A novel gene amplification system in yeast based on double rolling-circle replication

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Gene amplification 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. We describe a gene amplification system in *Saccharomyces cerevisiae* that is based on double rolling-circle replication utilizing break-induced replication. This system produced three types of amplification products. Type-1 products contain 5–7 inverted copies of the amplification marker, *leu2d*. Type-2 products contain 13 to ≈ 100 copies of *leu2d* (up to ≈ 730 kb increase) with a novel arrangement present as randomly oriented sequences flanked by inverted *leu2d* copies. Type-3 products are acentric multicopy minichromosomes carrying *leu2d*. Structures of type-2 and -3 products resemble those of homogeneously staining region and double minutes of higher eukaryotes, respectively. Interestingly, products analogous to these were generated at low frequency without deliberate DNA cleavage. These features strongly suggest that the processes described here may contribute to natural gene amplification in higher eukaryotes.

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Introduction

Gene amplification occurs in numerous organisms and has probably played an important role in genome evolution (Kimura and Ota, 1974; Schimke, 1982; Stark and Wahl, 1984; Dunham *et al.*, 2002). The ribosomal RNA genes (rDNA) are tandemly repeated in most eukaryotes (Petes, 1979) and must have been amplified at some point during evolution. Oncogene amplification is frequently observed in the process of cancer development (Stark and Wahl, 1984; Bishop, 1987; Lengauer *et al.*, 1998; Schwab, 1998). Gene

amplification also occurs when cultured cells respond to selection for drug resistance (Alt *et al.*, 1978; Wahl *et al.*, 1979) and when insects and plants acquire resistance to agricultural chemicals (Donn *et al.*, 1984; Mouches *et al.*, 1986; Field *et al.*, 1988). In bacteria, amplification can be an essential intermediate in the adaptive mutation phenomenology (Slechts *et al.*, 2003).

Models proposed to explain the amplification process include unequal sister-chromatid exchange, localized over-replication, rolling-circle replication, double rolling-circle replication (DRCR), extrachromosomal amplification and re-integration, and breakage–fusion–bridge (BFB) cycles (Windle and Wahl, 1992; Stark, 1993). Despite great effort, the molecular mechanism responsible for the majority of amplification events remains unknown. For example, the gene amplification occurring in tissue-cultured cells during selection for resistance to methotrexate (MTX) is believed to be initiated by the BFB cycles (McClintock, 1951; Smith *et al.*, 1992; Ma *et al.*, 1993). However, rather complicated products are produced, which may require additional kinds of amplification processes. The complexity of these structures has made it difficult to analyze the mechanism at a molecular level.

The mechanism responsible for amplification of rDNA is fairly well understood. In *Saccharomyces cerevisiae*, rDNA amplification is initiated by a double-strand break (DSB) produced when a DNA replication fork encounters a replication fork barrier (RFB) present in each rDNA unit (Kobayashi *et al.*, 1998, 2004). This double-strand end enhances unequal sister-chromatid recombination, resulting in rDNA amplification (Kobayashi *et al.*, 2004). The blocking of replication forks also causes gene amplification in *Escherichia coli* at a recombinational hotspot, named Hot DNA (Kodama *et al.*, 2002; Horiuchi and Fujimura, 1995 and references therein). However, known chromosomal amplifications in *E. coli* and yeast are invariably direct tandem copies, while mammalian cells show a mixture of direct and inverted repeats (Stark and Wahl, 1984; Windle and Wahl, 1992). Thus, understanding of amplification in higher eukaryotes might be enhanced by development of a microbial system that generates products analogous to those observed in tumors and cultured cells (Lengauer *et al.*, 1998).

The mechanisms underlying these gene amplifications in yeast and bacteria appear to be quite different. While yeast rDNA amplification occurs through unequal sister-chromatid recombination (Tartof, 1974; Kobayashi *et al.*, 2004), bacterial Hot amplification seems to proceed through rolling-circle replication (Kodama *et al.*, 2002). However, it is possible that both processes are initiated by break-induced replication (BIR) (Kraus *et al.*, 2001). While interchromatid unequal BIR could cause rDNA amplification in yeast, a similar intrachromatid BIR could cause the rolling-circle type amplification of Hot DNA in *E. coli*.

The DRCR model was first proposed by Fitcher (1986) for amplification of yeast circular 2 μ plasmid and experimentally

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confirmed by Volkert and Broach (1986). Later, a similar model was proposed by Hyrien *et al* (1988) to explain a case of drug resistance gene amplification. However, the former DRCR depends on a 2μ -specific site-specific recombination system (*FLP1/FRT*) and the latter model has not been examined experimentally. Thus far, no chromosomal amplification system has shown to rely on DRCR.

Here we describe a DRCR-based system of gene amplification utilizing BIR, as shown in Figure 1, and provide evidence that it might be generally applicable to gene amplification in higher eukaryotes.

Results

Description of the system for DRCR

The amplification system described below relies conceptually on BIR (Figure 1A). A DSB occurs between two sites (C and X) on a chromosome. The C end (the donor sequence) finds a homologous C sequence (the recipient sequence) on the sister chromatid, invades it to form a new replication fork and initiates replication. BIR was demonstrated in *E. coli* and in yeast (Asai *et al*, 1994; Morrow *et al*, 1997; Kraus *et al*, 2001), and we ascertained that it occurred in the experimental system described here (data not shown).

The BIR was then allowed to occur in a more complicated situation designed to amplify a sequence within a single chromosome by DRCR (Figure 1B). For this system, chromosome VI of LS20 strain (Butler *et al*, 1996), which lacks an HO site and has a chromosomal HO endonuclease gene (Kostriken *et al*, 1983) under the control of the GAL10 promoter, was modified as shown in Figure 2A. Cleavage

by HO endonuclease removes the *URA3* gene and separates this chromosome into two fragments, which include sequences for BIR-induced DRCR.

Note in Figure 2 the positions of three different sequences that are repeated on the modified version of chromosome VI. Sequences YF2 (1.1 kb; gray arrows) and YF4 (1.0 kb; white arrow) are in various orientations and they are flanked by the *leu2d* gene (black arrows). Following HO cutting, an YF2 sequence is near the end of one fragment and YF4 is near the end of the other; these will be the donor sequences. For each of these ends, each opposite fragment carries internally a copy of the same sequence that allows it to serve as a recipient. To establish DRCR, the orientation of the pairs of YF2 and YF4 on the chromosome is critical. While an inverted arrangement, named the RR-HO construct, is required for DRCR, a direct repeat arrangement, named FF-HO, is expected to cause recombination between either the pair of YF2 or the YF4 sequences, and lead to deletion of the region flanked by the respective pair. To select for *LEU2* gene amplification, this gene was supplied with a partially defective promoter, *leu2d* (1.6 kb). Strains with a single copy of the *leu2d* gene cannot grow in medium lacking leucine, but can grow if the *leu2d* allele copy number increases (Erhart and Hollenberg, 1983). For the RR-HO strain, HO cutting removes *URA3* and exposes the YF2 and YF4 donor sequences. Each end is expected to invade the homologous recipient sequence on the other fragment and initiate DRCR as shown in Figure 2A. The DRCR process would terminate by recombination between *leu2d* genes on each bidirectionally elongated arms (Figure 1B, bottom). Two control chromosomes had the same structures as those of FF-HO and RR-HO but lacked both HO sites, as shown in Figure 2B (constructs FF and RR).

Figure 1 Amplification through DRCR triggered by BIR. (A) BIR reaction. Two double-stranded DNA molecules have a region of homology (ABC). A DSB is marked with a red cross. See text for more details. (B) DRCR process. Induction of DSBs (red cross) results in the production of a pair of incomplete chromosomes with two termini, one a normal telomeric terminus (TEL) and the other donor sequence (two arrows with green asterisks). The latter will then be degraded by exonucleases, creating a 3' overhang that invades the corresponding recipient sequence (complementary arrows without asterisks), triggers BIR as in the insert box and initiates DRCR. An amplification selective marker, *leu2d*, is used to select amplified clones. The DRCR process would terminate by recombination between *leu2d* genes on each bidirectionally elongated arms. ARS: autonomously replicating sequence; CEN: centromere.

Amplification of the *leu2d* gene on a chromosome

We plated the four strains on leucine-omitted galactose plates and obtained *Leu*⁺ survivors. The RR-HO strain gave a 6.3- or 15.3-fold higher frequency of *Leu*⁺ survivors than those with FF-HO or RR structure, respectively (Figure 2C).

The structure and copy number of the *leu2d* genes were inferred by Southern hybridization to a *leu2d* probe following pulse-field gel electrophoresis (PFGE) of uncut DNA from *Leu*⁺ survivors (Figure 2D) and agarose gel electrophoresis of *Xho*I-digested DNA from them (Figure 2E). The *leu2d* probe detects chromosome III in addition to the modified chromosome VI, because the original *leu2* site and another *leu2* fragment used to displace the HO site in the *MAT* locus are located on chromosome III of the parental host strain, LS20. DNA bands seen in most colonies were explained by the chromosome III of LS20 strain (L) (see Materials and methods and Supplementary Figure 3A). The original structure and location of the amplification cassette (Figure 2G) could be ascertained in uninduced colonies (Pre), from which unrestricted DNA sample showed a band at 292 kb in Figure 2D corresponding to chromosome VI carrying the amplification cassette, and this cassette generated an 11.6 kb *Xho*I band in Figure 2E.

In contrast, HO-induced colonies under *Leu* selection (IND) showed various bands. The products seen in the colonies with HO-induced cutting can be classified into three characteristic types.

Type-1 clones (marked in blue) have the expected amplification product with multiple inverted copies of the *leu2d*

gene. Of these, the size of chromosome VI from sample #35 is longer than that of the remaining clones (Figure 2D), but the *Xho*I digestion patterns are similar, in that all show three *Xho*I fragments, 10.3, 6.4 and 4.4 kb (Figure 2E). The 10.3 and 4.4 kb bands in all of the type-1 samples are more intense than the 6.4 kb band. These data can be fully explained if we assume that the typical type-1 chromosome VI and the long (clone #35) chromosome VI have five and seven copies of the *leu2d* gene, respectively, with the structures shown in Figure 2H.

Type-2 products (red) appear to be extensively amplified. In four samples (#45, 56, 70 and 72), the original chromosome VI band disappeared and very dense DNA spots

appeared above the separation limit and at the well position (Figure 2D). In the other three samples (#49, 57 and 66), a single or a few bands were located at higher positions than chromosome VI (Figure 2D). The amplification in these type-2 clones seemed to occur within chromosome VI because a chromosome VI-specific probe, *RET2*, hybridized to the same chromosomal bands as the *leu2d* genes (Supplementary Figure 1). The sharpness of these chromosomal bands indicated that the large number of amplified *leu2d* repeats was rather stable. Figure 2D and Supplementary Figure 1 show chromosome VI remaining in the well, especially in samples #45, 56, 70 and 72. We speculate that when the copy number of *leu2d* is over a certain level (~60 copies), the chromosomal DNA may become entangled during DNA preparation in agarose plugs or as a result of extensive recombination between the amplified repeats during replication. The large bands above the separation limit in Figure 2D are most likely chromosome VI containing the highly amplified region, because these bands also hybridize with the *RET2* probe (data not shown). The large smear at around 225 kb may represent large extrachromosomal products as described below or circular molecules containing *leu2d* produced by recombination between direct repeats. *Xho*I digestion of all of these samples generated four strongly hybridizing bands of similar intensity, whose sizes are 4.4, 6.4, ~8.2 and 10.3 kb (Figure 2E). These bands could also be recognized with EtBr staining (data not shown). From the lengths of chromosome

Figure 2 Amplification of the *leu2d* gene on a chromosome. (A) Structure of the right terminus region of chromosome VI where an amplification cassette (*-leu2d*-YF4-HO-URA3-HO-YF2-*leu2d*-, with a gray background) was inserted. The components and the chain of reactions from the intact chromosome to DRCR are described in the text. This structure represents RR-HO (see panel B). HOcs: HO cutting site (red asterisk) (B) Structures of FF-HO, RR-HO, FF and RR constructs. The FF and RR constructs both lack HO cut sites. FF refers to a direct repeat orientation of each pair of YF2 and YF4 sequences, while RR refers to an inverted repeat orientation of that. The amplification cassettes are indicated with a gray background. (C) Frequency of *Leu*⁺ colony formation for each construct under induced conditions. The colonies were counted as described in Materials and methods. (D) Southern analysis of a representative sample of RR-HO survivors with the *leu2d* probe. PFGE was performed using the CHEF Mapper XA with a size range of 220–500 kb using the Auto Algorithm mode. The clone numbers are indicated above. The lanes marked in blue, red and green indicate type-1, -2 and -3 samples, respectively. The lanes marked in gray and black indicate samples with new fusion(s) of chromosomes VI and III accompanied by *Leu*⁺ recombination. See text for more details. L: LS20; Pre: preinduction conditions (cultured on Ura⁻ glucose plates); IND: induced conditions (cultured on *Leu*⁻ galactose plates). (E) Southern analysis of *Xho*I-digested DNA of the samples from (D) with the *leu2d* probe using 0.8% agarose gel electrophoresis. The fragment sizes (kb) from the type-2 products are indicated on the right. (F) Southern analysis of the samples from (D) by FIGE with the *leu2d* probe. The CHEF Mapper XA was used with a size range of 10–60 kb using the Auto Algorithm mode. The size (kb) of the type-3 product is indicated on the right side. (G) Structure of chromosome VI with an RR-HO construct. The expected size (kb) of the *Xho*I fragment that hybridizes with the *leu2d* probe is shown below. These illustrations are not to scale. The size (kb) of the undigested chromosome is indicated in green. (H) The predicted structure and *Xho*I restriction map of the type-1 products with five and seven copies of *leu2d*. The gray arrows above indicate a large inverted structure. (I) The predicted structure of the type-3 product. The relevant *Xho*I sites are shown. The gray arrows above indicate an inverted structure.

VI in Figure 2D, we estimate that clones #66, 49 and 57 have 13, 29 and 38–54 copies of the *leu2d* gene, respectively. Clone #56 has approximately 100 copies based on comparing the density of the *XhoI* bands to those seen in clone #57 in Figure 2E. Clones #45, 70 and 72 also seem to have 100 or more *leu2d* copies. The structure of the type-2 clones differs from that of type-1 clones in showing the 8.2 kb *XhoI* fragment, which is absent in type-1 clones. The characteristic structure of the type-2 product is described later.

Type-3 clones (green) are most frequent. Although the size of chromosome VI in these clones is the same as that of the parent strain (Figure 2D), *XhoI*-digested DNA included two extremely strongly hybridizing *leu2d* bands (6.4 and 4.4 kb) (Figure 2E), indicating extensive amplification. This suggested the presence in the type-3 clones of a new small chromosome that migrates faster than the shortest natural chromosome. To analyze this shorter chromosome, we subjected the clones to field inversion gel electrophoresis (FIGE), and probed this with *leu2d* (Figure 2F). The results show that type-3 clones have a very short minichromosome (about 23.5 kb length). We deduced the structure (Figure 2I) from restriction digestions using *Sall*, *PvuI* and *SmaI* and dot blot hybridization (see Supplementary Figure 2 for more details). The intense hybridization bands of the uncut samples and two extremely strongly hybridizing *XhoI* digestion bands (6.4 and 4.4 kb) indicate 10 or more copies of the minichromosome. The minichromosome was unexpected, because there is no known ARS site in this region. However, a recent report (Katou *et al*, 2003) and our dot blot experiments strongly suggested that the amplified minichromosome contained unidentified ARS sequence (Supplementary Figure 2B).

Among the remaining clones, the chromosome structure and *Leu*⁺ complementation of four clones (gray) and clone #40 (black) can be explained by the production of new fusion(s) of chromosomes VI and III accompanied by *Leu*⁺ recombination. Such fusion chromosomes could be produced if the exposed *leu2d* sequence from chromosome VI invaded the original *leu2* site on chromosome III (Supplementary Figure 3C and D).

The stability of each amplified product was examined with representative clones that were cultured under nonselective (leucine supplemented) conditions. The results indicated that the type-1 and -2 products were maintained stably for at least ≈ 20 generations, but the type-3 products were rather unstable (unpublished data); $\approx 50\%$ of the total population of cells harvested from the type-3 clones after ≈ 20 generations returned to *Leu* auxotrophy (unpublished data).

These amplified products did not affect any other chromosome as far as could be determined by checking the EtBr-staining gels. The RR-HO survivors showed various growth phenotypes on the selective plates. We numbered the RR-HO survivors from Figure 2D–F chronologically in order of appearance and observed their growth rates. The type-1 clones showed slow growth, the type-2 and -3 clones grew normally and the clones with the fusion chromosomes VI and III accompanied by *Leu*⁺ recombination formed colonies earlier and grew normally. The copy numbers of the *leu2d* gene may be insufficient in the type-1 clones but sufficient in the type-2 and -3 clones. It probably takes some time to generate each amplified product, because amplified colonies were formed later than the colonies with fusion chromosomes.

Detailed structural analysis of a type-2 product

To clarify the difference between the type-1 and -2 structures in more detail, *Sall*, *PvuI* and *SmaI* digestions were performed (Figure 3A). The bands with yellow asterisks were considered to be the fragments specific to the amplified products (Figure 3A). Restriction enzyme *Sall* cuts near the 3' end of the *leu2d* gene, *PvuI* cuts within the YF2 region and *SmaI* cuts within the YF4 region. In the amplified regions of the type-2 products, the two *Sall* fragments (5.9 and 8.9 kb named Sa1 and Sa2) were consistent with the expected type-1 structure, suggesting a regular inverted configuration of the *leu2d* genes and a constant length of the YF2 or YF4 regions (Figure 3A and C). However, the *PvuI* and *SmaI* patterns, with three fragments each (Pv1, 10–11 kb; Pv2, 14–15 kb; Pv3, 18–20 kb, and Sm1, 12–13 kb; Sm2, 14–15 kb; Sm3, 16–17 kb), were inconsistent with the type-1 structure, which is expected to produce 14.6 kb of *PvuI* and *SmaI* fragments. If we assume that Pv2 and Sm2 correspond to the expected 14.6 kb fragment, and that the total length of Pv1 and Pv3 (or Sm1 and Sm3) is twice that of Pv2 (or Sm2), the unexpected fragments could be explained by inversions of the YF2 and YF4 intervals. In other words, these results can be explained if the intervals between the *leu2d* genes are present in both orientations.

Figure 3D diagrams a type-1 structure, inverted intervals and restriction maps for four kinds of restriction enzymes. The vertical lines with a, b, ..., k and l indicate restriction cut sites from the type-1 structure, and the lines with a', b', ..., j' and k' indicate those from the inverted intervals. Expected fragment sizes are also indicated. Taking the second region from the left in the *PvuI* map as an example, *PvuI* digestion would produce a 14.6 kb fragment between sites b and c in the type-1 structure, an 18.9 kb fragment between sites b' and c when the YF2 region containing site b is inverted, a 10.4 kb fragment between sites b and c' when the YF2 region containing site c is inverted, or a 14.6 kb fragment between sites b' and c' when both YF2 regions are inverted. Similarly, *SmaI* digestion is expected to produce 12.6, 14.6 and 16.7 kb amplified fragments based on the orientation of the YF4 region. Furthermore, *XhoI* digestion is expected to produce 4.4, 6.4, 8.2 and 10.3 kb amplified fragments, in agreement with the result of Figure 2E. To ascertain whether this model is correct or not, we did dot blot experiments (Figure 3B). Amplified *Sall*, *PvuI* and *SmaI* fragments were extracted and hybridized with various diagnostic probes. The hybridization patterns of the extracted fragments were consistent with the structures we predicted. This indicates that the type-2 structure is characterized by inversion of the YF2 and YF4 regions. Unexpectedly, the Sm2 fragment in Figure 3B did not hybridize to the *leu2d* probe. However, when we reprobated this blot with a ³²P-labeled *leu2d* probe, the Sm2 fragment was detected as expected (data not shown). The relative intensities of the *PvuI* and *SmaI* fragments suggest the frequent inversion of YF2 and YF4 regions. In other words, the type-2 product may be regarded as a structural isomer of the type-1 product. The origin of the type-2 product is presented in Discussion.

Spontaneous gene amplification of the RR structure in the absence of DSB induction

We also analyzed the chromosome structure of three kinds of control survivors (Figure 4A and B). The FF-HO arrangement

Figure 3 Structural analysis of the type-2 product using restriction enzymes and the dot blot technique. **(A)** Restriction enzyme digestion patterns and Southern analysis of genomic DNA from a type-2 clone. *PvuI*- and *SalI*-digested DNA was subjected to 0.8% agarose gel electrophoresis, and *SmaI*-digested DNA was separated by FIGE with a switch time ramp of 0.1–0.8 s (linear shape), 180 cV (forward) and 120 cV (reverse) for 17 h at 20°C. After electrophoresis, the gels were stained with EtBr and subjected to Southern hybridization using *leu2d* as a probe. The bands with yellow asterisks are fragments specific to the amplified products, and are named as indicated to the right. These were gel-extracted for dot blot analysis. We regarded the bands marked with red asterisks as the amplified fragments, although many nonspecific signals were found in the *SalI* digestion. L: LS20; 2: type-2 product. **(B)** Dot blot analysis. The extracted restriction fragments from (A) were spotted on nylon membranes, and hybridized with the various probes indicated above the panels. The predicted structure and size of restriction fragments are indicated to the right of the panels. The same symbols are used as in Figure 2A. Red and blue bars indicate the position of the YF5 and YF6 probes, respectively. **(C)** Schematic representation of a type-1 structure. The restriction sites and the fragment sizes (kb) are shown below the structure. The same symbols are used as in Figures 2A and 3B. **(D)** Schematic representation of our prediction of the type-2 structure. A reference type-1 structure is shown above, with inverted YF2 and YF4 regions shown below. Restriction maps for four kinds of enzyme are illustrated at the bottom. The vertical lines with a, b, ..., k and l indicate restriction cut sites from the type-1 structure, and the lines with a', b', ..., j' and k' indicate those from the inverted regions. The possible fragment sizes, depending on the orientation of the YF2 and YF4 intervals, are also indicated. The same symbols are used as in Figures 2A and 3B.

Figure 4 Structural analysis of survivors from the control strains. (A) Southern analysis of a representative sample of FF-HO, FF and RR Leu^+ survivors with the *leu2d* probe. PFGE was performed as described in Figure 2D. The lanes marked in red and green indicate the samples with type-2-like and type-3-like products, respectively. The lanes marked in gray and black indicate samples with new fusion(s) of chromosomes VI and III accompanied by Leu^+ recombination. See text for more details. L: LS20; Pre: preinduction conditions; IND: induced conditions. (B) Southern analysis of *XhoI*-digested DNA from the samples from (A) with the *leu2d* probe using 0.8% agarose gel electrophoresis. (C) The predicted structure of the type-3-like product, and a model for the mechanism of its production. The symbols are identical to Figure 2A. The predicted DSB site is marked with a red cross. The gray arrows above indicate an inverted structure.

is not expected to initiate DRCC due to the orientation of YF2 and YF4, and the FF and RR arrangements lack both HO sites (Figure 2B). The HO-induced FF-HO or FF survivors had structures that could be explained either by fusion chromosomes as described above (gray or black) (Supplementary Figure 3C and D) or by aberrant structures caused by some unknown rearrangement.

Surprisingly, gel patterns seen in RR survivors were completely different from those of FF-HO and FF survivors, but were quite similar to the gel patterns of RR-HO survivors (Figure 4A and B). Among the four clones analyzed, two (78 and 80) were highly amplified and the other two clones (77 and 79) seem to have type-3-like minichromosomes.

In the former two samples, the original chromosome VI bands disappeared and very dense DNA spots were seen above the separation limit and in the well as in type-2 clones. Several *XhoI* DNA bands were generated, three of which (4.4, 6.4 and 8.2 kb) were also seen in the type-2 clones, although the intensities of these bands varied.

The clones (#77 and 79) showed two strongly hybridizing *XhoI* bands (4.4 and 11–12 kb) (Figure 4B) although these clones have the original size of chromosome VI (Figure 4A), suggesting that these clones have minichromosomes as the type-3 clones. We deduced the type-3-like structure (Figure 4C, bottom) from restriction digestions using *SalI*, *PvuI* and *SmaI* and dot blot hybridization (data not shown). This structure could be explained by a spontaneous DSB that occurred at the YF2 and subsequent intramolecular BIR between the YF2 sequences (Figure 4C).

These analyses strongly suggest that the RR configuration *per se* gives the genome the ability to amplify genes without artificial induction of DSB.

Discussion

Our amplification system yielded two types of highly amplified products. One type (type 2) is a novel chromosomal product in yeast, whose amplified size in some clones accounts for more than 70% of chromosome VI. The other (type 3) is a multicopy linear extrachromosomal molecule.

Interestingly, analogous products were also generated spontaneously at low frequency without HO cleavage. Analysis of these spontaneous products implied that a spontaneous DSB could trigger amplification. Furthermore, our results are consistent with data from many other researchers suggesting that DSB and/or an inverted repeat structure are required for gene amplification (Coquelle *et al*, 1997; Albrecht *et al*, 2000; Singer *et al*, 2000; Tanaka *et al*, 2002).

The explosive chromosomal amplification seen in the type-2 products has not been previously observed in yeast, although extrachromosomal products similar to the type-3 products have been demonstrated. In *S. cerevisiae*, gene amplification has been observed in a few cases. Copper-resistant strains carry chromosomal tandem repeats (≈ 15 copies) of the *CUP1* gene (Fogel and Welch, 1982). Antimycin A-resistant mutants carry repeats of the *ADH4* gene either in the chromosome or as extrachromosomal, linear elements with an inverted structure analogous to the type-3 products found here (Walton *et al*, 1986; Dorsey *et al*, 1992). The *DFR1* gene, corresponding to the higher eukaryotic *DHFR* gene, is amplified as a circular, extrachromosomal element carrying an inverted repeat of the *DFR1* genes (Huang and Campbell, 1995). In *Schizosaccharomyces pombe*, the *sod2* amplicon, conferring Li resistance, is also found as linear, extrachromosomal elements resembling the type-3 product (Albrecht *et al*, 2000).

Figure 5A and B illustrates a proposed model for the amplification mechanisms in the RR-HO strain. The formation of the type-1 product can be easily explained with our

Figure 5 Model describing the amplification process for the three amplification types, and gene amplification models in nature. (A, B) Two pathways are proposed that differ in the initial reaction of the broken ends. Type-1 and -2 products are proposed to result from the two DSBs initiating BIR and thus DRCR (A), while type-3 products are proposed to result from degradation of one broken end and the subsequent intramolecular BIR (B). A detailed description of these models is presented in the text. The same symbols are used as in Figure 2A. (C, D) The DRCR process is proposed to start in higher eukaryotes by replication fork arrest and subsequent BIRs (C), or, alternatively, by the arrangement that several rounds of BFB cycle produce and a subsequent BIR (D), facilitating gene amplification. A detailed description of this model is presented in the text. Two pairs of small arrows (gray and white) indicate two pairs of sequences with homology in (C).

DRCR model as shown in Figure 5A. If strand invasion occurs at two sites simultaneously, DRCR is expected to proceed, leading to the formation of type-1 products (pathway A1). Since the two long stretches bidirectionally elongated by the BIRs have the identical sequence in opposite orientation, they form a giant palindromic structure (see Figure 2H). Such a long palindromic sequence may be unstable because we have not obtained any of these amplification products with more than eight copies of *leu2d*. Our model for the formation of the type-2 product via DRCR is shown in Figure 5A. The DRCR and the following inversion of YF2 and YF4 regions (pathway A2) would form the type-2 products. The mechanism of the inversions is discussed later. Our model for the formation of the type-3 product is shown in Figure 5B. If one broken end is degraded before it invades the homologous region, the exposed *leu2d* gene can initiate an intramolecular BIR, resulting in the formation of the type-3 minichromosome. As described in Results, this minichromosome surely contains an unidentified ARS site, but lacks a centromere. Therefore, the multicopy minichromosome is expected to segregate unequally and thus be unstable.

We think that the DRCR model could provide explanations of gene amplification occurring in tumors and cultured cells. If replication forks from an origin nearby the gene to be amplified are arrested and subsequent DSBs facilitate BIRs

between sequences with homology, as shown in Figure 5C, DRCR can start, generating an amplification product with an inverted array. Alternatively, the structure that is produced after several rounds of BFB cycle, followed by DSB formation, has the potential to promote the formation of an arrangement analogous to the amplification cassette, resulting in DRCR and explosive amplification, as shown in Figure 5D.

We used the *leu2d* gene to select for amplification. In our system, all type-1 clones, which have 5–7 copies of the *leu2d* gene, showed slow growth in the absence of leucine, while the type-2 and -3 amplified clones with more than 13 copies showed nearly normal growth. The copy number of the *leu2d* gene in type-2 products varies from 13 to probably more than 100. Our DRCR model is consistent with these type-2 amplification products, because the amplification is expected to proceed extensively in a very short period of time and terminate randomly, and the final amplification level can far exceed that required for complementation.

Interestingly, results of structural analyses of the type-2 product suggest that the amplified sequences have been rearranged, as shown in Figure 3D. In yeast, repeated sequences generally seem to be very unstable. We propose that the introduction of rearrangement into the repetitive sequence stabilizes repeated structures in the genome. In particular, we propose that the giant palindromic structure formed by DRCR is very unstable. Thus, we presume that it is important for the YF2 and YF4 regions to arrange in random orientations as described in Results to stably maintain a large number of repeated sequences by disrupting the giant palindromic structure. There are two possible pathways for introducing rearrangement of the two YF fragments: (1) after a DRCR event, the giant palindromic structure could induce genomic instability and may consequently lead to random inversion when the length of the palindrome is over a certain level and (2) inversion may occur in coordination with DRCR.

Microscopic observations using FISH technology revealed two distinct types of amplification in cell lines and tumor cells: homogeneously staining region (HSR) and double minutes (DMs) (Schimke, 1982; Hamlin *et al*, 1991; Hahn, 1993; Stark, 1993). HSR involves amplification that occurs within the chromosome, and the products form a repeated array of multiple copies and are relatively stable, while DMs are multicopy extrachromosomal elements containing a few selected genes and are commonly acentric and thus unstable. Although DMs are thought to be circular (Hahn, 1993), this has not been rigorously proved yet. These two types of amplification products are analogous to the type-2 and -3 amplification products in yeast cells in the present work, respectively. A type-2 product containing 100 copies of *leu2d* consists of a 730 kb amplified repeated array, while the rest of chromosome VI comprises 275 kb, and the type-3 product exists as a multicopy (i.e. 10 copies) minichromosome carrying two copies of *leu2d* gene each, as shown in Figure 6 to scale.

The similarity between the amplification products in our system and the HSR and DMs amplification seen in tissue culture suggests that higher eukaryotic amplification occurs via pathways similar to those seen in our yeast amplification system. If this is true, it should be possible to use this yeast amplification arrangement in higher eukaryotes to greatly improve the frequency of amplifications in these systems. Richardson *et al* (1998) indicated that high-frequency BIR

Figure 6 Schematic representation of the two characteristic types of amplification products. Gene amplification in our system formed two characteristic types of products. Gray and red thick lines indicate chromosome VI and amplified regions, respectively. Black arrowheads indicate *leu2d* genes. The type-2 product diagramed here contains 100 copies of *leu2d*, which occupies 730 kb. These type-2 and -3 products resemble HSR and DMs, respectively. The size of the rest of chromosome VI is 275 kb. These illustrations are to scale.

occurred interchromosomally during the repair of an I-SceI-induced DSB on a chromosome in an embryonic stem cell line. These data strongly suggest that the basic recombination systems are present in mammalian cells and utilization of this amplification system in mammalian cells has the potential to provide great savings of time and energy in the overproduction of recombinant proteins for medical uses.

Materials and methods

Strains and plasmids

Yeast strain LS20 was provided by Dr D Butler and used as the parental host strain, whose genotype is *mat, his3, ade2, can1, trp1, ura3, leu2, lys5, cyh2r, ade3::GalHO* (Butler *et al*, 1996). Previously, in order to delete the *MAT* locus, another *leu2* fragment was used to artificially displace the *HO* site (Sandell and Zakian, 1993), and therefore both this and the original *leu2* mutation are located on chromosome III (Supplementary Figure 3A).

Plasmids containing the cassettes for the four LS20 derivative strains, FF-HO, RR-HO, FF and RR, shown in Figure 2B, were constructed as indicated in Supplementary Figures 4 and 5A–C.

GalHO induction

Induction of the *HO* endonuclease gene was carried out as follows. Cells were grown in synthetic complete (SC; Adams *et al*, 1997), –Ura, 2% glucose liquid medium to mid-log phase, harvested by

centrifugation, washed twice with sterile distilled water and plated at various dilutions onto SC, 2% galactose medium without uracil, leucine or both. Cells were also plated onto SC, –Ura, 2% glucose medium to measure the number of viable cells. Cells were grown at 25°C. Colonies on galactose plates were counted after 4 or 5 days of growth.

PFGE and Southern analysis

Cells were embedded in agarose plugs as previously described (Smith *et al*, 1988). The gel plugs were then treated with 5 mg/ml proteinase K at 30°C for 2 days and washed twice with TE buffer containing 0.1 mM PMSF, twice with TE buffer and once with 1 × restriction enzyme buffer.

PFGE and FICE were carried out in 1% agarose gels with 0.5 × TBE buffer, using CHEF Mapper XA (Bio-Rad) and FICE Mapper (Bio-Rad) as described in the figure legends. DNA samples digested with restriction enzymes were separated using conventional gel electrophoresis in 0.8 or 1.0% agarose gels in 1 × TAE buffer at 0.9 V/cm for 16 h.

Southern blot hybridization was performed according to Sambrook *et al* (1989), using Hybond-N⁺ membrane (Amersham Bioscience). Hybridization was performed with fluorescein-labeled probes. Fluorescein-labeled *leu2d* and *RET2* probes were prepared using the Gene Images random-prime labeling module (Amersham Bioscience) and detected using the Gene Images CDP-Star detection module (Amersham Bioscience) and a luminescent image analyzer (LAS1000plus, Fujifilm).

Dot blot analysis

PvuI and *SalI* fragments from type-2 or -3 products were subjected to 0.8% agarose gel electrophoresis and *SmaI* fragments from type-2 or -3 products were separated by FICE as described in the figure legends. Bands in the gel were extracted with a QIAquick Gel Extraction Kit (QIAGEN). They were spotted on Hybond-N⁺ membranes and hybridized with various fluorescein-labeled probes. The probes for TEL and ARS were amplified by PCR using pSI as the template (primers, AGAGTTTCTT-TCTTGAGGG and AGTGGTGAATCCGTTAGCGA for TEL, and TTTACTTGACG-ACTT GAGGC and AGCGGAGGTGTGGAGACAAA for ARS). The plasmid pSI was constructed by disrupting one of the two *EcoRI* sites on the plasmid pNo42IR (Butler *et al*, 1996), which was kindly provided by Dr DK Butler. The *leu2d*, YF2, YF4, YF5 and YF6 PCR products described above were used as the probes. The labeling, detection and image analysis were performed as above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- Adams A, Gottschling DE, Kaiser CA, Stearns T (1997) *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Albrecht EB, Hunyady AB, Stark GR, Patterson TE (2000) Mechanisms of *sod2* gene amplification in *Schizosaccharomyces pombe*. *Mol Biol Cell* **11**: 873–886
- Alt FW, Kellems RE, Bertino JR, Schimke RT (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* **253**: 1357–1370
- Asai T, Bates DB, Kogoma T (1994) DNA replication triggered by double-stranded breaks in *E. coli*: dependence on homologous recombination functions. *Cell* **78**: 1051–1061
- Bishop JM (1987) The molecular genetics of cancer. *Science* **235**: 305–311
- Butler DK, Yasuda LE, Yao MC (1996) Induction of large DNA palindrome formation in yeast: implications for gene amplification and genome stability in eukaryotes. *Cell* **87**: 1115–1122
- Coquelle A, Pipiras E, Toledo F, Buttin G, Debatisse M (1997) Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. *Cell* **89**: 215–225
- Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide-resistant alfalfa cells: an example of gene amplification in plants. *J Mol Appl Genet* **2**: 621–635

- Dorsey M, Peterson C, Bray K, Paquin CE (1992) Spontaneous amplification of the *ADH4* gene in *Saccharomyces cerevisiae*. *Genetics* **132**: 943–950
- Dunham MJ, Badrane H, Ferea T, Adams J, Brown PO, Rosenzweig F, Botstein D (2002) Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **99**: 16144–16149
- Erhart E, Hollenberg CP (1983) The presence of a defective *LEU2* gene on 2 μ DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number. *J Bacteriol* **156**: 625–635
- Field LM, Devonshire AL, Forde BG (1988) Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem J* **251**: 309–312
- Fogel S, Welch JW (1982) Tandem gene amplification mediates copper resistance in yeast. *Proc Natl Acad Sci USA* **79**: 5342–5346
- Futcher AB (1986) Copy number amplification of the 2 micron circle plasmid of *Saccharomyces cerevisiae*. *J Theor Biol* **119**: 197–204
- Hahn PJ (1993) Molecular biology of double-minute chromosomes. *BioEssays* **15**: 477–484
- Hamlin JL, Leu TH, Vaughn JP, Ma C, Dijkwel PA (1991) Amplification of DNA sequences in mammalian cells. *Prog Nucleic Acid Res Mol Biol* **41**: 203–239
- Horiuchi T, Fujimura Y (1995) Recombinational rescue of the stalled DNA replication fork: a model based on analysis of an *Escherichia coli* strain with a chromosome region difficult to replicate. *J Bacteriol* **177**: 783–791
- Huang T, Campbell JL (1995) Amplification of a circular episome carrying an inverted repeat of the *DFR1* locus and adjacent autonomously replicating sequence element of *Saccharomyces cerevisiae*. *J Biol Chem* **270**: 9607–9614
- Hyrien O, Debatisse M, Buttin G, de Saint Vincent BR (1988) The multicopy appearance of a large inverted duplication and the sequence at the inversion joint suggest a new model for gene amplification. *EMBO J* **7**: 407–417
- Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**: 1078–1083
- Kimura M, Ota T (1974) On some principles governing molecular evolution. *Proc Natl Acad Sci USA* **71**: 2848–2852
- Kobayashi T, Heck DJ, Nomura M, Horiuchi T (1998) Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. *Genes Dev* **12**: 3821–3830
- Kobayashi T, Horiuchi T, Tongaonkar P, Vu L, Nomura M (2004) *SIR2* regulates recombination between different rDNA repeats, but not recombination within individual rRNA genes in yeast. *Cell* **117**: 441–453
- Kodama K, Kobayashi T, Niki H, Hiraga S, Oshima T, Mori H, Horiuchi T (2002) Amplification of Hot DNA segments in *Escherichia coli*. *Mol Microbiol* **45**: 1575–1588
- Kostriken R, Strathern JN, Klar AJ, Hicks JB, Heffron F (1983) A site-specific endonuclease essential for mating-type switching in *Saccharomyces cerevisiae*. *Cell* **35**: 167–174
- Kraus E, Leung WY, Haber JE (2001) Break-induced replication: a review and an example in budding yeast. *Proc Natl Acad Sci USA* **98**: 8255–8262
- Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* **396**: 643–649
- Ma C, Martin S, Trask B, Hamlin JL (1993) Sister chromatid fusion initiates amplification of the dihydrofolate reductase gene in Chinese hamster cells. *Genes Dev* **7**: 605–620
- McClintock B (1951) Chromosome organization and genic expression. *Cold Spring Harbor Symp Quant Biol* **16**: 13–47
- Morrow DM, Connelly C, Hieter P (1997) 'Break copy' duplication: a model for chromosome fragment formation in *Saccharomyces cerevisiae*. *Genetics* **147**: 371–382
- Mouches C, Pasteur N, Berge JB, Hyrien O, Raymond M, de Saint Vincent BR, de Silvestri M, Georgiou GP (1986) Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex mosquito*. *Science* **233**: 778–780
- Petes TD (1979) Yeast ribosomal DNA genes are located on chromosome XII. *Proc Natl Acad Sci USA* **76**: 410–414
- Richardson C, Moynahan ME, Jasini M (1998) Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev* **12**: 3831–3842
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Sandell LL, Zakian VA (1993) Loss of a yeast telomere: arrest, recovery and chromosome loss. *Cell* **75**: 729–739
- Schimke RT (1982) Studies on gene duplication and amplification, A historical perspective. In *Gene Amplification*, Schimke RT (ed) pp 1–6. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
- Schwab M (1998) Amplification of oncogenes in human cancer cells. *BioEssays* **20**: 473–479
- Singer MJ, Mesner LD, Friedman CL, Trask BJ, Hamlin JL (2000) Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. *Proc Natl Acad Sci USA* **97**: 7921–7926
- Slechta ES, Bunney KL, Kugelberg E, Kofoed E, Andersson DI, Roth JR (2003) Adaptive mutation: general mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*. *Proc Natl Acad Sci USA* **100**: 12847–12852
- Smith CL, Klc SR, Cantor CR (1988) Pulse-field gel electrophoresis and the technology of large DNA molecules. In *Genome Analysis*, Dacis KE (ed) pp 41–112. Oxford, UK: IRL Press
- Smith KA, Stark MB, Gorman PA, Stark GR (1992) Fusions near telomeres occur very early in the amplification of CAD genes in Syrian hamster cells. *Proc Natl Acad Sci USA* **89**: 5427–5431
- Stark GR (1993) Regulation and mechanisms of mammalian gene amplification. *Adv Cancer Res* **61**: 87–113
- Stark GR, Wahl GW (1984) Gene amplification. *Annu Rev Biochem* **53**: 447–491
- Tanaka H, Tapscott SJ, Trask BJ, Yao MC (2002) Short inverted repeats initiate gene amplification through the formation of a large DNA palindrome in mammalian cells. *Proc Natl Acad Sci USA* **99**: 8772–8777
- Tartof KD (1974) Unequal mitotic sister chromatid exchange as the mechanism of ribosomal RNA gene magnification. *Proc Natl Acad Sci USA* **71**: 1272–1276
- Volkert FC, Broach JR (1986) Site-specific recombination promotes plasmid amplification in yeast. *Cell* **46**: 541–550
- Wahl GM, Padgett RA, Stark GR (1979) Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphonacetyl)-L-aspartate-resistant hamster cells. *J Biol Chem* **254**: 8679–8689
- Walton JD, Paquin CE, Kaneko K, Williamson VM (1986) Resistance to antimycin A in yeast by amplification of *ADH4* on a linear, 42 kb palindromic plasmid. *Cell* **46**: 857–863
- Windle BE, Wahl GM (1992) Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutat Res* **276**: 199–224