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The genomes of higher organisms contain significant amounts of repetitive sequences which, in general, are unstable. At present, neither the physiological function(s) of these repeated sequences, nor the mechanism producing them and controlling instability are fully understood. To clarify these aspects, we are pursuing several lines of research using *Escherichia coli*, *Saccharomyces cerevisiae* and Chinese Hamster Ovary (CHO) cells. In 2008 we discovered a new role of rDNA in yeast and reported a relationship between condensin associating with the chromosome and gene transcription. More specifically, we discovered a new condensin recruiting system, consisting of the following four proteins, Tof2p, Csm1p, Lrs4p and Fob1p, which recruit condensin protein complexes to the *RFB* site in rDNA repeat units. In addition, we also developed a new system of gene amplification via DRCR (double rolling circle replication) in yeast using Cre-*lox* site-specific recombination. From previous and present results, we concluded that DRCR is an amplification mechanism actually used at least in budding yeast. We are investigating the possibility that DRCR might also act in gene amplification in higher eukaryotes as well.

I. Analysis of mechanisms maintaining repeated structures of ribosomal RNA genes

In most eukaryotic organisms, the rDNAs are clustered in long tandem repeats on one or a few chromosomes. Although the total number of these rDNA repeats appears to be maintained at a level appropriate for each organism, genes with such a repeated structure are in general thought to be unstable because of a high frequency of recombinational events. Thus, it might be expected that organisms must have developed systems to regulate recombination within rDNA repeats.

In the yeast *Saccharomyces cerevisiae*, 200 copies on average are tandemly arrayed in a central position on the longest chromosome (XII). Recombinational events within the rDNA repeats in normal growing yeast cells appear to be mostly mediated by a *FOB1*-dependent system. *FOB1* is the gene required for replication fork blocking activity at replication fork barrier (*RFB*) sites, for rDNA region-specific recombination and for expansion/contraction of rDNA repeats. The latter two activities are likely to be triggered by double-strand breaks at the *RFB* site and repair of the break via gene conversion. Thus, *FOB1*-dependent recombination apparently contributes to the maintenance of average copy number of rDNA. However, in *Δfob1* cells, the repeats are still maintained without any fluctuation of copy number, suggesting that another, hitherto unknown, system acts to prevent contraction of the number of repeats.

In order to understand this putative alternative system, we collected a number of mutants in which the rDNA copy number decreased drastically under *Δfob1* conditions. We have found that mutations of genes encoding condensin caused this phenotype, suggesting that, in addition to its role in condensation and separation of chromosomes in M phase, condensin influences the maintenance of repeated rDNA structures. Each gene encoding a condensin subunit is known to be essential for growth, but the mutants isolated here are of leaky type. Analyzing the condensin and *fob1* double mutants and examining specific interactions between condensin and rDNA regions revealed that (1) in the double mutants, the copy number of rDNA in the mutant dramatically decreased; (2) the condensin complex associated with the *RFB* region in a *FOB1*-dependent manner; (3) the association between condensin and *RFB* was established during S phase and was maintained until anaphase; (4) double mutants showed slow growth which may be caused by a defect in the separation of the long rDNA array in anaphase. These results strongly suggest that *FOB1*-dependent condensin association with the *RFB* region is required for efficient segregation of rDNA repeat regions.

II. Mechanism of condensin recruitment onto *RFB* sites located within the tandem rDNA repeat in budding yeast.

The primary functions of mitotic chromosome condensation are to reduce the length of the chromosomes to prevent truncation during cell division and to ensure proper segregation of sister chromatids. The compaction ratio of mitotic chromosomes relative to double-stranded DNA ranges from ~160-fold in budding yeast to ~10000 – 20000-fold in mammalian chromosomes. Condensin is a multi-subunit protein complex that plays a central role in mitotic chromosome condensation and segregation. In vertebrates, condensin is distributed in the axial part over the whole length of condensed chromosomes, but this had only been shown at the resolution of light microscopy. The sites where condensin acts on the chromatin and the molecular mechanisms of condensin recruitment thereto had largely remained elusive. As described above, we found that condensin localized to the *RFB* site in a Fob1-dependent manner during S-phase. To date, this Fob1p-dependent condensin localization is the only example of condensin association with a specific DNA site in a specific protein factor-dependent manner. Towards understanding chromosome condensation at the level of molecular resolution, we have studied mechanisms of condensin localization at the *RFB* site. Recently, we discovered that condensin could bind to a short DNA fragment containing *RFB* sequences, even if the sequence was inserted at an ectopic chromosomal site. This indicates that the *RFB* site itself acts as a site recruiting condensin onto chromatin.

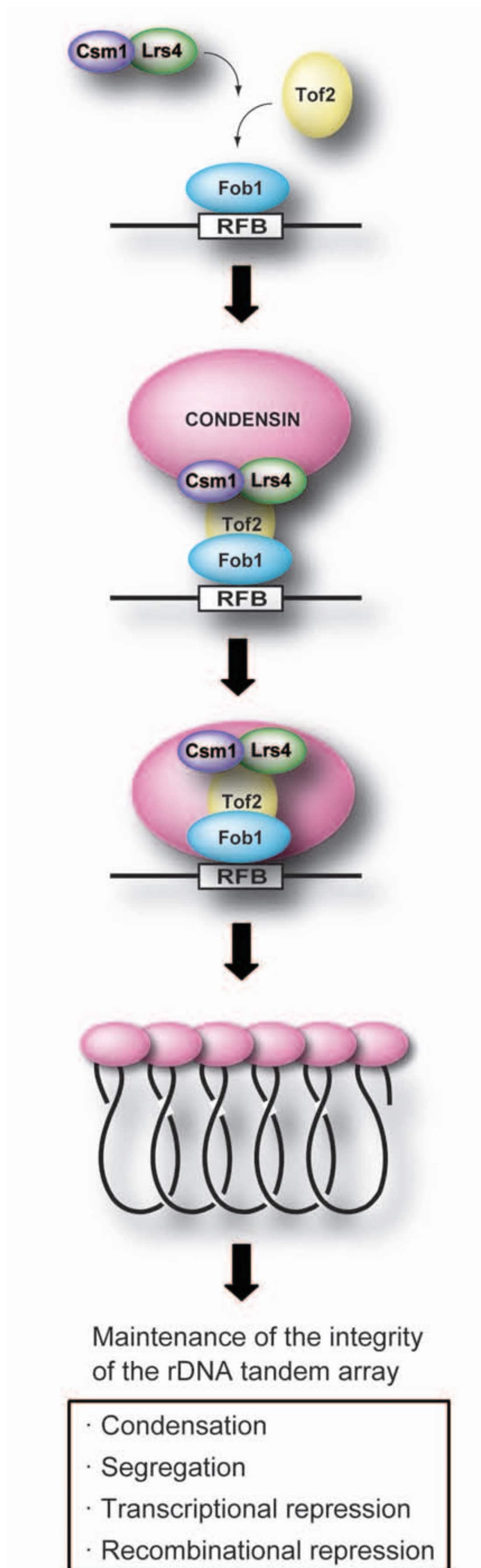

Figure 1. Model for condensin recruitment to the RFB site, contributing to maintenance of the integrity of long rDNA repeats.

Analysis of the relationship between condensin recruitment to the *RFB* site and Fob1p-dependent replication fork blockage at the *RFB* site demonstrated that those two events were completely independent phenomena. Instead, we identified three additional protein factors, Tof2p, Csm1p, and Lrs4p, necessary for both *FOB1*-dependent condensin recruitment to the *RFB* site and for ensuring the faithful segregation of long rDNA repeats. We also found ordered binding of Fob1p, Tof2p, Csm1p/Lrs4p, and condensin complexes at the *RFB* site. Finally, *in vivo* interactions between Csm1p, Lrs4P and multiple subunits of condensin were detected. These results suggest that condensin is recruited to the *RFB* site by the sequential interactions of Fob1p, Tof2p, Csm1p, Lrs4p, and finally condensin, to ensure the proper segregation of long rDNA tandem arrays (Figure 1). (Johzuka, K. and Horiuchi, T. The *cis*-element and factors required for condensin recruitment to chromosome. *Mol. Cell*, in press)

Ⅲ. Gene amplification can be induced via excessive silencing caused by over-expression of Sir2p and Sir3p in *Saccharomyces cerevisiae*

Gene amplification is involved in various biological phenomena such as cancer development and drug resistance. Furthermore, amplification can be the first step in the evolution of a new gene because the extra copies are free to acquire new functions. However, the nature of the events causing gene amplification is poorly understood. We focused on gene silencing, an epigenetic mechanism for controlling of gene expression, and examined whether gene silencing affects gene amplification. A construct with the potential to induce gene amplification was inserted near a telomeric region in *Saccharomyces cerevisiae*, at which gene silencing was enhanced by *SIR2* or *SIR3* overexpression. This construct contains two markers, *URA3* and *leu2d*. The enhanced gene silencing is expected to suppress *URA3* expression and to lead to copy number increase that confers a growth advantage on organisms maintained in medium lacking uracil. We used the amplification marker, *leu2d* to detect the amplification of *URA3* with great sensitivity. The *leu2d* gene has only a slight transcriptional activity and complements leucine auxotrophy only when amplified. *SIR3* overexpression using the plasmid (YRp-*SIR3*) gave 700-fold higher frequency of Leu^+ survivors than the control. About 50% Leu^+ clones having a construct with inverted repeats contained intra-chromosomal products (>100 copies), and 16% Leu^+ clones contained extra chromosomal products (~20 copies). The remaining 32% of Leu+ clones contained 2-3 copies of the inserted structure. On the other hand, *SIR2* overexpression could cause gene duplication, although at low frequency. In addition, a type of chromosomal translocation was observed through introduction of an extra copy of *SIR2* (total 2 copies per cell) into cells harboring a construct with deliberate DNA cleavage. These results suggest that excessive levels of gene silencing might generate selective pressure and promote amplification of genes.

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

In addition to rDNA gene amplification in eukaryotes, there is another type of gene amplification which is involved in various biological phenomena, such as cancer development and drug-resistance. However, the mechanism is largely unknown because of the complexity of the amplification process. Previously, we developed a gene amplification system in *S. cerevisiae* that is based on double rolling-circle replication (DRCR), utilizing break-induced replication (BIR). This system produced two types of amplification products. Type-1 products contain 13 to ~100 copies of the amplification marker, *leu2d* (up to ~730 kb increase) with novel arrangements present as randomly-oriented sequences flanked by inverted *leu2d* copies. Type-2 products are acentric multi-copy mini-chromosomes, each carrying two *leu2d* copies. Structures of type-1 and -2 products resemble those of homogeneously staining regions (HSR) and double minutes (DMs) of higher eukaryotes, respectively. Interestingly, products analogous to these were generated at low frequency without deliberate DNA cleavage (*EMBO J* 24, 190-198 (2005)).

If DRCR were 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 recombination occurs between un-replicated and replicated regions during replication, the fork will replicate these already-replicated regions again, and that the Cre recombination system would make this process more efficient, as shown in Figure 2(a). Furthermore, a combination of the processes, as shown in Figure 2(b), could efficiently induce gene amplification through DRCR. In fact, this system produced two kinds of products: highly amplified (>100 copies) chromosome products and acentric multi-copy extra-chromosomal products. Structures of these products resemble those of HSR and DMs of higher eukaryotes, respectively. From previous and present results, we concluded that DRCR is indeed an amplification mechanism in budding yeast and could be naturally initiated in the presence of the structural reguirement.

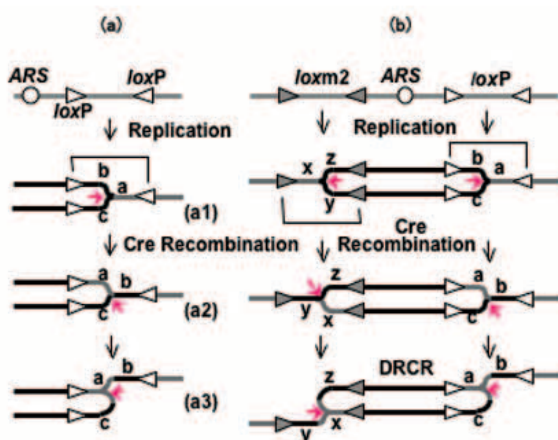

Figure 2. DRCR induced by the Cre-*lox* system
(a) Cre-*lox*-dependent reversal of replication orientation. (a1) When the replication fork passes between a pair of *lox* sites, Cre recombination occurs between them, as shown in (a1). Recombination changes replication orientation from un-replicated DNA (parental DNA strand) to replicated DNA (one of the sister chromatids) as shown in (a2 and a3), because DNA strand a and b are identical. (b) DRCR is induced by Cre-dependent combinational recombination of two pairs of *lox*P and *lox*m2 as shown in (b). Recombination cannot occur between *lox*P and *lox*m2.