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The Pattern of Gene Amplification Is Determined by the Chromosomal Location of Hairpin-Capped Breaks

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SUMMARY

DNA palindromes often colocalize in cancer cells with chromosomal regions that are predisposed to gene amplification. The molecular mechanisms by which palindromes can cause gene amplification are largely unknown. Using yeast as a model system, we found that hairpin-capped double-strand breaks (DSBs) occurring at the location of human *Alu*-quasipalindromes lead to the formation of intrachromosomal amplicons with large inverted repeats (equivalent to homogeneously staining regions in mammalian chromosomes) or extrachromosomal palindromic molecules (equivalent to double minutes [DM] in mammalian cells). We demonstrate that the specific outcomes of gene amplification depend on the applied selection, the nature of the break, and the chromosomal location of the amplified gene relative to the site of the hairpin-capped DSB. The rules for the palindrome-dependent pathway of gene amplification defined in yeast may operate during the formation of amplicons in human tumors.

INTRODUCTION

Amplification of chromosomal regions plays an important role in tumor pathogenesis. Increase in copy number of oncogenes can promote initiation and progression of a variety of solid tumors, while amplification of genes that modify or detoxify drugs can cause resistance to chemotherapeutic agents (Albertson et al., 2003; Fletcher, 2005; Naeem, 2005). Two types of amplification events have been detected cytogenetically: extra- and intrachromosomal amplicons (Debatisse and Malfor, 2005; Stark et al., 1989; Windle and Wahl, 1992). Extrachromosomal amplicons or double minutes (DM) have up to several hundred copies of a genomic segment and form mini-chromosomes with inverted symmetry. Intrachromosomal amplicons, also described as abnormally banded or homogeneously staining regions (HSR), are head-to-tail or tail-to-tail tandem repeats, which have ten or fewer copies at early stages of amplification. Some gene amplifications are accompanied by aneuploidy, deletions, or translocations (Albertson et al., 2003; Fletcher, 2005; Naeem, 2005).

Current models propose that DSBs initiate the process of gene amplification. In yeast and mammalian cells, DSBs induced with I-*Sce*I or HO endonucleases increase the frequency of extrachromosomal and intrachromosomal amplification (Coquelle et al., 2002; Pipiras et al., 1998; Watanabe and Horiuchi, 2005). Amplification is also triggered by DNA-damaging agents, which can directly or indirectly cause DSBs (Kuo et al., 1994; Paulson et al., 1998; Poupon et al., 1996; Yunis et al., 1987). In addition, in human and rodent cells, chromosomal regions containing fragile sites, which are natural hot spots for breakage and recombination, are highly susceptible to amplification and are found to frame the early amplicons (reviewed in Debatisse and Malfor, 2005) DSBs can trigger gene amplification through a variety of mechanisms, including unequal sister-chromatid exchange, rolling-circle replication, break-induced replication, foldback priming, and the breakage-fusion-bridge (BFB) cycle (Kobayashi et al., 2004; Kraus et al., 2001; McClintock, 1941; Rattray et al., 2005; Watanabe and Horiuchi, 2005).

The BFB cycle (McClintock, 1941) is the most popular model to explain intrachromosomal amplification. In cancer cells, HSRs are often organized as an inverted ladder associated with a deletion that spans from the amplicon toward a telomere (Debatisse and Malfor, 2005). According to the BFB model, such a complex rearrangement results from the following repeating cycle: an initial DSB; replication of the broken molecule; fusion of sister chromatids; formation of a bridge during anaphase; and asymmetrical breakage due to mechanical tension, which generates one chromatid with an inverted repeat at the broken end. Although the fusion of broken chromatids has not yet been directly demonstrated, this step seems likely because mammalian cells have a robust nonhomologous end joining machinery. Anaphase bridges are often observed in cells undergoing gene amplification, implicating

BFB cycles during this process (Coquelle et al., 1997; Ma et al., 1993; Shimizu et al., 2005; Toledo et al., 1993). In addition, end-to-end chromosome fusions are well documented in cells with telomere dysfunction due to the recognition of unprotected chromosome ends as DSBs (Chan and Blackburn, 2004).

DNA palindromes are abundant in human cancer cells and often colocalize with the chromosomal regions that are predisposed to gene amplification (Tanaka et al., 2005). Palindromic sequences are implicated in early steps of gene amplification and are hot spots for other types of gross chromosomal rearrangements (GCRs) in many organisms (Fried et al., 1991; Zhou et al., 2001 and references therein). However, the molecular mechanisms by which palindromes cause GCRs in eukaryotic genomes are poorly understood. Previous studies in *Saccharomyces cerevisiae* showed that a quasipalindrome comprised of two human *Alu* repeats induces DSBs terminated by covalently closed hairpins. Unprocessed DSBs lead to the formation of acentric and dicentric rearranged molecules characterized by inverted symmetry (Lobachev et al., 2002). In the present study, we investigated the fate of these rearranged intermediates and determined how they can be directed into chromosomal aberrations. We have found that chromosomal arm loss and extrachromosomal and intrachromosomal gene amplification events are different consequences of the hairpin-capped breaks. Using the yeast model system, we define a novel, palindrome-dependent pathway of gene amplification that can mimic the formation of oncogene amplicons in humans.

RESULTS

Experimental System

To characterize the GCRs that result from secondary structure-mediated DSBs, we developed an experimental system based on the loss of the *CAN1* and *ADE2* genes and the amplification of *CUP1* and *SFA1* genes located on chromosome V (Figure 1). Two sets of haploid yeast strains were constructed where the left arm of chromosome V in the region of the *CAN1* gene was modified. A *LYS2* cassette containing homologous and homeologous inverted or direct *Alu* repeats was placed centromere-proximal to *CAN1*, such that the region between *LYS2* and the telomere does not contain essential genes and can be deleted. The *Alu* repeats are 320 bp long and separated by a 12 bp spacer. One hundred percent, ninety four percent, and eighty six percent identical inverted *Alu* repeats (*Alu*-IR) were used as a source of hairpin-capped breaks (Lobachev et al., 2000, 2002). As a control, 100% identical direct *Alu* repeats that cannot form a secondary structure were inserted at the same chromosomal location. The *ADE2* gene was moved telomere-distal to *CAN1*, while *CUP1* and *SFA1* genes were positioned either telomere-proximal (Figure 1, TP strains) or telomere-distal (Figure 1, TD strains) to *CAN1.*

This system allows for selection of two types of GCRs. First, a hairpin-capped break can cause deletion of the chromosome V region, including *CAN1* and *ADE2*, resulting in canavanine-resistant red colonies ($Can^R Ade^-$). Second, breakage at the location of the inverted repeat can cause amplification of the telomere-proximal or telomere-distal regions, including *CUP1* (encoding copper chelatin) and *SFA1* (encoding formaldehyde dehydrogenase) genes that serve as convenient gene dosage markers (Resnick et al., 1990; van den Berg and Steensma, 1997). Clones carrying amplified *CUP1* are initially selected on medium containing a high concentration of copper, and copper-resistant colonies (Cu^R) are replica plated onto media with a high concentration of formaldehyde, which selects for amplification of *SFA1*.

Inverted *Alu*s Strongly Induce Chromosome Arm Loss Characterized By Terminal Deletion and Adjacent Inverted Duplication

The rates of GCRs were scored in strains carrying homologous or homeologous *Alu*-IRs and in a control strain with direct repeated *Alu* sequences. The rate of *CAN1* region loss was nearly 25,000-fold higher in strains containing 100% homologous inverted repeats than in control strains with direct repeats (see Table S1 in the Supplemental Data available with this article online). The majority of $Can^R Ade^-$ colonies were small in size (Figure 2A). Forty to sixty percent of the cells in these colonies were "large budded," suggesting ongoing DNA damage in these isolates (Figure 2B). These colonies, when subcultured, gave rise to both small and normal colonies (Figure 2C). For small-sized colonies, ethidium bromide-stained CHEF gels showed that chromosome V was undetectable or had diminished intensity (data not shown), but multiple bands of different sizes (Figure 2D; I-S1 and I-S2 isolates) were revealed by hybridization with the chromosome V-specific probe. In each of the derived normal-sized colonies, a discrete band was detected (Figure 2D; I-1 to I-12 isolates). The cells in these colonies were morphologically normal (data not shown). These results suggest that arm-loss events lead to the generation of a mixed population of cells carrying unhealed broken chromosome Vs. These broken chromosomes are detected by DNA-damage checkpoints, resulting in the characteristic G2/M arrest phenotype. In the small colonies, the lack of a single species of healed chromosome also results in the absence of a discrete chromosome V band. Subsequent repair of the broken molecules leads to the formation of rearranged chromosomes (as described below) and recovery from the arrest, resulting in normal-sized colonies.

Most of the rearranged chromosome Vs in $Can^R Ade^-$ normal-sized isolates were equal to or larger than the unrearranged chromosome V. This result suggests that the loss of the 42 kb telomeric region adjacent to the DSB site was accompanied by a gain of genetic material from elsewhere (right panel in Figure 2D). To determine the structure of GCRs in these strains, we examined genomic DNA from 18 independent $Can^R Ade^-$ isolates using

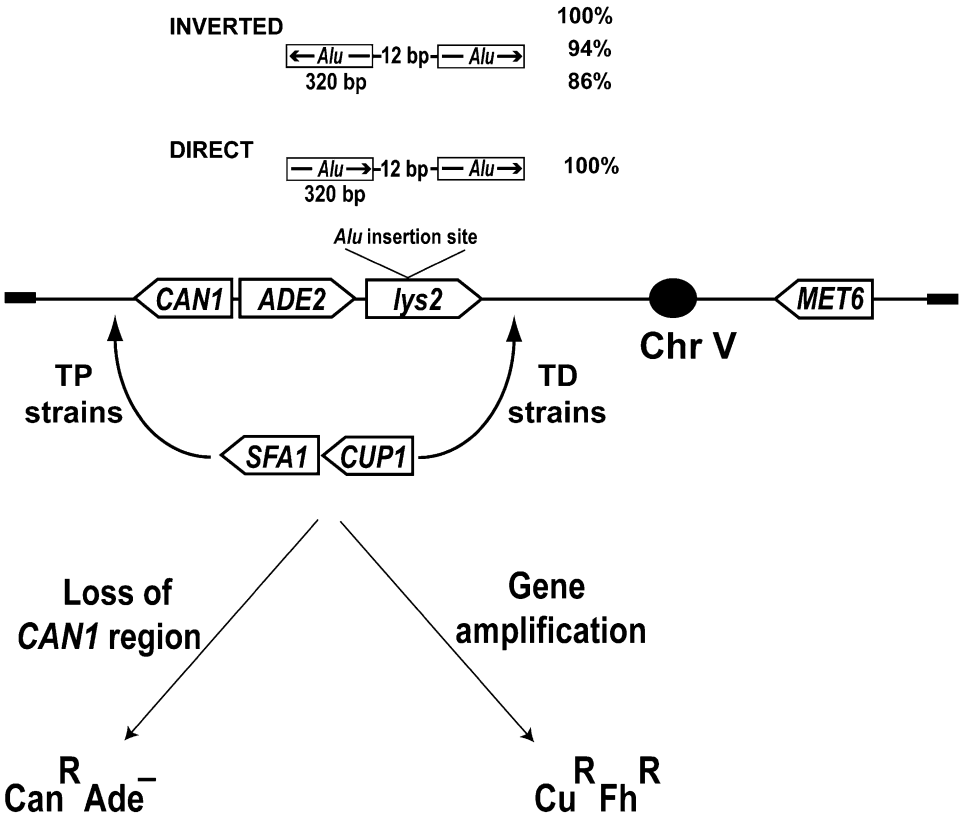

Figure 1. Experimental Systems to Study GCRs Resulting from Hairpin-Capped DSBs

TP and TD strains were used to detect deletions or amplifications. Selection for canavanine-resistant strains that are also Ade$^-$ results in isolates with a 42 kb telomeric deletion. Selection for copper- and formaldehyde-resistant derivatives (Cu^RFh^R) results in either extrachromosomal amplifications (in TP strains) or intrachromosomal amplifications (in TD strains) of the regions adjacent to the *Alu* repeats.

comparative genomic hybridization (CGH) on microarrays (Figure 2E). Based on the CGH and CHEF data, four classes of GCRs were identified. Class I (I-9) had a chromosome V that was about 40 kb smaller than the wild-type (Figure 2D) and had a terminal deletion of V with a breakpoint near the *CAN1* locus (Figure 2E). This pattern can result from a DSB near the *Alu*-IR, followed by resection of the broken end and de novo addition of telomeric sequences. The initiating DSB for I-9 and all of the other rearrangements is likely to reflect processing of an extruded cruciform containing the *Alu* repeats (Figure 3).

In class II isolates (I-4, I-7, I-10, I-11, I-13, I-14, I-18) chromosome V had a deletion of the region of centromere-distal to *CAN1* and a duplication of about 30 kb located centromere-proximal to *CAN1* (Figure 2E). In six of the seven class II isolates, chromosome V was slightly smaller than the wild-type V, suggesting that the duplication of 30 kb is within chromosome V, since the duplication of 30 kb compensates for the deletion of 40 kb. For six of the seven class II isolates, the duplication breakpoint is within a region of about 1 kb containing *YELWdelta1*, *YELWdelta2*, and *PAU2* (a repetitive sequence found near most telomeres; Rachidi et al., 2000). As in class I, the likely initiating event for the class II isolates is a DSB at the extruded *Alu* cruciform, followed by replication of the resulting hairpin-capped molecule, generating a dicentric chromosome (Figure 3). The frequent involvement of the repetitive elements in the resulting rearrangement in the class II isolates as well as in classes III and IV (see below) might indicate that the dicentric breaks preferentially near the region of the chromosome with the delta elements and *PAU2*. A more likely alternative, however, is that the position of the anaphase break is random, but the subsequent resection of the DSB end continues until repetitive sequences are exposed. The resulting broken end is either capped by de novo telomere addition or repaired by break-induced replication (BIR) (Malkova et al., 1996) utilizing *PAU2*-related or delta-related repeats located near the telomeres of other chromosomes (Figure 3). It is interesting to note that GCRs in other studies frequently involve repetitive elements such as delta elements (Lemoine et al., 2005; Putnam et al., 2005; Umezu et al., 2002).

In most of the class III isolates (I-1, I-2, I-6, I-8, I-12, I-15, I-17), there was a 30 kb duplication adjacent to the deletion, as for class II isolates. In addition, a region of variable

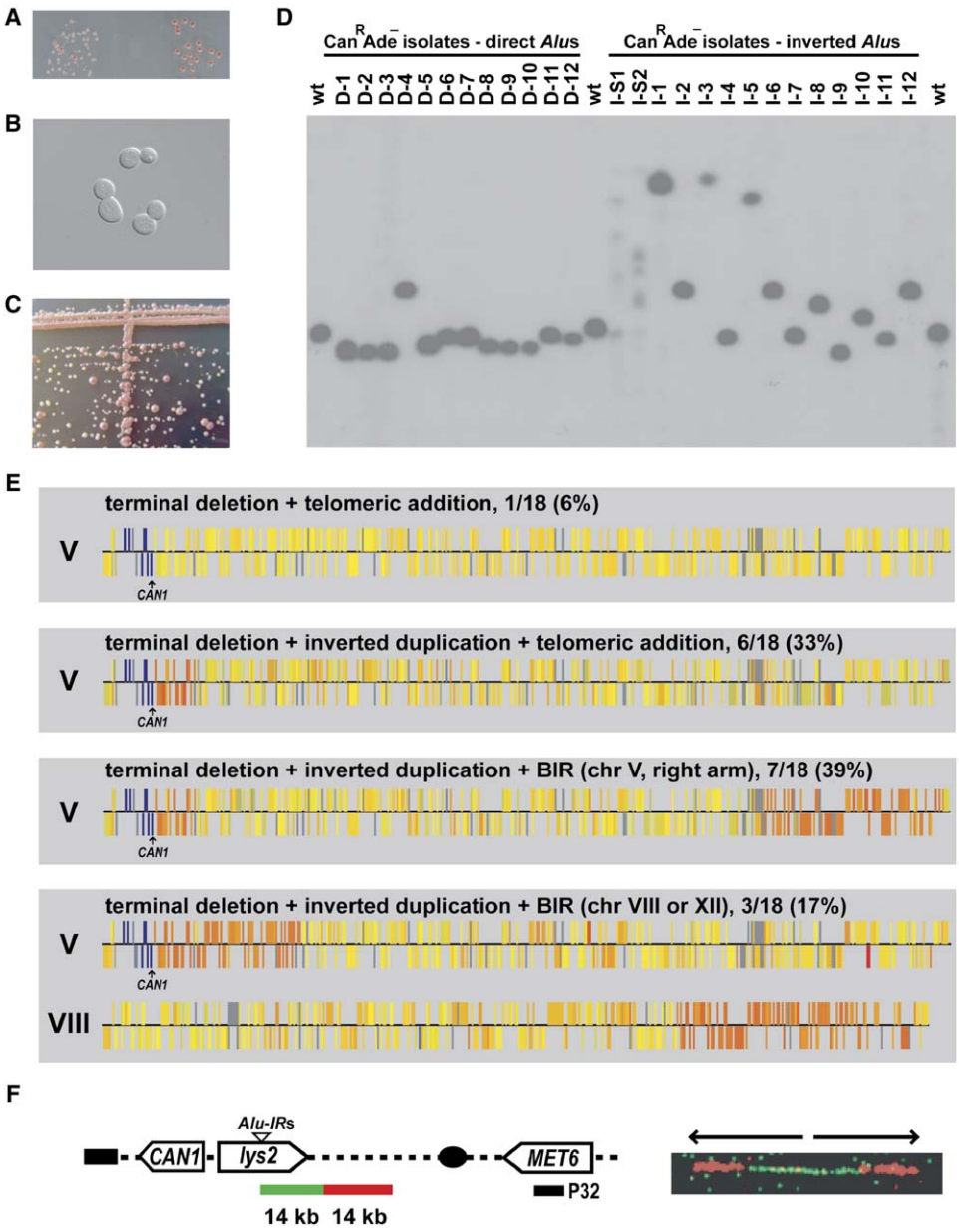

Figure 2. Analysis of Arm-Loss Events Triggered by Hairpin-Capped DSBs

(A) Small-sized colonies of primary Can^{R}Ade$^-$ isolates from TP strains. Can^{R}Ade$^-$ colonies arising from the TP strain (left side of the plate) were grown adjacent to colonies from the isogenic strain with point mutations in the *CAN1* gene (right side of the plate).

(B) Cells of the primary Can^{R}Ade$^-$ isolates are often arrested with large buds, characteristic of a DNA-damage checkpoint response.

(C) The primary Can^{R}Ade$^-$ isolates give rise to a heterogeneous population of small- and normal-size colonies.

(D) Analysis of rearranged chromosome Vs in Can^{R}Ade$^-$ isolates by CHEF and Southern blotting. Lanes D-1 to D-12 are Can^{R}Ade$^-$ isolates from TP strains with direct *Alu*s. Lanes I-S1 and I- S2 are small-size primary Can^{R}Ade$^-$ isolates, and lanes I-1 to I-12 are normal-sized Can^{R}Ade$^-$ isolates from TP strains with inverted *Alu*s. Lanes labeled with "wt" are TP strains with the wild-type chromosome V. Chromosome V was detected using a *MET6*-specific probe.

(E) Microarray analysis of GCRs stimulated by hairpin-capped DSBs. The DNA microarrays contained almost all yeast genomic ORFs. Color coding is as follows: gray, absent on the array; yellow, single-copy sequences; red, repeated sequences; blue, deletions. Only those chromosomes that had a deletion or duplication are shown in this figure. Complete data for these experiments is online at https://genome.unc.edu/cgi-bin/SMD/publication/viewPublication.pl?pub_no=47. From the top panel to the bottom, the classes of GCRs (number of isolate in parentheses) are as follows: class I (I-9), class II (I-7), class III (I-12), and class IV (I-16).

(F) The duplicated regions adjacent to terminal deletions are organized as inverted repeats. The left panel shows the regions on chromosome V that are fluorescently-labeled for FISH, and ^{32}-P-labeled for Southern analysis. The right panel is an example of an inverted duplication visualized by molecular combing (isolate I-8). Arrowheads above the panel depict the repeat units in the amplicon.

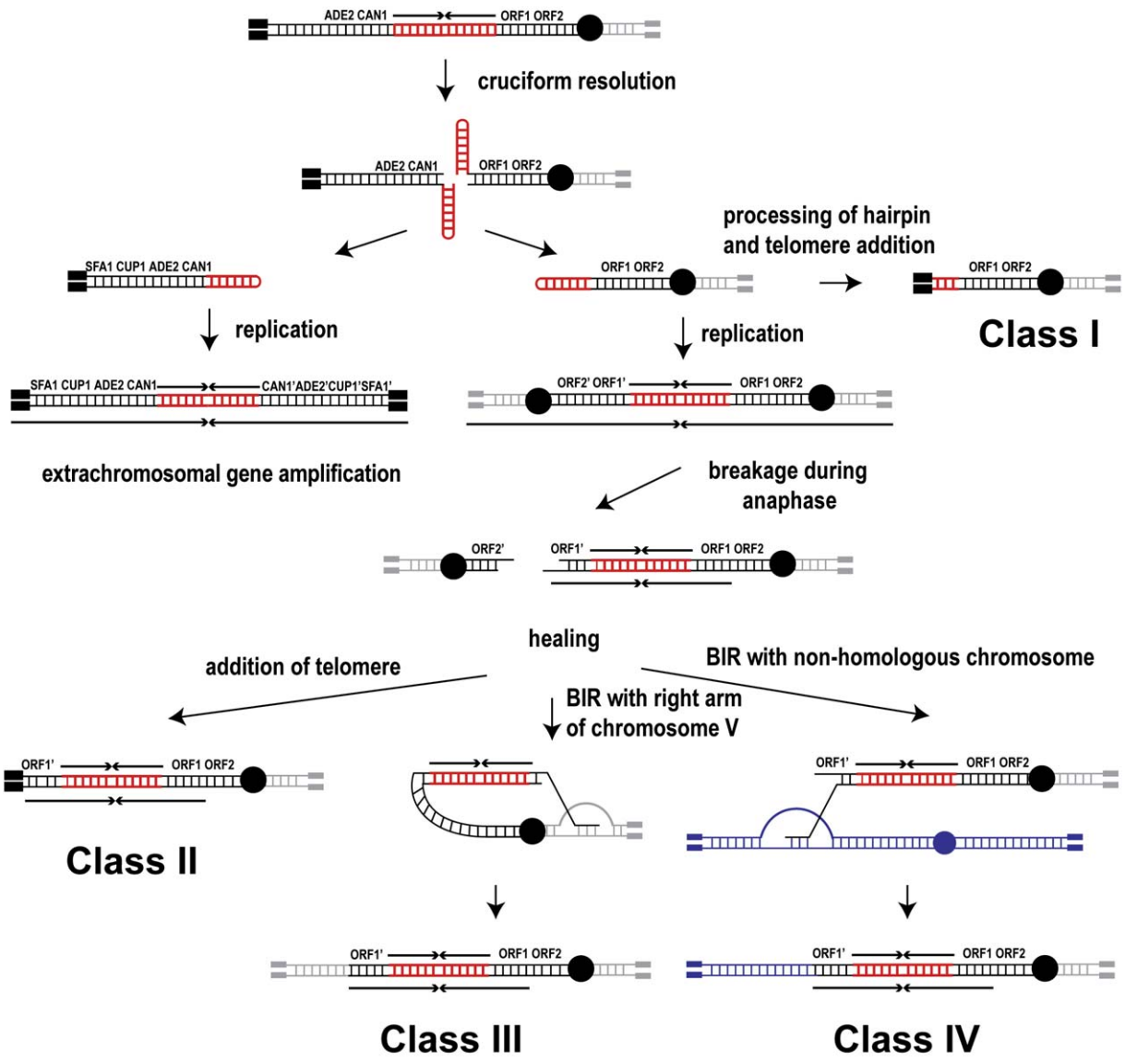

Figure 3. Model for Chromosomal Rearrangements Triggered by Hairpin-Capped DSBs

The *Alu*-IRs are depicted (not to scale) as red color. Telomeres (filled rectangles) and centromeres (filled circles) are also shown. Right arm of chromosome V is represented by the color gray. A nonhomologous chromosome is indicated by the color blue. A detailed description is presented in the text. Classes I–IV refer to the various classes of chromosomes with a deletion of *CAN1*, as described in the text. Intrachromosomal amplification is not shown in this figure but is presented in Figure 7.

size on the right arm of chromosome V was duplicated. In the isolate shown in Figure 2E (I-12), the duplicated region was about 130 kb, with a breakpoint near *YERCTy1-1*; three other class III isolates (I-6, I-8, and I-17) shared this breakpoint. In two other isolates (I-2 and I-15), the duplicated region was at *YERCTy1-2*. Thus, one simple way of explaining most of the class III isolates is that the delta elements near the breakpoint of the 30 kb duplication (*YELWdelta1* and *YELWdelta2*) initiate a BIR event with a delta or Ty element located on the right arm of chromosome V (Figure 3).

In class IV isolates, a deletion of the sequences centromere-distal to *CAN1* is adjacent to a duplication of 30 kb (I-3) or 100 kb (I-5 and I-16); both of these breakpoints are near delta elements (*YELdelta1,2* and *YELdelta4-6*, respectively). In addition, in these isolates, duplications of sequences derived from another chromosome were observed: chromosome XII in I-3 and I-5 and chromosome VIII in I-16 (shown in Figure 2E). The breakpoints of the duplications on the other chromosomes are near delta elements (delta elements 9-12 on chromosome XII for I-3, delta elements 18 and 19 for I-5, and delta elements 10

and 11 for I-16). The simplest explanation of these strains is that the delta elements at the breakpoints of chromosome V were involved in a BIR event using delta elements on another chromosome.

PCR was used to confirm the structure of GCRs in one class III isolate (I-6) and two class IV isolates (I-3 and I-16) (Figures S1A and S1B). CHEF gel analysis and Southern blots were also carried out for the class IV isolates using probes for *MET6* and the translocation donor chromosome (Figure S1C). Previous studies demonstrated BIR events involving very large duplications (Malkova et al., 1996) and nonreciprocal translocations mediated by transposable elements (Lemoine et al., 2005).

The model shown in Figure 3 predicts that the intrachromosomal duplication at the left end of chromosome V in class II, III, and IV isolates will form a large (30–100 kb) quasipalindrome with the inverted *Alu* repeats in the middle. This prediction was confirmed in two ways. First, DNA fiber fluorescent in situ hybridization (FISH) and molecular combing (Conti et al., 2001) were used to examine the DNA of one class II isolate (I-7), three class III isolates (I-6, I-8, and I-12), and two class IV isolates (I-5 and I-10). Two 14 kb chromosomal DNA regions centromere-proximal to the *Alu* repeats (positions shown in Figure 2F) were PCR amplified and used as probes for this analysis. One fragment was labeled with biotin-tagged dUTP and the other with digoxigenin-tagged dUTP. Chromosomal DNA from each strain was stretched on siliconized cover slips and hybridized to the labeled probes, and the probes were detected with green and red fluorescent-conjugated antibodies to biotin or digoxigenin, respectively. Each isolate had a palindromic pattern (shown in Figure 2F for I-8), in which a green region was flanked by two red regions approximately half the size of the green region. The structure was also confirmed by Southern analysis (Figure S2).

In summary, these results show that hairpin-capped breaks primarily induce GCRs that have a very specific pattern: terminal deletion coupled with an adjacent inverted duplication (large quasipalindromes). This pattern of chromosomal rearrangements is different from GCR events that result from spontaneous or damage-induced breaks analyzed in other yeast studies where *CAN1* was used as a reporter (Myung and Kolodner, 2003; Putnam et al., 2005 and references therein).

Chromosome Rearrangements in Strains with Direct *Alu* Repeats

The GCRs described above were compared with GCRs in 12 $Can^{R}Ade^{-}$ clones isolated from a control strain with direct *Alu* repeats. Chromosome V structure was determined in these 12 clones by CHEF gel analysis, Southern blot hybridization (Figure 2D), CGH (Figure S3), and DNA combing (Figure S3). This analysis identified four classes of rearrangements involving the left arm of chromosome V. In contrast to the GCR isolates from strains with inverted *Alu*s, most (8 of 12) of the isolates obtained from strains with directly repeated *Alu*s had terminal deletions of the left arm of chromosome V (including the *CAN1* region) with no associated duplication (top panel in Figure S3A). In such isolates (D-1, D-2, D-3, D-5, D-8, D-9, D-10, D-12), it is likely that the deleted chromosome is capped by a telomere. One of the isolates (D-7) had a 20 kb interstitial deletion (second panel from the top in Figure S3A). The remaining three isolates resemble class II and class III isolates from strains with the inverted *Alu* repeats. D-6 and D-11 had a duplication of about 30 kb adjacent to a terminal deletion of about 40 kb (third panel from top in Figure S3A) and D-4 had a terminal deletion, an adjacent duplication of 30 kb, and a duplication of about 130 kb from the right arm of chromosome V near *YERCTy1-1*. Molecular combing showed that the 30 kb duplications in D-4, D-6, and D-11 were inverted repeats (D-6 in Figure S3B).

How are inverted repeats generated from yeast strains with direct *Alu* repeats? It is possible that a spontaneous DSB centromere-proximal to *CAN1* triggers the resection of the broken end. The processed single-strand end can fold back on itself (utilizing very short inverted repeats), priming DNA synthesis to form a dicentric in an intramolecular reaction (Rattray et al., 2005). It should be stressed that large inverted duplications are approximately 10^{5}-fold more frequent in strains with inverted *Alu* repeats than with direct *Alu* repeats. Table S2 summarizes the data on the $Can^{R}Ade^{-}$ isolates derived from strains with the direct and inverted *Alu* repeats.

Hairpin-Capped Breaks Trigger a Palindrome-Dependent Recurring Chromosome Instability

Since hairpin-capped breaks at *Alu*-IRs often generate GCRs with 30 or 100 kb quasipalindromes, we hypothesized that these large quasipalindromes could also cause hairpin-capped DSBs that would trigger a new round of chromosome V rearrangements. We observed previously that one phenotypic manifestation of hairpin-capped DSBs is the occurrence of small colonies containing a high percentage of large-budded cells. Thus, we used these criteria to follow the fate of the individual palindromes by isolating on nonselective medium-small, non-*petite*, colonies in the progeny of $Can^{R}Ade^{-}$ clones. Depending on the GCR isolate, the frequency of the occurrence of the small colonies varied from 1% to 10%. The structure of chromosome V in normal-sized colonies derived from small colonies was assessed by CHEF analysis, Southern blot with *MET6*, and CGH (Figure 4). The results showed that chromosome V containing a 30 kb quasipalindrome with added telomeric sequences at the end can give rise to differently-sized chromosomes that have either changed the length of the quasipalindromic sequence (35 or 100 kb) and/or acquired telomeres via BIR. The derivative chromosome V containing a 100 kb quasipalindrome was also unstable, producing a next generation of chromosomes with changes in size and structure (Figure 4). These results show that hairpin-capped breaks cause ongoing chromosome instability.

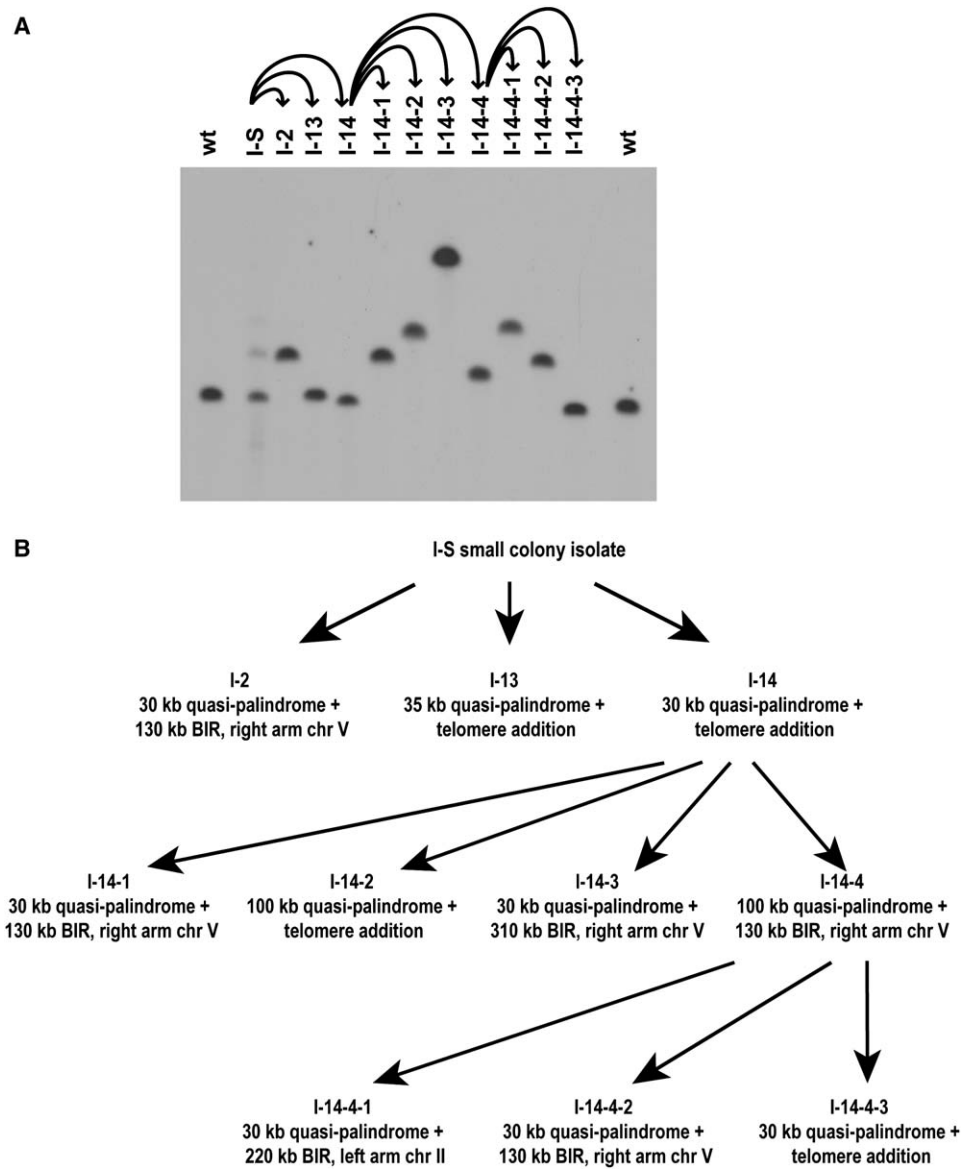

Figure 4. Recurring Instability of Chromosomes with Large Quasipalindromes

(A) Analysis of chromosome V rearrangements by CHEF and Southern blotting. The DNA samples are derived from the following strains: lane I-S (small colony of a primary $Can^R Ade^-$ isolate); lanes I-2, I-13, and I-14 (normal-sized colonies derived from I-S); lanes I-14-1 to I-14-3 (colonies derived from I-14); and lanes I-14-4-1 to I-14-4-3 (colonies derived from I-14-4). Lanes labeled "wt" contain samples from the progenitor TP strains. Arrows above the lanes indicate the origin of the specific isolate.

(B) Chromosome rearrangements in strains derived from I-S. The sizes of the palindromes and the presumptive nature of stabilizing the broken end are indicated. The sizes of the palindromes and the extent of the BIR-related duplications are based on CGH analysis.

Extrachromosomal Amplicons Resulting from Hairpin-Capped Breaks Are Linear Inverted Dimers

A hairpin-capped break at the *Alu*-IR is expected to split chromosome V into acentric and centromere-containing fragments (Figure 3). In the TP strains, the *CUP1* and *SFA1* gene dosage markers were inserted on the left arm of chromosome V, telomere-proximal to the break (Figure 1). GCRs with amplified *CUP1* and *SFA1* were isolated by selecting for resistance to copper and formaldehyde. Both homologous and homeologous *Alu*-IRs greatly induced amplification of *CUP1* and *SFA1* (Table S1). $Cu^R Fh^R$ colonies occurred rarely in the progeny of strains containing direct repeats (2×10^{-9}). The rate of amplification increased 11,000-, 2,000-, and 250-fold in strains with 100%, 94%, and 86% identical *Alu*-IRs, respectively.

GCRs from $Cu^R Fh^R$ colonies were analyzed using CHEF analysis, Southern blot and CGH (Figure 5). All $Cu^R Fh^R$ clones from strains with *Alu*-IRs (IA-1 to IA-12) had

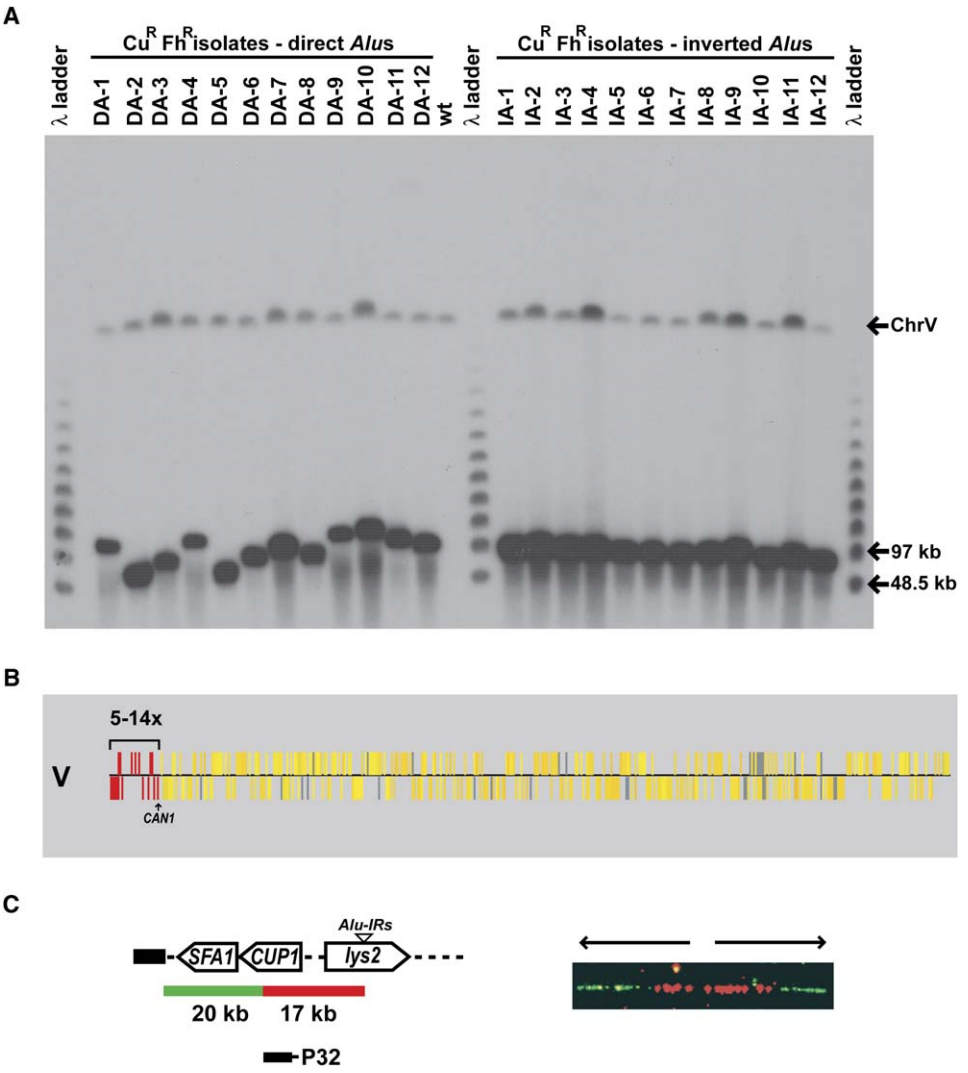

Figure 5. Analysis of Extrachromosomal-Amplification Events Induced by Hairpin-Capped DSBs

(A) Analysis of karyotypic changes in Cu^RFh^R isolates derived from TP strains by CHEF and Southern blotting. Lanes DA-1 to DA-12 have DNA samples from Cu^RFh^R isolates generated from TP strains with direct *Alu*s, and lanes IA-1 to IA-12 contain DNA samples from Cu^RFh^R isolates of TP strains with *Alu*-IRs. The lane labeled "wt" contains DNA from the progenitor TP strain. A 48.5 kb lambda ladder was used as a molecular-size standard. The CHEF gel was hybridized simultaneously with *CUP1*- and lambda-specific probes.

(B) CGH analysis of an extrachromosomal amplification in an Cu^RFh^R isolate of a TP strain with *Alu*-IRs. The amplified region is bracketed, with the degree of amplification in different isolates varying between 5 and 14.

(C) The extrachromosomal amplicons are arranged as inverted dimers. The right panel is an example of an amplicon visualized by molecular combing.

extrachromosomal amplicons that were approximately twice as large as the 42 kb fragment that would be expected as a result of breakage at the location of the inverted *Alu*s. The size of these amplicons was also about twice as large as the amplified region detected with CGH analysis (Figure 5B). No size changes were detected for all 16 chromosomes (including chromosome V). *CUP1* and *SFA1* were amplified up to 5- to 13-fold, as determined by Southern blot or by microarray using the Cluster Along Chromosomes (CLAC) program (Wang et al., 2005). Extrachromosomal amplification was sometimes accompanied by the nondisjunction of chromosome V and/or II (data not shown). Molecular combing (Figure 5C) and Southern analysis (Figure S2) showed that the extrachromosomal amplicons were inverted dimers containing the *Alu* quasipalindrome at the center of symmetry. This structure suggests that the extrachromosomal amplicons were generated by duplication of an unprocessed acentric hairpin-capped fragment resulting from resolution of the cruciform (Figure 3).

The structure of amplicons in strains with direct *Alu* repeats (presumably reflecting spontaneous DSBs) was different. In these strains (DA-1 to DA-12), the amplicons were variable in size (Figure 5A). Although we have not

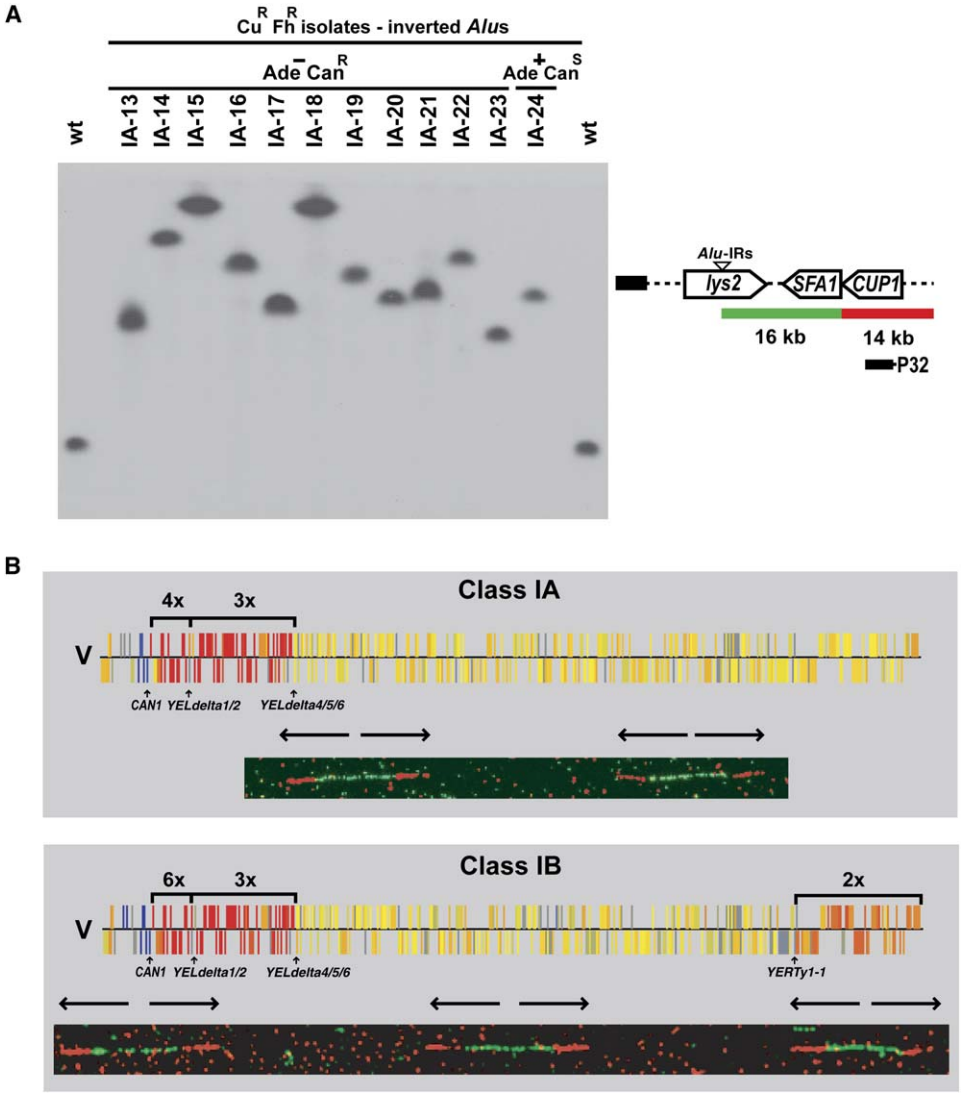

Figure 6. Analysis of Intrachromosomal Amplification Stimulated by Hairpin-Capped DSBs

(A) The left panel is a Southern analysis of a CHEF gel containing DNA from Cu^RFh^R isolates of TD strains with *Alu*-IRs (lanes IA-13 to IA-24). The gel was hybridized with a *CUP1*-specific probe.

(B) CGH and the FISH/molecular combing analyses of two class I isolates. The upper isolate is class IA (IA-17) and the bottom isolate is class IB (IA-16). In the microarray depiction, bracketed regions indicate differential amplification of the two segments within that isolate. The positions of the *CAN1* gene and repetitive elements near the junctions of the amplification segments are indicated.

analyzed these events in detail, only 3 of the 12 amplicons had an inverted repeat structure (data not shown). In addition, about 10% of the clones were disomic for chromosome V. Hence, the frequency of extrachromosomal palindromic amplicons was approximately 46,000-fold higher in strains with inverted *Alu* repeats than in strains with direct *Alu* repeats.

Intrachromosomal Amplification is an Alternative Outcome of the Repair of Hairpin-Capped Broken Molecules

To determine if the centromere-containing broken molecules, which were formed as a result of hairpin-capped DSB, had the potential for gene amplification, we created TD strains, where the *SFA1* and *CUP1* were placed centromere-proximal to the *Alu* quasipalindrome (Figure 1). In the TD strain with direct *Alu* repeats, the Cu^RFh^R isolates were rare and were usually a consequence of the duplication of chromosome V or extrachromosomal amplification of broken fragments of the left arm of chromosome V (data not shown). In contrast, in Cu^RFh^R isolates from strains with *Alu*-IRs, chromosome V was larger than the wild-type chromosome, and no extrachromosomal bands were detected (Figure 6A), indicating intrachromosomal amplification. The chromosomes containing the intrachromosomal amplicons were highly

unstable and upon propagation often gave rise to secondary rearrangements (data not shown).

There were three classes of the intrachromosomal amplicons. The majority (96%) of the Cu^RFh^R isolates were Ade^-Can^R, suggesting a telomere-proximal deletion. This conclusion was confirmed by microarray analysis (Figure 6B) of 11 isolates. In class I isolates (9 of 11), the telomere-proximal deletion bordered the amplified region on the left arm of chromosome V, and the amplified region corresponded to a 100 kb block with a breakpoint near the *YELCdelta4*, *YELWdelta5*, and *YELWdelta6* elements. Within the 100 kb block, two levels of amplification were detected: 30 kb deletion-proximal region (3–6 copies) bordering *YELCdelta1* and *YELCdelta2* and a less amplified adjacent 70 kb region (2–4 copies) (Figure 6B). The presence of delta elements at the borders of the amplicon and the differentially-amplified regions within the amplicon strongly suggests the involvement of homologous recombination in the generation of these events.

There were two types of class I isolates. In class IA strains (IA-17 and IA-23), there were no additional amplifications detected by microarray (upper panel in Figure 6B). In class IB strains (IA-13 to IA-16, IA-19 to IA-21), sequences from the right arm of chromosome V were amplified. The breakpoint of this amplification was near *YERTy1-1* (lower panel in Figure 6B). It is likely that classes IA and IB represent two different mechanisms of stabilizing the end of a broken DNA molecule with class IA reflecting de novo telomere addition and class IB reflecting acquisition of a telomere by a BIR event involving the right arm of chromosome V. Although for most of the class I isolates, the sizes of the chromosome Vs were as expected if the amplifications were intrachromosomal, for two isolates (IA-15 and IA-17), the chromosome Vs were larger than estimated based on the regions amplified; we have not determined the source of the extra DNA in these isolates.

In the class II isolates (IA-18 and IA-22), a 44 kb region centromere-proximal to the terminal deletion was amplified 3- to 4-fold. The boundary of the amplification events in these clones was near *GDA1*, a region lacking repetitive elements. In both class II isolates, chromosome V was larger than expected. A small fraction (4%) of the Cu^RFh^R isolates were Ade^+Can^S (class III). We examined only one of these isolates (IA-24) by microarrays, and we found that, similar to the class I isolates, there was an amplification of a 100 kb region spanning from the *CAN1* locus up to the *YELCdelta4*, *YELWdelta5*, and *YELWdelta6* cluster. However, the telomere-proximal region was not deleted, since it was detected by Southern blot with an *ADE2* probe (data not shown).

The genomic DNA of six Cu^RFh^R isolates (IA-13, IA-16, IA-17, IA-20, IA-22, IA-24) was analyzed by molecular dynamic combing and dual-color FISH (Figure 6). In all six Cu^RFh^R clones, the amplified copies were organized as inverted repeats, usually separated by about 70 kb of unlabeled DNA. Southern blot hybridization and restriction analysis showed that *Alu*-quasipalindromes are present in the center of the amplified units (Figure S2). The structure of the intrachromosomal amplicons derived from the *Alu*-IR strains is strikingly similar to that observed for HSRs in chromosomes of human cancers (Debatisse and Malfor, 2005).

We suggest that the first steps in the intrachromosal amplification process for class I isolates are similar to those shown in Figure 3. The inverted *Alu* sequences extrude as a cruciform that is processed to two hairpin-capped molecules. The centromere-containing fragment is replicated, and the resulting dicentric chromosome breaks. In class I events, the DSB occurs near the delta 4 element on the right arm generating a 100 kb duplication (Figure 7). The delta 4 element at the end of the chromosome invades one of the pairs of delta 1, 2 elements, setting up a rolling-circle replication intermediate. This intermediate will produce tandem 130 kb repeats (two copies of the 30 kb repeat separated by one copy of the 70 kb region). This reaction might be terminated by a DSB break within the circular part of the replication structure or by a break at the replication fork. The broken end can be stabilized either by telomere addition (class IA) or by a BIR using the delta elements of *YERTy1-1* as a template (class IB). The class II and class III isolates are not explained by the model shown in Figure 7 and will be the subjects of future experiments. Table S3 summarizes data on the isolates with intrachromosomal gene amplifications.

Elevated Levels of Deletions and Amplifications in *mre11* Strains

Our previous study (Lobachev et al., 2002) showed that Mre11 complex is not required to make the DSB at the extruded cruciform, but it is required to process the resulting hairpins. In the absence of Mre11p, an elevated level of chromosomes with inverted duplications was observed. Consistent with that, in the present study, the *mre11* mutation caused ~5-fold increase in the rate of arm loss and amplifications in *Alu*-IR strains (100% identity) (Table S1). The observation that all three types of rearrangements were affected to similar extents is consistent with the hypothesis that all are initiated by the same event: a hairpin that is generated by resolution of an extruded cruciform. In the absence of Mre11p, unprocessed hairpin-capped molecules generate acentric and dicentric intermediates that ultimately lead to GCRs.

DISCUSSION

This study presents detailed structural analysis of GCRs resulting from hairpin-capped breaks occurring at the inverted *Alu*s. In three separate types of experiments, we selected for loss or amplification of markers located centromere-distal or centromere-proximal to the quasi-palindrome. Depending on the nature of the selection and the chromosomal location of a DSB, different chromosome rearrangements were recovered that resembled GCRs in cancer cells. Our study demonstrates that palindromic sequences that can adopt hairpin and cruciform secondary

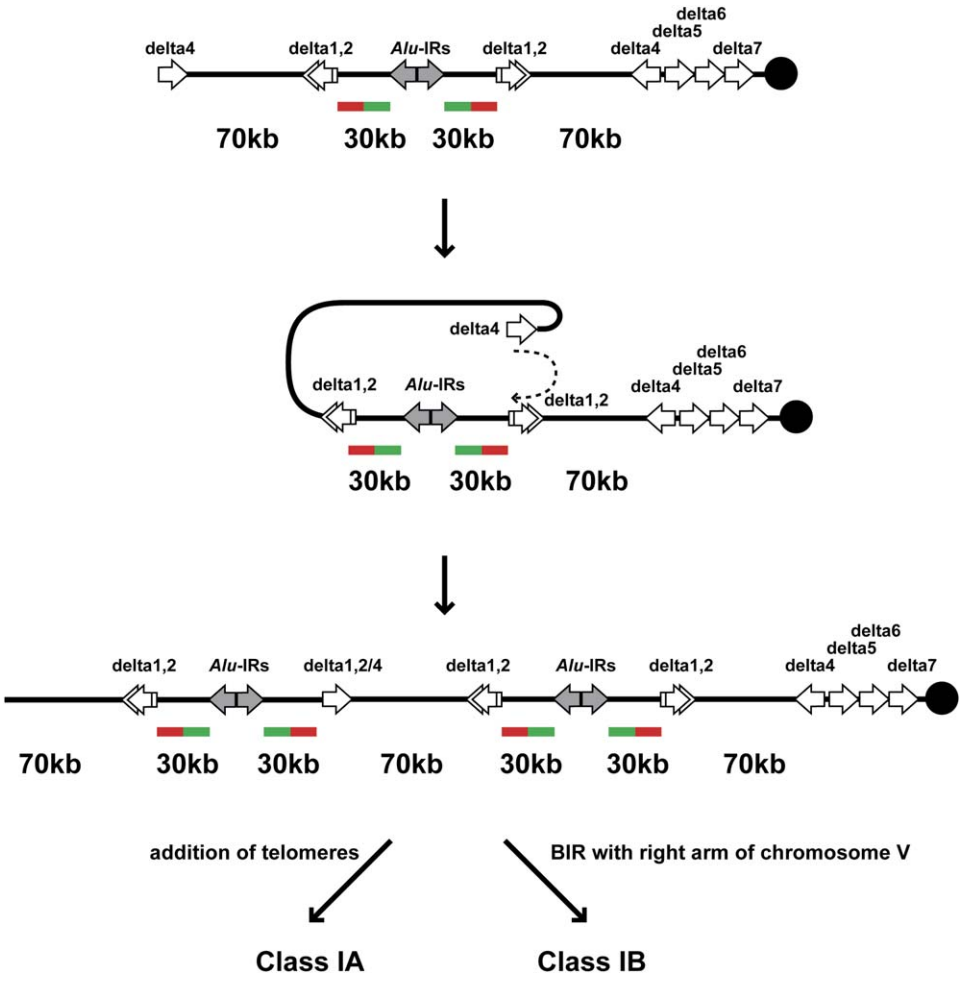

Figure 7. Model for Generating Intrachromosomal Amplicons

Arrows indicate the orientation and location of the delta elements on the left arm of chromosome V. *Alu*-IRs located at the center of symmetry of the duplications are shown as solid gray arrows, and the centromere is depicted as a filled circle. A derivative of chromosome V with a palindrome centered on the *Alu* repeats can be generated by the pathway shown in Figure 3. In this particular derivative, the DSB resulting from resolution of the dicentric chromosome is near delta 4. The red and green regions indicate the positions of the fluorescent probes described in Figure 6. As indicated by the dashed arrow, delta 4 invades one of the two delta 1 elements, setting up a BIR. In the orientation shown, a rolling-circle replication intermediate would result. Continued synthesis would produce tandem arrays of 60 kb (containing the inverted pairs of labeled segments) separated by 70 kb. DNA synthesis would continue to produce longer arrays until the rolling-circle intermediate is broken. The broken end could then be healed by telomeric addition (class IA events) or by delta-mediated BIR using the right arm of chromosome V (class IB events). In this figure, the 30 kb segment is amplified four times, while the 70 kb segment is amplified three times (similar to the isolate shown in the upper half of Figure 6B).

structures are potent sources of GCR events, including gene amplification.

Mechanisms of GCRs Triggered by Hairpin-Capped DSBs

Previously we showed that an *Alu*-IR inserted into chromosome II led to DSBs (Lobachev et al., 2002), and the resulting hairpin-capped ends required Mre11p and Sae2p for their processing. Unprocessed hairpin-capped molecules accumulated in mutants defective in the endonuclease function of the Mre11 complex and frequently gave rise to large acentric and dicentric inverted duplications. This study examines the types of GCR events associated with processing of the inverted duplications. All the GCRs can be explained by a single initiating event: processing of an extruded cruciform to generate two hairpin-capped broken ends (Figure 3). If neither end is processed by the Mre11 complex, the broken molecules cannot participate in homologous recombination or nonhomologous end-joining (NHEJ) or be stabilized by the addition of telomeres. Consequently, two palindromic dimers will be produced: (1) an acentric fragment that includes *CAN1* and *ADE2* and (2) a dicentric fragment with a deletion from *LYS2* to the left telomere region of chromosome V (Figure 3). In experiments selecting for loss of *CAN1*, only one of these products will be detected. As shown in

Figure 3, the dicentric fragment with the terminal deletion would be expected to break, resulting in a duplication of the sequences adjacent to the deletion (Haber et al., 1984; Kramer et al., 1994). To generate a stable chromosome, the broken end would have to acquire a telomere. This process could involve either de novo telomere addition by telomerase, as observed in previous studies (Kramer and Haber, 1993; Pennaneach et al., 2006), or repair by homologous recombination utilizing microhomology or a large repetitive sequence (for example, a delta or Ty element) to initiate BIR. Our analysis suggests that both de novo telomere additions and BIR repair the broken ends.

In all of the Cu^RFh^R clones isolated from TP strains (*CUP1* and *SFA1* located centromere-distal to the inverted repeats), the *CUP1* and *SFA1* amplicons formed large (~80 kb) palindromic extrachromosomal molecules (Figure 5), as expected from the mechanism shown in Figure 3. In contrast, for a majority of the Cu^RFh^R clones isolated from TD strains (*CUP1* and *SFA1* located centromere-proximal to the inverted repeats), the amplicons were located on the centromere-containing portion of chromosome V. The resulting rearranged chromosome had a deletion of the DNA distal to the inverted repeats and tandem arrays of an inverted repeat containing the selectable markers (Figure 6). We suggest that a dicentric molecule is a common intermediate for this rearrangement and for the chromosome deletions. After breakage of the dicentric, the broken DNA could invade a homologous repeat on the same chromosome arm, which could produce tandem repeats by rolling-circle DNA replication (Figure 7).

Palindrome Regeneration Cycle Leading to Continuing Genetic Instability

We showed that *Alu*-IRs induce arm loss events coupled with the formation of large (more than 30 kb) quasipalindromes (Figure 2). It is important to point out that such chromosomes will be unstable because of the existence of the large quasipalindromes. It should be noted that the breakpoints of the resulting GCRs do not colocalize with the initial hairpin-capped break site. Instead, the breakpoints are where the repair of the molecules broken during anaphase was initiated. The sequence that triggered the primary DSB and resulting GCR is at the center of the duplication.

Other studies show that long palindromic duplications can be generated by mechanisms that involve very short (4–12 bp) inverted repeats (Albrecht et al., 2000; Maringele and Lydall, 2004; Rattray et al., 2005). It is possible that the rare inverted duplications identified in the strains with direct *Alu* repeats occur via a related mechanism. Nevertheless, large palindromic regions will initiate iterative cycles of genome instability, leading to a wide variety of chromosomal aberrations, as shown in Figure 4.

DM versus Homogeneously Staining Regions

We have presented evidence that hairpin-capped breaks can lead to both extra- and intrachromosomal amplification. The nature of the amplicons depends on the chromosomal location of the amplified gene relative to the DSB. The chromosomal structure with the order, telomere/amplified gene/hairpin-capped DBS/centromere results in a DM-like amplicon. In contrast, the telomere/hairpin-capped DBS/amplified gene/centromere arrangement yields intrachromosomal amplicons with an inverted ladder-like structure. The structural organization of the amplicons identified in this work bear striking similarity to DMs and HSRs detected in human cancer cells. Based on this correlation, we propose that the rules of the palindrome-dependent amplification as seen in yeast may also operate in higher eukaryotes. We found that extrachromosomal amplicons (up to 14 copies) resulting from hairpin-capped DSBs were linear dimers. These amplicons most likely arise from missegregation of the acentric fragments during mitotic divisions. As shown in the study by Kaye et al. (2004), the acentrics are highly prone to missegregation. It is interesting to note that the DM in our system were not accompanied by arm loss. This indicates that the hairpin-capped DSB formed during G2 or S, after the synthesis of *Alu*-IRs region. In some cases, extrachromosomal amplification was associated with nondisjunction of chromosomes V and/or II. Similarly, trisomy is frequently found in cancer cells carrying DM (Naeem, 2005).

Intrachromosomal amplicons resulting from processing of hairpin-capped DSBs were usually coupled with loss of the telomere-proximal region. A similar pattern has been described for HSRs in tumors. This structure is most frequently explained by the BFB cycle (Debatisse and Malfor, 2005). The key step in the BFB model is the repetitive formation of dicentrics through sister-chromatid fusions via NHEJ. This conclusion is somewhat controversial since a mammalian cell line deficient in NHEJ had an elevated level of gene amplification (Mondello et al., 2001). In this study, it is unlikely that dicentrics are caused by fusion of broken sister chromatids since NHEJ is inefficient in yeast cells compared to mammalian cells (Krogh and Symington, 2004). In addition, the palindrome-mediated DSB obviates the requirement for sister-chromatid fusions by generating a broken molecule terminated with a hairpin. Consistent with our model, we were unable to detect intrachromosomal amplification events in *Δrad52* strains containing inverted *Alu* repeats, while the *Δdnl4* mutants had approximately the same rate of amplification as wild-type strains (data not shown).

Implications for Human Genome Stability

We find that both homologous and homeologous inverted *Alu* repeats are strong inducers of GCR (Table S1); the latter class of *Alu* repeats are found in the human genome (Lobachev et al., 2000; Stenger et al., 2001). These repeats, therefore, represent a potential threat to the integrity of the human genome, especially in mutant backgrounds that promote rearrangements (for example, in mutants with defective Mre11 complex). The mechanisms of GCRs described in this study can apply not only to inverted repeats, but also to other repeats that can adopt

stable hairpin or cruciform structures, such as certain trinucleotides (CAG/CTG or CCG/CGG repeats) or AT- and GC-rich minisatellites. These types of repeats are often found at rare fragile sites in humans (Sutherland, 2003).

Terminal deletions, duplications, translocations, amplifications, and more complex rearrangements are frequently found in leukemias, lymphomas, and sarcomas (Albertson et al., 2003; Fletcher, 2005; Naeem, 2005). Our results demonstrate that, in palindrome-mediated rearrangements, the sequence that triggers GCR is located in the center of the duplicated or amplified regions. We propose that the specific patterns of GCR described in our study (terminal deletions coupled with adjacent duplications) can serve as biomarkers in cancer genomic studies to reveal the causative sequence of rearrangements.

EXPERIMENTAL PROCEDURES

Strains

All strains in this study were isogenic to KS520 (*MATa*, *his7-2*, *leu2-3,112, trp1-Δ*, *ura3-Δ*, *lys2-Δ, ade2-Δ, bar1-Δ, sfa1-Δ, cup1-1-Δ*, *yhr054c-Δ, cup1-2-Δ*). Details of the constructions of the TP and TD strains are given in the Supplemental Experimental Procedures.

Genetic Techniques

The rates and 95% confidence intervals of the arm loss and gene amplification were estimated in fluctuation tests using at least 14 independent cultures (Lobachev et al., 1998). The canavanine-containing media was made with a low concentration of adenine (5 mg/l) to allow color detection. Copper and formaldehyde plates were prepared from SD complete media with final concentration of 700 μM $CuSO_4$ solution and 2 mM formaldehyde solution, respectively. To select for amplification events, the Cu^R colonies were replica plated to freshly-made formaldehyde plates. After 2 days of incubation, these plates were replica plated again to formaldehyde plates to verify the growth.

Structural Analysis of the Genome Rearrangements

Chromosome aberrations were characterized using CHEF gels, Southern Blot Hybridization, CGH analysis, DNA combing, and FISH. The detailed description of these techniques can be found in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include three figures and three tables and can be found with this article online at http://www.cell.com/cgi/content/full/125/7/1283/DC1/.

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