HSRs. Rather, all the chromosomal signals appeared as ladders (Type 2 HSR; Fig. 5B and C). The frequency of signals at DMs was higher when Dox was present during the cell selection process (Fig. 5C). Furthermore, the expression of d2EGFP was slightly higher in cells selected in the presence of Dox compared to the absence of Dox. These results could reflect an increased frequency of cells with DMs when cell selection was performed in the presence of Dox, or that expression from Type 2 HSRs is more efficient than from Type 1 HSRs.

Efficient Amplification and Expression of the IR/MAR Plasmid in CHO Cells

The most frequently used cell line for protein production is the CHO-K1 cell line. We co-transfected CHO-K1 cells with pTet-ON, and pDHFR.TRE-d2EGFP (which contain the IR/MAR sequences) or pV.TRE-d2EGFP (which does not contain the IR/MAR sequences), and selected stable transformants in the presence of BS. FISH analysis revealed that the IR/MAR-plasmid was amplified even in CHO-K1 cells, and formed long HSRs, short HSRs, or extra-chromosomal DM-like material (representative images appear in Figs. 6A–C). The length of the HSRs generated in CHO cells was generally shorter than those generated in COLO 320 cells.

The presence of Dox during the selection process prevented the generation of both long



C	Type 1	Type 2	DM
Dox (-)	28.0% (40/143)	0% (0/143)	13.3% (19/143)
Dox (+)	0% (0/148)	25.7% (38/148)	23.0% (34/148)

Fig. 5. Continuous activation of a plasmid-encoded TRE-promoter prevented the generation of large HSRs in COLO 320DM cells. COLO 320DM cells were co-transfected with a mixture of pTet-ON and pDHFR.TRE-d2EGFP. Stable transformants were selected in the presence of 5 μg/ml BS without Dox [Dox (−)] or in the continuous presence of Dox [Dox (+)]. Five weeks after transfection, metaphase chromosome spreads were prepared from the transformants, and plasmid sequences were detected using FISH analysis. Chromosomal HSRs appeared as either a continuous array (Type 1; **A**) or a ladder (Type 2; **B**). Selection in the absence or presence of Dox led to the preferential appearance of Type 1 or Type 2 HSRs, respectively (**C**). The expression of d2EGFP in the polyclonal transformants was also analyzed (**D**, **E**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

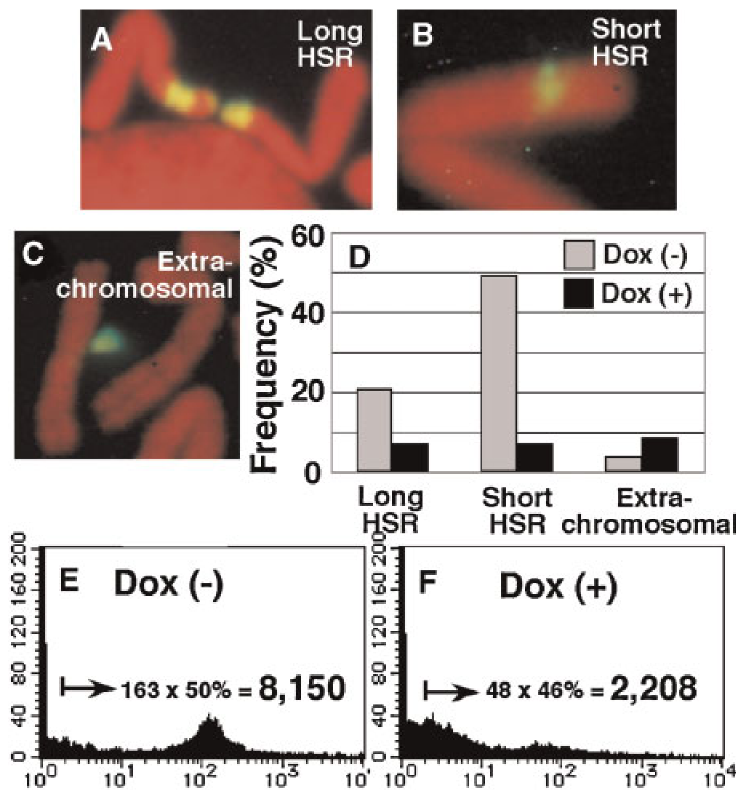
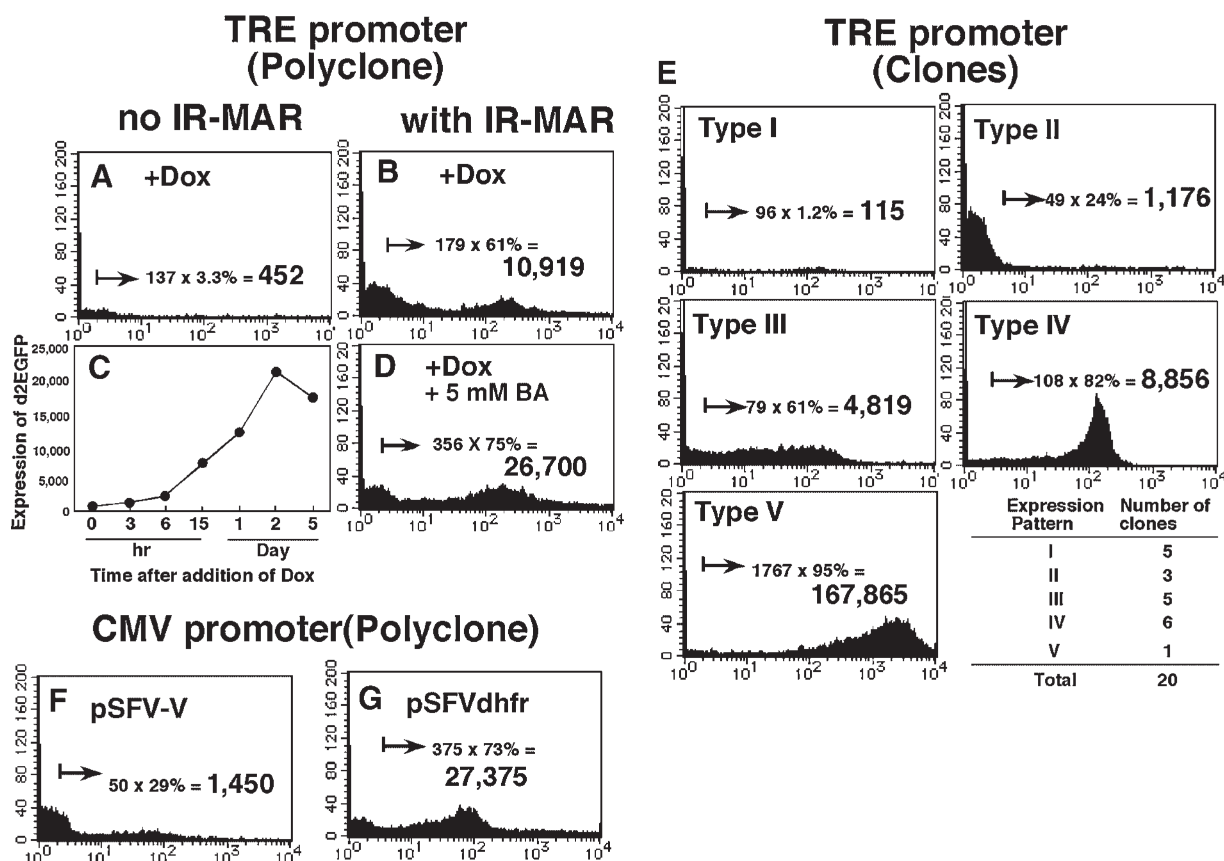


Fig. 6. Continuous activation of a plasmid-encoded TRE-promoter prevented the extensive amplification in CHO-K1 cells. CHO-K1 cells were co-transfected with a mixture of pTet-ON and pDHFR.TRE-d2EGFP. Stable transformants were selected in the presence of 5 μg/ml BS without Dox [Dox (−)] or in the continuous presence of Dox [Dox (+)]. Five weeks after transfection, metaphase chromosome spreads were prepared from the transformants, and plasmid sequences were detected using FISH analysis. In the transformants, long HSRs (**A**), short HSRs (**B**), or extrachromosomal amplification (**C**) were apparent. Both long and short HSRs appeared with higher frequency in transformants selected in the absence of Dox, compared to the presence of Dox (**D**). The expression of d2EGFP was higher in transformants obtained in the absence of Dox (**E** and **F**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and short HSRs (Fig. 6A,B,D). As in COLO 320DM cells, the frequency of extrachromosomal amplification was higher when cells were selected in the presence of Dox (Fig. 6C,D). Relative d2EGFP expression was approximately fourfold higher in cells selected in the absence of Dox (Fig. 6E,F). This result may simply reflect the copy number of the plasmid in these cells.

We then addressed on protein production from the plasmid sequence in the trasnformants selected in the absence of Dox. FACS analysis revealed that the relative level of d2EGFP was more than 20-fold higher in the IR/MAR-transformants (Fig. 7B) compared to transformants that carried an IR/MAR-deficient plasmid (Fig. 7A). Expression was inducible, and reached a peak 2 days after the addition of Dox (Fig. 7C). We also found that expression was further increased by the addition of sodium butyrate to the cells (Fig. 7D). Sodium butyrate is an inhibitor of histone deacetylase, and has been reported to reactivate the expression of silenced transgenes [Chen et al., 1997].

We obtained 20 independent clones of pDHFR.TRE-d2EGFP-transformed CHO-K1 cells, and classified these clones into five types, based on their pattern of d2EGFP expression. We also documented the frequency of occurrence of each type (Fig. 7E). The results indicated that using a simple procedure, we could easily obtain high protein-producing clonal cell lines with the IR/MAR-plasmid system. The expression of d2EGFP was heterogeneous, even within a clonal cell population. It was also the case for the transformant clones from COLO 320DM cells (see Fig. 4). It suggested that there were epigenetic effects on expression from the tandem plasmid repeat.



Expression Pattern	Number of clones
I	5
II	3
III	5
IV	6
V	1
Total	20

Fig. 7. IR/MAR plasmid-mediated amplification results in elevated protein production in CHO-K1 cells. CHO-K1 cells were co-transfected with a mixture of pTet-ON and either pV.TRE-d2EGFP (**A**) or pDHFR.TRE-d2EGFP (**B**–**D**). Stable transformants were selected in the presence of 5 μg/ml BS for 4 weeks. The expression of d2EGFP was analyzed 2 days (A, B) or at the indicated times (C) after the addition of 1 μg/ml Dox, or 2 days after the addition of both 5 mM sodium butyrate and 1 μg/ml Dox (D). From the polyclonal transformants shown in panel B, 20 clones were isolated (**E**). Clones were incubated with Dox for 2 days, and the expression of d2EGFP was analyzed by FACS. Expression patterns were classified into five types (type I to V), and the incidence of each pattern is summarized in the Table in E. CHO-K1 cells were co-transfected with pCMV-d2EGFP and pSFV-V (**F**) or pSFVdhfr (**G**). Stable transformants were selected in the presence of 5 μg/ml BS for 4 weeks, and d2EGFP expression was analyzed by FACS.

Expression Driven by the CMV Promoter was Increased by Co-Amplification of an IR/MAR-Bearing Plasmid

CHO-K1 cells were co-transfected with a plasmid encoding d2EGFP under the control of the constitutively active CMV-promoter (pCMV-d2EGFP) and either an IR/MAR-containing plasmid (pSFVdhfr), or one lacking IR/MAR sequences (pSFV-V). We obtained stable transformants, and analyzed their plasmid sequences by FISH. We found that 44 and 3% of the stable transformants carrying an IR/MAR-bearing plasmid formed HSRs or DMs, respectively, while no prominent structures were observed in transformants carrying the plasmid that lacked IR/MAR sequences. FACS analysis revealed that relative d2EGFP expression was more than 20-fold higher in the former transformants, compared to the latter (Fig. 7G compared with F).

DISCUSSION

Underlying the fact that gene amplification plays a role in malignant transformation of mammalian cells is the idea that the amount of gene product increases in proportion to the copy number of the amplified gene. With some exceptions, this has indeed been shown to be the case [Myllykangas and Knuutila, 2006], and this was true in the current study, in human colorectal carcinoma COLO 320 cells (Fig. 2). However, when gene amplification was induced using an IR/MAR plasmid-based system, gene expression was suppressed. The silencing that we observed in the tandem plasmid repeat is typically referred as RIGS [Garrick et al., 1998;

Henikoff, 1998; McBurney et al., 2002]. RIGS has been frequently related to a host protection mechanism for inactivating foreign sequences. It has been suggested that RIGS is mediated by RNA-interference [Martienssen, 2003], and methylation of target sequences [Morris et al., 2004], which in turn, results in the recruitment of several heterochromatin proteins. In the current study, we found that of 5-aza, which prevents DNA methylation, increased gene expression, particularly from HSRs. We also found that 5-aza alone or in combination with Dox induced fragmentation of long HSRs, generating extrachromosomal DMs, short HSRs, and multiple HSRs in a chromosome. Expression from DMs was higher than from HSRs, suggesting that the increase in gene expression induced by 5-aza may be the result of excision of the plasmid repeat into an extrachromosomal environment. DNA hypomethylation has frequently been related to a recombinogenic or clastogenic outcomes [Perry et al., 1992; Rizwana and Hahn, 1999; Ramirez et al., 2003]. Furthermore, re-expression of a cluster of silenced transgenes was associated with 5-aza-induced intrachromosomal rearrangement of transgenes, which suggested again the connection of DNA methylation and a host protection mechanism for inactivating foreign sequences [McBurney et al., 2001].

We found that continuous activation of the TRE-promoter by rtTA/Dox suppressed the formation of large HSRs. This was not the case for the SRα promoter driving the transcription of the *BS* gene, which should have been continuously active during cell selection, as the cells acquired BS-resistance. Furthermore, a plasmid bearing the constitutively active CMV promoter appeared to be amplified in long HSRs in CHO cells (data not shown). Therefore, long HSRs do appear in the presence of transcription. One explanation for our results may be that the VP16 acidic activation domain of rtTA exerts a particularly strong effect on chromatin structure, causing it to open and become active, and thus activation of the TRE-promoter has a more pronounced effect on HSR structure than other promoters. A more precise understanding of the effect of different promoter types on the formation of HSRs awaits future study. Nonetheless, the above finding was consistent with the finding that the Dox appeared to act cooperatively with 5-aza to induce fragmentation of large HSRs. Active transcription of the plasmid repeat could induce frequent DSBs and/or recombination due to a direct interaction of the transcriptional machinery with the replication fork [Shimizu et al., 2003]. Thus, the continuous presence of Dox during the selection of transformants might result in fragmentation of the HSRs, and suppress the formation of long HSRs. Such a process would be greatly accelerated if DNA methylation levels decreased for any reason. These results may have important implications for our understanding of oncogenesis-associated gene amplification, as transcriptional activation in concert with fluctuations in DNA methylation levels may cause the interconversion of extrachromosomal and intrachromosomal gene amplification, as well as influence the overall extent of gene amplification.

Several published studies have shown that regions initially amplified by BFB cycles can delete out to form DMs [Toledo et al., 1993; Coquelle et al., 1998; Coquelle et al., 2002] and that double-strand breaks can promote this conversion [Coquelle et al., 2002]. We have also shown here that the HSR generated by IR/MAR-plasmid through BFB cycle was fragmented to form DMs by synergetic action of DNA methylation inhibition and transcriptional activation. The transcriptional activation may increase the frequency of double strand break by collision with the replication fork [Shimizu et al., 2003]. Whereas, published studies have also shown that DMs can integrate to create HSRs [Windle et al., 1991; Coquelle et al., 1998], and regions amplified by BFB cycles can also be transferred to other chromosomes through inter-chromosome fusion [Murnane, 2006]. Our current result also suggested that the DMs excised from HSR might generate multiple HSR in a single chromosome or in different chromosomes. Thus, our model system using IR/MAR plasmid appeared to mimic the interconversion of intrachromosomal and exrtachromosomal gene amplification that was observed in malignant cells, and it further suggested that DNA methylation and transcription may play a pivotal role in this process.

On the other hand, amplification of endogenous genes in hamster cells commonly occurs at the site of the gene through BFB cycles, while high-copy amplification in human cells is seen as DMs or HSRs at sites other than where the gene was originally located (for a review, see [Murnane and Sabatier, 2004]). One study has

found that this difference is due to the fact that initial amplification by BFB cycles rapidly converts to DMs in human cells [Singer et al., 2000]. Our system using IR/MAR-plasmid also generated DMs far more frequently in human COLO 320DM cells than hamster CHO-K1 cells. COLO 320DM cells had multiple DMs before the transfection of IR/MAR-plasmid, and the plasmid was amplified either at the pre-existing DMs or at the new DMs that was generated by the plasmid. Thus, the amplification at DMs not necessarily depends on the presence of pre-existing DMs. Because we showed the interconversion of intrachromosomal and exrtachromosomal gene amplification by modulation of gene expression and DNA methylation, the promoter activity and/or the DNA methylation activity may differ between human and hamster cells.

Recently, it was reported that the presence of a replicator sequence in a transgene could prevent gene silencing [Fu et al., 2006]. This was a remarkable finding, as DNA replicator sequences may improve the efficiency and safety of gene therapy. The results were obtained using cells bearing a single copy transgene generated by Cre-mediated cassette exchange, and the human *β-globin* IR as a replicator. We previously showed that a plasmid bearing the same *β-globin* IR was efficiently amplified, and generated DMs and HSRs in COLO 320DM cells that were transfected using lipofection [Shimizu et al., 2003]. On the other hand, we also showed that a plasmid bearing the *DHFR* 4.6 kbp IR (*Ori β*) or the c-*myc* 2.4 kbp IR was consistently and efficiently amplified [Shimizu et al., 2001a, 2003, 2005; Shimizu and Shingaki, 2004; Bosisio et al., 2006]. Both of these IRs have been shown to act as replicators [Trivedi et al., 1998; Malott and Leffak, 1999; Altman and Fanning, 2001; Liu et al., 2003; Altman and Fanning, 2004]. Thus, plasmids bearing replicator sequences were, if transfected into cells using lipofection, amplified in animal cells and adopted a tandem repeat structure. Furthermore, our data presented here demonstrated that the expression of a tandem plasmid repeat was usually suppressed, even if the sequence contained a replicator. Therefore, prevention of silencing via a replicator sequence, particularly in applications such as gene therapy, should be applied carefully in order to prevent amplification.

The IR/MAR-gene amplification method is very easy and efficient. Any sequence can potentially be amplified to a few thousand copies per cell by co-transfection with the IR/MAR-plasmid, followed by one-step antibiotic selection. In the current study, we demonstrated the relationship between gene expression and gene amplification in this system, and suggested how this method may be applied to protein overproduction. Traditionally, the most widespread method for protein overproduction uses the amplified *DHFR* structural gene and selection by Mtx (reviews in [Wurm, 2004]). However, it is highly time-consuming and labor-intensive, and the effect of gene amplification on protein production was a ten to twenty-fold increase, at most [Wurm, 2004]. Therefore, the IR/MAR-gene amplification method may provide a novel, easy, and efficient protein production system.

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