DIVISION of GENOME DYNAMICS †

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Gene amplification provides host organisms with an abundance of gene products. One example is the high copy number of rDNA (rRNA gene), which enables a large amount of protein production. On the other hand, drug-resistance genes can become amplified increasing their effectiveness. These two forms of gene amplification produce products that differ radically in structure. The former is a direct repeat product and the latter is an inverted repeat product. We previously clarified the mechanism of the former type of amplification and here have shed light on the latter.

We have carried out an amplification experiment using yeast on the assumption that double rolling circle replication (DRCR) is a core reaction in gene amplification. We induced DRCR by BIR (break induced replication) and obtained two gene amplification products, HSR (homogeneous staining region) and DMs (double minutes) (Watanabe and Horiuchi, *EMBO J*. 2005). To further confirm this result, we carried out an experiment in which DRCR was induced by two entirely different methods, the Cre-*lox* system and homologous recombination. We obtained the gene products HSR and DMs in either case respectively. Furthermore, using Chinese Hamster Ovary (CHO) cells, we induced DRCR by the Cre-*lox* system and obtained two gene products. In addition, scattered products, as are frequently observed in cultured cells, are obtained. Finally, we found that inversion occurred frequently in the inverted repeat structure of the products and is dependent on the DRCR process itself.

I. Construction of a new gene amplification system via DRCR (double rolling circle replication) using Cre-*lox* site-specific recombination

Previously, we developed a gene amplification system in *S. cerevisiae* that is based on DRCR, utilizing break-induced replication (BIR) (Watanabe and Horiuchi, *EMBO J*. 2005). This system produced two amplification products resembling the HSR and DMs of higher eukaryotes. If DRCR is an actual gene amplification mechanism in yeast, a quite different initiation reaction, which can induce DRCR, should produce amplification products resembling HSR and DMs. Thus, we tried to construct a new DRCR amplification system that is induced by another process, Cre-*lox* site-specific recombination. We first predicted that, if Cre recombination occurs between the two *lox* sites, one present on the replicated and the other on the un-replicated region, as shown in Figure 1 (a), the replication fork should switch the template from the parental (un-replicated) to the sister–chromatid (replicated) DNA strands. Furthermore, a

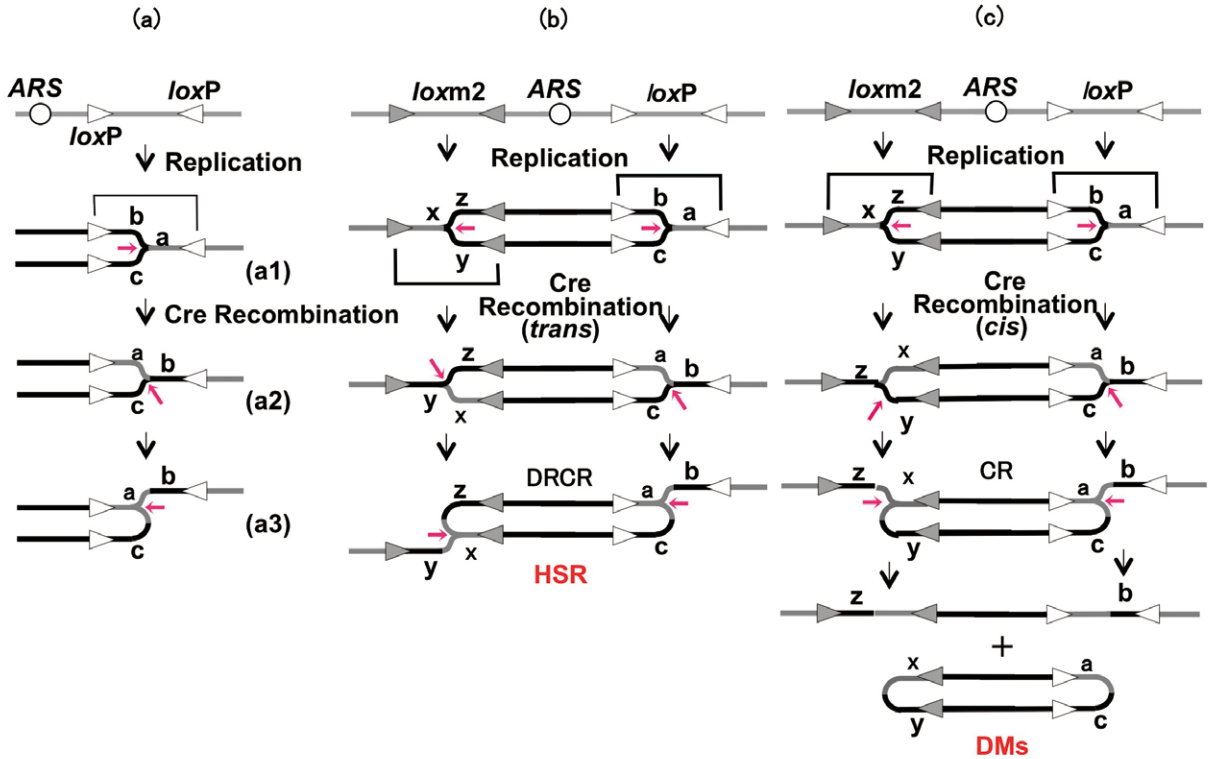

Figure 1. Cre-*lox* can initiate template switching, DRCR and CR (convergent replication).

†: This laboratory was closed on 31 March, 2011.

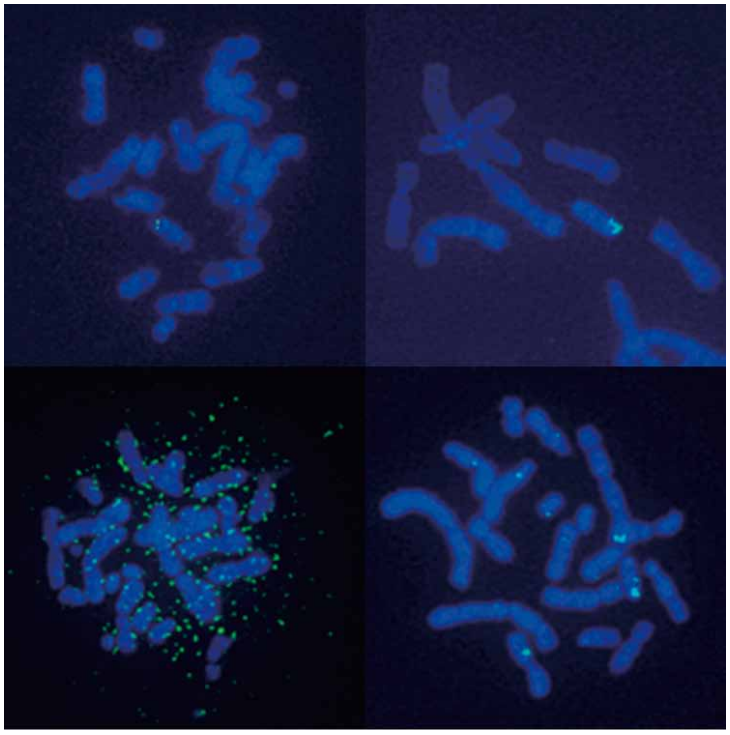

Figure 2. Three gene amplification products in CHO cells. (top left) control cell, (top right) HSR type, (bottom left) DMs type, (bottom right) Scattered type.

combination of the recombination processes, as shown in Figure 1 (b) and (c), could efficiently induce gene amplification through DRCR or CR (convergent replication). In fact, this system produced two kinds of gene product: highly amplified (>100 copies) chromosome HSR products and acentric multi-copy extra-chromosomal DMs products. The structures of these products resemble HSRs and DMs of higher eukaryotes, respectively. From previous and present results, we concluded that DRCR is indeed an amplification mechanism in budding yeast and can be naturally initiated if some structural requirements are satisfied.

Next, we constructed a similar amplification system in CHO cells, based on DRCR. This system also produced intra- and extra-chromosomal amplification products resembling HSRs and DMs (Figure 2. top right, bottom left). The amplified regions of HSR-type products undergo intensive rearrangement seen in mammalian gene amplification. Furthermore, the CHO system produces scattered-type amplification seen in cancer cells. This system can serve as a model for amplification of oncogene and drug-resistance genes, and may improve the productivity and ease of use of amplification systems that are widely used for making pharmaceutical proteins in mammalian cells (manuscript submitted for publication).

II. Mechanism of oncogene-type amplification under natural conditions

Site-specific recombination consists of two elements, a short specific sequence (*cis*-element) and a specific protein (*trans*-element) which recognizes the specific sequence and recombines efficiently between them. On the other hand, general recombination consists of a long non-specific sequence and several sets of recombination proteins (Rec proteins), which recognize homologous sequences and recombine between them. However, homologous recombination can take the place of site-specific recombination by replacing a short specific sequence with a long non-specific sequence. This indicates that if a *lox* sequence is replaced by a long sequence, homologous recombination can induce DRCR. Replacement of the sequence alone should initiate DRCR by itself, in the absence of any site-specific recombination protein factors. We confirmed this expectation as follows: we created a →←→← structure, called FAIR (Four Alternate Inverted Repeat) at the right end of chromosome VI and an amplification selective marker, *leu2d*, inserted within the FAIR structure. This yeast strain was plated on agar without leucine, Leu^+ clones grew out and their chromosomal structure analyzed. As expected, two types of amplification were observed, HSR- and DM -type, both with the expected repeated structure. The implications of these results are very important, namely, that if FAIR structures form under natural conditions, gene amplification will occur. In fact, there are studies in which FAIR structures were observed at an initial stage of drug-resistance gene amplification. Furthermore, there is data suggesting that the BFB cycle can be an initial step of amplification. From these previous and our present results, all steps of oncogene-type gene amplification can be deduced as follows: a double strand break or recombinational template switching spontaneously occurs on a chromosome, and the BFB cycle initiates. As a result, a di-centric chromosome is produced and ds-breakage occurs again. The chromosome structure after two cycles of BFB is a perfect FAIR structure. Thus, this can initiate DRCR and gene amplification starts.

Using budding yeast, we here demonstrate that the FAIR structure has the potential to induce DRCR. Thus, we believe that the basic mechanism of oncogene-type gene amplification has been determined. However, gene amplification may also be induced by the BFB cycle. Especially in higher eukaryotes, there are a large number of transposable elements, among which there should be FAIR structures. Thus, it is not surprising that DRCR initiates without any double-stranded breaks. It is very interesting to ask the question whether or not this type of gene amplification does indeed occur (manuscript in preparation).

III. Double rolling circle replication (DRCR) is recombinogenic.

Homologous recombination plays a critical role in maintaining genetic diversity as well as genome stability. Interesting examples showing hyper-recombination are found in nature. In chloroplast DNA (cpDNA) and the herpes simplex virus (HSV) genome, DNA sequences flanked by inverted repeats undergo inversion very frequently,

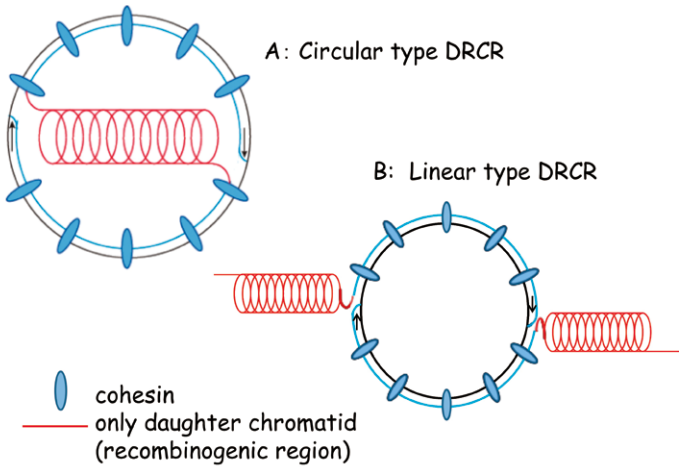

Figure 3. An "only daughter chromatid" model
A. circular-type DRCR, B. Linear-type DRCR

suggesting hyper-recombinational events. However, the mechanisms responsible for these events remain unclear. We previously observed very frequent inversion in a designed amplification system based on double rolling circle replication (DRCR). Utilizing the yeast 2μ plasmid, the genome of which is known to be replicated by DRCR and the gene amplification system in yeast, we demonstrate that DRCR is closely related to hyper-recombinational events. Inverted repeats or direct repeats inserted into these systems frequently caused inversion or deletion/duplication, respectively, in a DRCR-dependent manner. These results suggest that DRCR is involved in chromosome rearrangement associated with gene amplification and the replication of cpDNA and HSV genomes. We propose a model (Figure 3), termed the "only daughter chromatid" (red lines) model, in which DRCR inevitably produces only daughter chromatids and is markedly activated recombinationally (manuscript in press).