Viewpoints

Origin-Dependent Inverted-Repeat Amplification: A Replication-Based Model for Generating Palindromic Amplicons

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Introduction

Models proposed to explain the generation of palindromic (or quasipalindromic) structures during segmental amplification almost invariably begin with double-stranded DNA breaks that are repaired in different ways—for example, by end-to-end fusion at short inverted repeats, by non-allelic homologous recombination at low copy repeats, or by break-induced replication at regions of microhomology. However, the specific class of amplicons that consists of interstitial inverted triplications has no completely satisfactory explanation. By examining the molecular structure of a specific amplicon in *Saccharomyces cerevisiae*, we derived a model that does not require an initiating double-stranded break but rather invokes an underappreciated potential error in replication to explain the generation of an initial hairpin-capped linear intermediate. The model furthermore can explain the final structure and the pathway for forming this and other types of amplicons in both yeast and humans. Because the model requires the presence of both an origin of replication and short, closely spaced, flanking inverted repeats, we call this model Origin-Dependent Inverted-Repeat Amplification (ODIRA).

Background

Exposure to environmental stress often selects for cells that have amplified genes involved in the amelioration of that stress. Sometimes the connection between the gene amplification and stress makes intuitive sense, such as the amplification of the gene for dihydrofolate reductase when yeast or mammalian cells are treated with methotrexate [1,2]; in other cases, the link is less obvious [3]. To explain the mechanisms involved in the localized amplification of specific genomic loci, models have been proposed based on the DNA structures of the end products of amplification [4–6]. Many of the current models begin with a double strand DNA

(dsDNA) break and implicate DNA fusions (either homologous or non-homologous), Break-Induced Replication (BIR), Micro-homology/Microsatellite-Induced Replication (MMIR), and/or inverted or directly repeated sequences that adopt unusual secondary structures for their repair [4]. From the molecular analysis [7] of a yeast strain that contains amplified copies of the gene for the high affinity sulfur transporter, *SUL1* [3], we derived a new general model that explains the generation of interstitial tandem inverted repeat arrays of chromosome segments in yeast and in human cancers, and of de novo congenital inverted triplications and other chromosomal rearrangements. We propose that cells commit a singular error in replication: the ligation of the nascent leading strand to the nascent lagging strand at the replication fork. This model can potentially explain the origin of many palindromic rearrangements and their structural, enzymatic, and genetic requirements.

A Unique Class of Genomic Rearrangements

In the past dozen years, examples of patients with various types of developmental and physical abnormalities have been found to harbor de novo triplications with an inverted central copy for regions of chromosomes 2, 3, 5, 7, 9, 10, 12, 13, and 15 [8–16]. In three of the cases, where the parent of origin could be determined, the inverted triplication was found to be

composed of alleles from both homologs of one of the parents in a 2:1 ratio, consistent with the hypothesis that the event occurred in a meiotic or a pre-meiotic division [8,14,15]. In all cases of de novo triplications with an inverted central copy, the distal portion of the chromosome was retained. This finding appears to eliminate models such as the Breakage-Fusion-Bridge model of McClintock [17], at least in their simplest forms, as models that invoke a dsDNA break cannot easily explain the retention of distal sequences.

The *SUL1* Amplicon: A Yeast Model for Human Inverted Triplications

Subjecting wild-type yeast to long-term growth in medium that is limiting for sulfur selects for cells that have amplified the *SUL1* (high affinity sulfur transporter) locus along with variable amounts of flanking DNA [3]. To understand the chromosomal structure of one particular amplification event, Araya et al. [7] sequenced the genome of a strain selected under sulfur limitation. The novel junction sequences they identified—occurring at 7-bp, closely spaced, inverted repeats (Figure 1A) in the nearby genes *CTP1* and *PCA1* (Figure 1B)—allowed them to deduce the structure of the amplicon: a 5× tandem array of alternating head-to-head/ tail-to-tail copies of an approximately 11-kb region that contains *SUL1* and the

adjacent origin of replication, *ARS228* (Figure 1C). Southern blot analysis confirmed the inverted nature of the 11-kb tandem repeats of the *SUL1* locus [7], and array comparative genome hybridization (CGH) confirmed the retention of distal chromosome II sequences [3]. This *SUL1* amplicon therefore contains several important features of the human inverted triplication syndromes and provides a model for understanding the formation of this type of amplification event.

Applying Existing Models to Explain the *SUL1* Amplicon

Many models have been proposed to explain genomic rearrangements. Such models include recombination, repair or replication mechanisms that invoke an initial dsDNA break or the 3′ end of single-stranded DNA (ssDNA) [4]. The presence of short inverted repeats flanking the rearrangement breakpoints of the *SUL1* amplicon might suggest mechanisms that involve the formation of hairpins through intrastrand annealing in the exposed ssDNA in one of the parental strands at a replication fork or the extrusion of a cruciform in duplex DNA, leading ultimately to a hairpin-capped dsDNA break. Replication of the hairpin-capped linear would generate an isodicentric chromosome (with the hairpin at its center) that would then be subject to Breakage-Fusion-Bridge (BFB) cycles [17]. Upon capture of a telomere at the resulting break, a stable chromosome with alternating head-to-head and tail-to-tail repeats and a terminal deletion would be recovered. However, comparing this proposed structure to the chromosome actually recovered in the haploid strain used for the sulfur-limited selection, it is clear that BFB cycles cannot readily explain this particular *SUL1* amplification event as the distal sequences were retained. In addition, BFB cannot easily explain the human triplications with an inverted center copy as BFB is inherently an intrachromosomal event and the three triplications where the parent of origin was studied clearly included DNA from both homologs of one of the parents. We explored all of the existing models in a similar way, but were unable to explain simultaneously the generation of an uneven number of copies
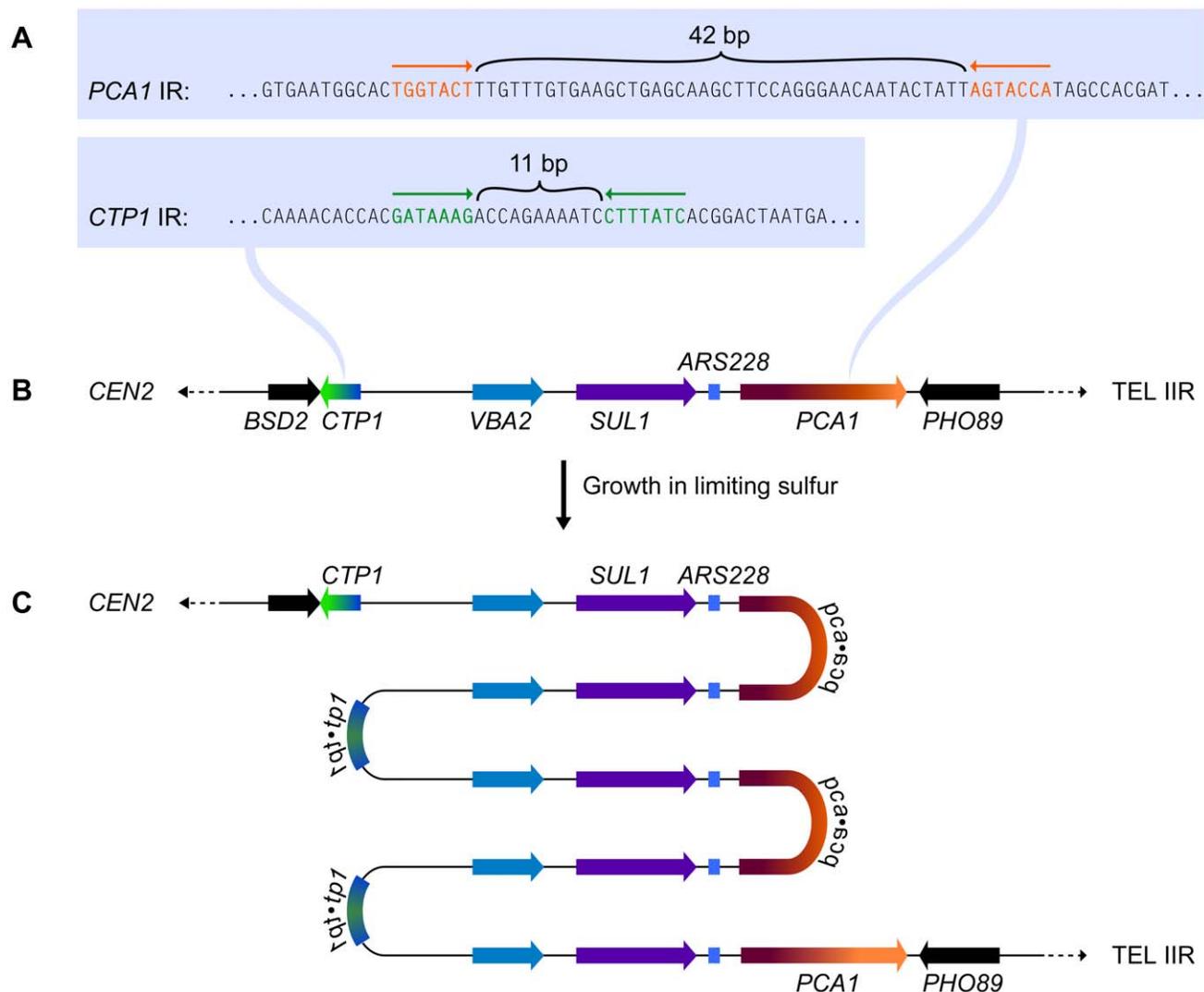
Figure 1. The chromosomal context for *SUL1* amplification. (A) The inverted repeat sequences in *CTP1* and *PCA1* that define the breakpoints of a specific *SUL1* amplification event [7]. (B) The structure of the wild type *SUL1* locus that includes the nearby origin of replication, *ARS228*. (C) The inferred structure of the head-to-head/tail-to-tail 5× *SUL1* amplification product recovered after selective growth of a haploid yeast strain in medium limiting for sulfur.
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of amplified genes in an alternating head-to-head/tail-to-tail tandem configuration, the perfect reuse of both proximal and distal break sites, the retention of distal chromosomal segments, and the creation of genetically mixed amplicons through any of the existing models that used a DNA break as the initiating event.

An Origin-Dependent Inverted-Repeat Amplification Model Explains the *SUL1* Amplicon

Intrigued by the inclusion of a potential origin of replication (*ARS228*) in the *SUL1* amplicon (Figure 1), we wondered whether the presence of bidirectional forks might play a role in the amplification process beyond the proposed mechanisms of break-induced replication (BIR), microhomology-microsatellite-induced replication (MMIR), fork stalling and template switching (FoSTeS), or serial replication slippage (SRS) [4,18–20]. The short inverted repeats and their close spacing (i.e., within the size of eukaryotic Okazaki fragments) could permit an aberrant replication intermediate to form if one or both of the replication forks regressed by just a few base pairs. In this scenario, the 3′ end of the leading strand of a replication fork initiated at the origin *ARS228* (Figure 2Ai and Aii) becomes detached from the leading strand template after synthesis of the second copy of the short inverted repeat has occurred. The detached end then anneals to its complement in the single-stranded portion of the lagging strand template (Figure 2Bi, Bii, and Biii). In this new location, the 3′ end primes synthesis on the lagging template (Figure 2Biv) and becomes ligated to the adjacent Okazaki fragment of the lagging strand, creating a continuous DNA strand between the two nascent strands at the fork—a "closed" fork (Figure 2Bv).

We have illustrated the aberrant event occurring at the oppositely oriented forks on both sides of *ARS228* (Figure 2Aiii), thereby generating a self-complementary, circular DNA intermediate that is annealed to the two parental strands but with "closed" forks that are unable to progress into the adjacent chromosomal regions. To complete replication of the chromosome and permit segregation of the two parental chromosome strands, an approaching fork from a nearby origin on either or both sides of the closed loop would facilitate the branch migration or fork reversal at the closed forks through a combination of both topological and enzymatic forces [21,22]. As the advancing forks replicate through the region of

the annealed circular molecule (Figure 2Aiv and Av), a linear duplex with hairpins at both ends (a "dog bone"; Figure 2Avi) is released. Because the displaced fragment contains the origin *ARS228*, the "dog bone" could be converted to a dimeric circular molecule by replication in the next S-phase (Figure 2Avii).

Up to this point there are three interesting features of the model: first, within the dimeric circle are the inverted *SUL1* genes and the rearrangement breakpoints that satisfy the sequencing results of Araya et al. [7]; second, there were no dsDNA breaks, hairpin cleavages, or DNA repair processes required; and third, the presence of the dimeric circle confers a selective advantage on the cell because that cell now has three copies of *SUL1*. Missegregation of the dimeric circle can cause multiple copies of the circle to accumulate, providing a further selective advantage. At some later time, the amplification event can be stabilized by the integration of one of the dimeric plasmids back into the chromosomal *SUL1* locus by conventional homologous recombination (Figure 2C). To achieve the five copies of *SUL1* [7], two independent integrations of the inverted-repeat, dimeric circular molecule would be required. Other ways to generate the 5× copies include extrachromosomal concatemerization of the dimeric molecule—by the rolling circle model proposed by Futcher for the yeast 2-micron plasmid [23]—before integration into the chromosome or by unequal sister chromatid recombination after the initial integration of the dimeric circle. In the case of the human triplication disorders, the "dog bone" intermediate generated from one homolog in a division prior to meiosis would replicate to generate the dimeric inverted circle and then integrate by homologous recombination into the other homolog during meiosis to generate the observed 2:1 allele ratio in the inverted triplication chromosome.

In comparison to existing models, this new model is relatively simple, requiring only a single type of error in replication to generate the extrachromosomal intermediate in amplification. The model demands (1) that the amplicon contain an origin of replication, both to generate the self-complementary single-stranded circular intermediate and to convert it to an extrachromosomal dimeric, inverted-repeat plasmid; (2) that pairs of inverted repeats flank the origin in close enough proximity to each other that each pair could lie within a single Okazaki-sized single-stranded gap; and (3) that the

dimeric plasmid integrates into a chromosome by homologous recombination. The fact that the creation and integration of the circular intermediate do not need to occur in the same cell cycle greatly increases the chances of recovering the final chromosomal amplicon. The high density of potential origins in the yeast and human genomes (yeast, one every ~20 kb, OriDB at http://www.oridb.org/index.php; human, one every ~68 kb; [24]) and the frequency of closely spaced (≤65 bp) inverted 7 bp repeats (one every ~250 bp; BJB, unpublished; based on random scans of 100 kb segments of the yeast genome using the "Palindrome" program at http://mobyle.pasteur.fr/cgi-bin/portal.py?form=palindrome) suggest that amplification by this mechanism need not be limited to specific loci.

Extension to Other Amplification and Genome Rearrangement Events in Yeast and Humans

The simplicity of our model makes it appealing, but is there existing evidence to support it? We have characterized a second independent *SUL1* amplicon that mimics the features of the sequenced *SUL1* amplicon [7], but with different potential inverted repeats at the junctions (C. Payen and M. J. Dunham, unpublished results). A search of the literature failed to uncover any model that includes all of the features we have described; however, strand switching from leading to lagging at a replication fork has been proposed to occur in bacteria [25,26]. We found reports that transformation of both yeast and mammalian cells with hairpin capped linear molecules ("dog bones") resulted in the expected dimeric inverted circular plasmids after replication in vivo [27,28]. We have independently confirmed that ARS fragments capped with hairpins are converted to palindromic dimers in yeast (M. M. Walker, M. K. Raghuraman, and B. J. Brewer, unpublished results). This dimerization preserves the terminal sequences of the capped ARS fragments, distinct from the dimerization of uncapped linear fragments reported by Kunes et al. [29]. We also searched for more examples of gene amplification that could be explained by our model and found several instances in both yeast and mammalian cells where the end products or the intermediates are consistent with such a ligation of leading to lagging strands at a replication fork.

The first example involves amplification of the gene for dihydrofolate reductase
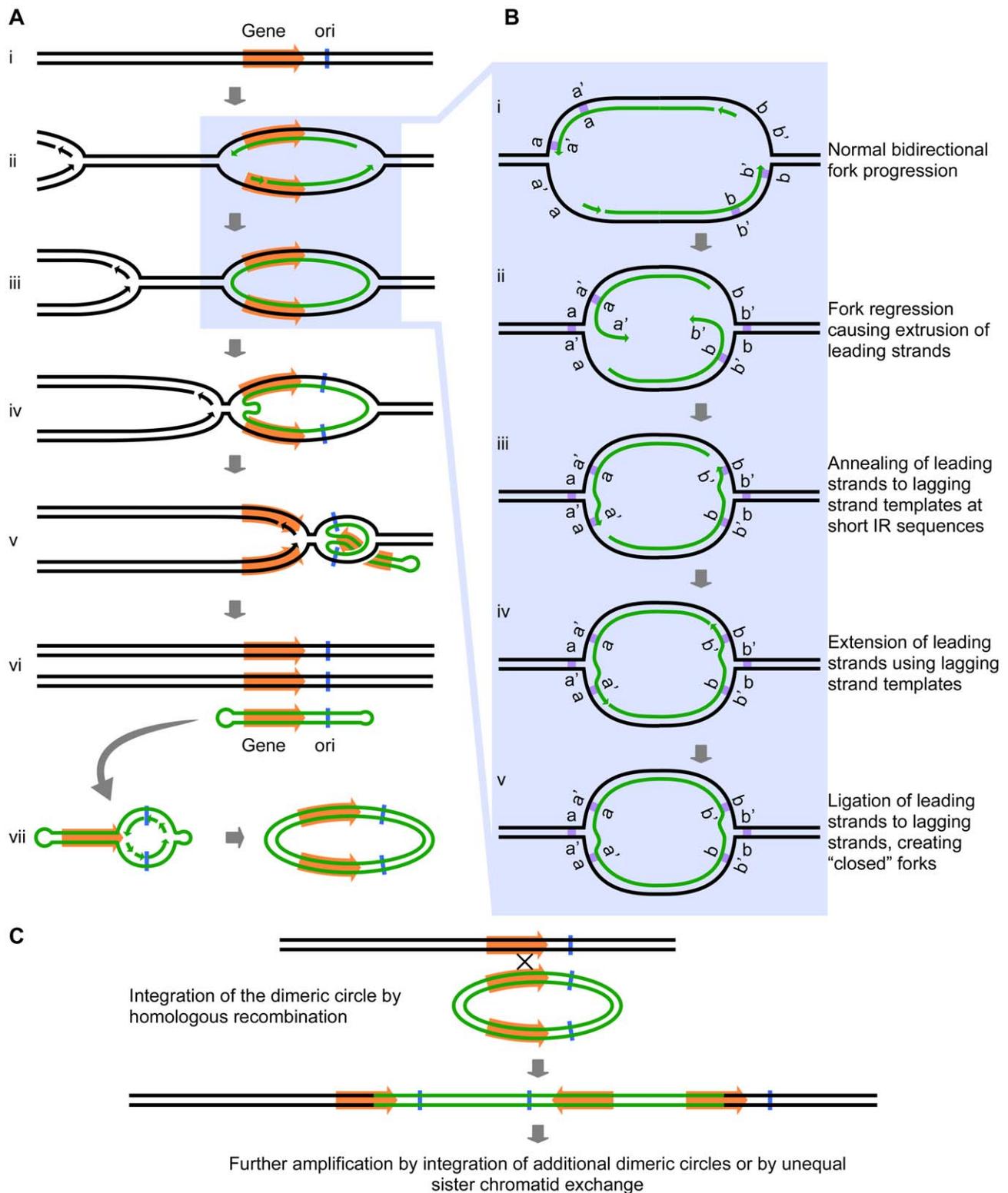
Figure 2. The Origin-Dependent Inverted-Repeat (ODIRA) model for Amplification of chromosomal segments. (A) An overview of the release of a closed circular, self-complementary intermediate that arises from aberrant replication. (B) Details of the mechanism that leads to ligation of the leading and lagging strands at short, closely spaced, inverted repeats (IRs; labeled as a a′ and b b′). (C) Replication and reinsertion of the inverted dimeric amplicon into the genome by homologous recombination. See text for detailed explanations.

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(*DFR1* in yeast and *DHFR* in Chinese hamster ovary cells). A subset of independent *DHFR* amplification events in Chinese hamster ovary cells contains chromosomally integrated repeats of alternating orientations that include one or more replication origins in each repeat [2], a pattern very similar to the chromosomally amplified *SUL1* locus of yeast. The structures of yeast chromosomally amplified *DFR1* amplicons were not determined; however, one methotrexate-resistant survivor maintained the amplified copies of *DFR1* as extrachromosomal 11-kb circular molecules composed of an inverted dimer of the *DFR1* gene and the adjacent origin of replication *ARS1524* [1]. While the authors did not sequence the junctions, several examples of short inverted repeats occur in the genome at the margins of the amplified region. In all respects, this circular inverted dimer of *DFR1* exactly conforms to the expelled and replicated, extrachromosomal molecule predicted by our model (Figure 2Avii and Figure 3-I).

A second example from the yeast literature is the amplification of the *ADH4* gene in *adh1* cells that had been treated with antimycin A [30,31]. In this case, the amplicon was most frequently found as an acentric isochromosome (Figure 3-II) of approximately 40 kb encompassing the terminal ~20 kb of the left arm of chromosome VII. In this terminal segment of chromosome VII are two potential origins of replication (likely ARSs at 8 and 17 kb on chromosome VII; OriDB, http://www.oridb.org/) that lie on either side of the *ADH4* gene. The junctions of independent isolates were mapped by restriction digestion and Southern blotting and found to lie in a roughly 2-kb region [30] that contains more than ten pairs of interrupted short inverted repeats.

A third example of an extrachromosomal inverted amplicon similar to the case of *ADH4* just described was generated from an artificial construct on the left arm of chromosome V in haploid strains of yeast [32]. The *CUP1* and *SFA1* genes, along with inverted human *Alu* sequences separated by a 12-bp spacer, were inserted near the *CAN1* gene, and clones resistant to copper and formaldehyde were selected. The most common amplicon recovered consisted of an ~80-kb inverted dimeric linear (Figure 3-II) with the *Alu* sequences at the center and a copy of the native origin, *ARS504*, near each telomere. It should be noted that in all cases, the cells also retained a full-length copy of chromosome V. The implication

of this finding is that during the generation of the *CUP1/SFA1* isochromosome, chromosome V did not suffer a double-stranded break.

The generation of the extrachromosomal *DFR1*, *ADH4*, and *CUP1/SFA1* amplicons can be easily explained by our model, as each contains one or more origins of replication and appropriately placed short inverted repeats. Amplification of *DFR1* would require "closure" at both of the diverging forks (Figure 3-I), similar to what we have proposed for *SUL1*, while amplification of *ADH4* or *CUP1/SFA1* would require only a single event in the fork moving toward the centromere (Figure 3-II). In all four of these cases an acentric, extrachromosomal, nearly perfect palindromic DNA molecule, either circular or linear, is the result. Similar examples can be found in mammalian cells: hairpin-capped linear fragments are maintained as palindromic extrachromosomal tiny episomes (ETEs; [28]); double-minute chromosomes [33], isochromosomes [34], and homogeneously staining regions (HSRs; [35,36]) with inverted repeat architecture have all been recovered from tumor cell lines; and well-characterized isochromosomes in humans occur at regions that contain large inverted repeats [37–39].

While many of the extrachromosomal molecules described so far lack centromeres, we wish to point out that our model could also provide the starting point for the Breakage-Fusion-Bridge cycle described by Barbara McClintock [17] if just the fork moving away from a centromere were to experience ligation of the leading strand to the lagging strand (Figure 3-III). Subsequent expulsion and replication of this hairpin would create an isodicentric chromosome that would form a bridge at anaphase, be broken at cytokinesis, and undergo repair by fusion in the next cycle. The chromosome becomes stable when the broken end acquires telomeric sequences either by de novo telomere addition or by recombination with another chromosome [40]. There are many examples of such chromosomes in the clinical literature of de novo chromosomal abnormalities [41]—chromosomes that end in an inverted duplication but are missing the terminal portion of the original chromosome (also known as an "inv dup del" chromosome).

Our model also provides a second way to generate "inv dup del" chromosomes without cycles of BFB (Figure 3-IV). After generating a closed fork at the telomere-proximal fork, the fork proceeding toward the centromere could suffer a single-stranded break in one of the parental

strands at the fork. Subsequent resolution of the closed fork by replication/branch migration and addition of a telomere to the broken end would generate the same structure that is usually attributed to breakage of isodicentric chromosomes by BFB. It is interesting that "inv del dup" chromosomes were the predominant class of rearranged chromosomes found by Narayanan et al. [32] when selecting for the loss of a marker that was distal to the *Alu* inverted repeats on yeast chromosome V.

Replication Delays and Gene Amplification

In our model for inverted amplicons, a closed fork can form when both copies of a short inverted repeat lie within a single-stranded gap on the lagging strand of a replication fork. Therefore, as long as the space between the inverted repeats is not greater than the Okazaki gap on the lagging strand, both the length of the inverted repeat and the amount of time the repeats persist in a single-stranded form would influence the probability of forming a closed fork at that particular position. The 320-bp inverted *Alu* sequences that were placed centromere proximal to the *SFA1* and *CUP1* genes on the left arm of yeast chromosome V increased the formation of extrachromosomal amplicons between 250- and 11,000-fold, depending on the percent identity between the repeats, and a 25,000-fold increase in the formation of "inv dup del" chromosomes when the repeats were perfect matches [32]. The degree of identity between the repeated *Alu*s would certainly influence the probability of cross-fork annealing, but the presence of the inverted repeat has also been shown to slow progression of the replication fork as much as 6-fold [42], providing more time for the fork to "close."

Other methods of slowing fork progression would also be expected to increase the formation of closed forks. Two recent yeast papers describe dicentric, palindromic chromosomes that were created by interfering with replication fork progression in yeast. Mizuno et al. [43] placed inverted replication termination sequences at the *ura4+* locus of fission yeast and followed the fate of the chromosome over time after induction of the fork-blocking factor Rts1. Both acentric and dicentric palindromic chromosomes were generated at high frequency at inverted repeats within their artificial construct, but, to their surprise, no double-stranded breaks
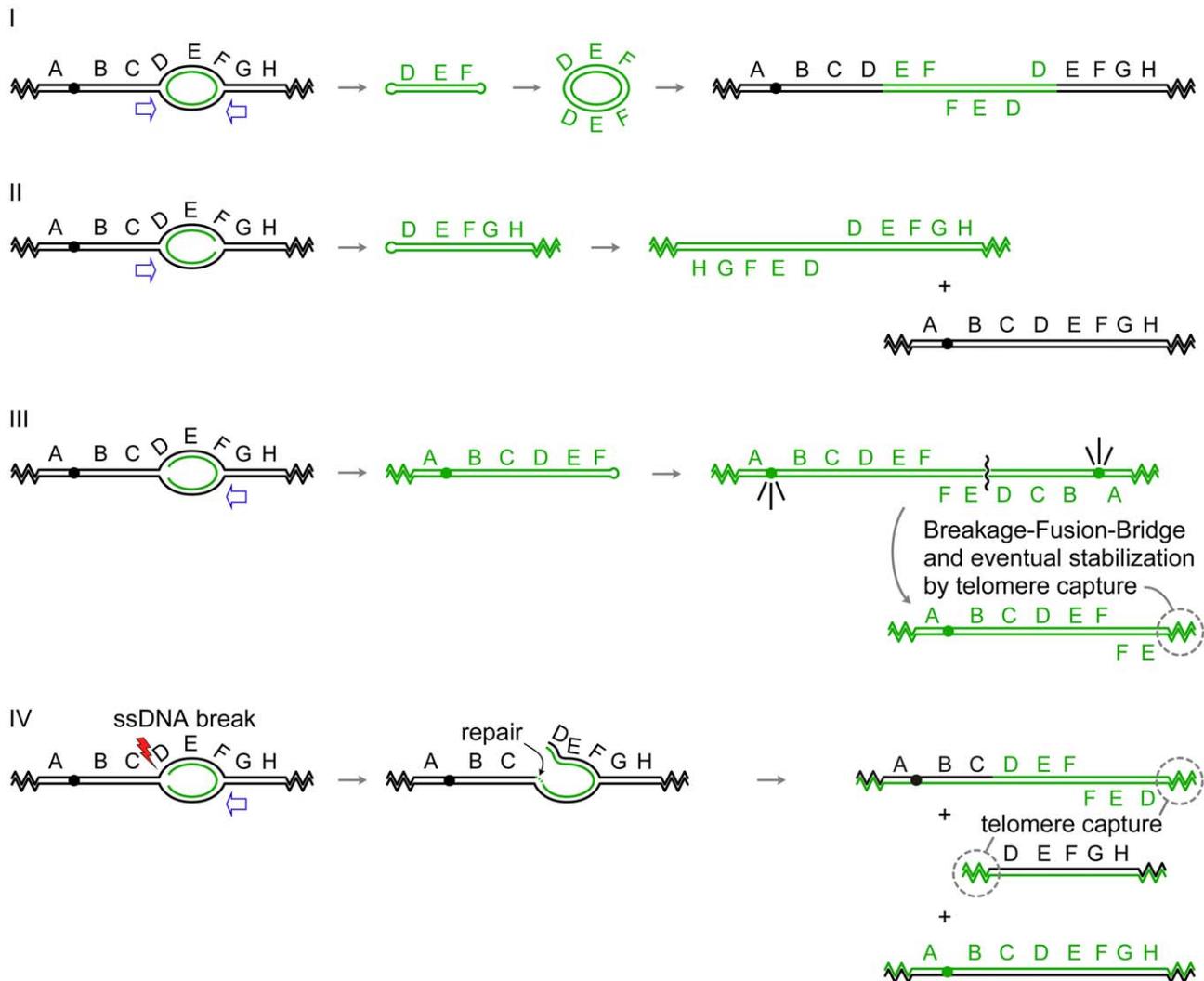
Figure 3. Palindrome formation and resolution by ODIRA generates a range of amplification products. In each of the examples I–IV, initiation of replication from an origin near the sequence labeled E generates bidirectional forks that have progressed through the flanking sequences D and F. In the four scenarios depicted, either one or both of the replication forks becomes "closed" by ligation of the leading strand to the lagging strand. Open arrows indicate the direction that flanking replication forks move to expel the "closed" fork intermediate by branch migration. (I) In this example, both forks "close." Replication forks approaching from either direction (open arrows) will release the hairpin-capped linear that contains genes D, E, and F. Subsequent replication of this "dog bone" molecule generates a dimeric, palindromic circular molecule that can reintegrate through homologous recombination into the original chromosome, or into a homolog if one is present, or by random integration elsewhere in the genome. These steps are described in detail in Figure 2. (II) In this example, the fork closest to the centromere "closes" while the telomere proximal fork remains active, fusing with distal replicons to allow replication of segments G, H, and the right telomere. The fork moving outward from the centromere (open arrow) will dislodge a fragment that has a hairpin at one end and a telomere at the other. Subsequent replication results in an isochromosome containing genes D–H. These acentric chromosomes are relatively stable in yeast [30,32], and in human cells stability can be improved by the acquisition of a neocentromere [52] or by chromosome tethering [53,54]. (III) In this example, only the distal fork closes. Completion of replication by a fork from the telomere proximal side of the closed fork releases a similar intermediate as in (II). Replication of the fragment with a hairpin near gene F generates an isochromosome that contains the centromere and genes A–F. Cycles of BFB will occur until the break is healed by the addition of a telomere. (IV) This example begins as the one in (III); however, the fork near gene D suffers a ssDNA break. Repair of this break by ligation of the broken strand to the nascent strand generates a branched intermediate that can be resolved by a fork moving in from the region of genes G and H. Two aberrant chromosome fragments are generated by this resolution, both requiring telomere addition to become resistant to nucleases. The fragment containing the centromere is an "inv dup del" chromosome indistinguishable from a chromosome generated by BFB (III). The fate of the acentric fragment is expected to be similar to that of an acentric isochromosome.
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were detected as a precursor. Paek et al. [44] obtained an isodicentric palindromic version of the budding yeast's chromosome VII at naturally occurring inverted repeats by disrupting replication in a checkpoint mutant. By studying the formation of the isodicentric chromosome in various mutant strains, they ruled out any involvement of double-stranded break repair pathways, post-replication repair, and break-induced replication. The authors of both papers suggested that some form of aberrant template switching was involved, although the models they proposed were topologically complex and/or lacked specific details. Our model of origin-dependent, inverted-repeat amplification could be the common mechanism

that explains both of these chromosomal rearrangements.

Applying replication stress to mammalian cells in culture, in the form of carcinogens and/or mutagens, has been found to cause the release of circular inverted-repeat intermediates from the genome. For example, Cohen et al. [45] found that the origin region of an integrated SV40 genome in a Chinese hamster cell line is expelled from the chromosome as a circular inverted repeat amplicon. They also observed, under the same experimental treatments, extrachromosomal circular molecules of genomic DNA [46] that we predict would also contain an origin of replication. Cohen et al. [45] proposed a mechanism called "U-turn" replication in which the leading strand folds back on itself and primes a second strand using the nascent leading strand as template. Although they did not specifically predict the importance of an origin of replication or the short, closely spaced inverted repeats, or suggest how the open end of the hairpin would be repaired, their U-turn model proposed that the hairpin essentially replicates itself out of the chromosomal context. The open end is then sealed by some unspecified mechanism and the hairpin capped linear molecule is subsequently replicated to create the dimeric, inverted, circular molecule.

More recent studies of human cancers by genome-wide analysis of palindrome formation revealed a widespread increase in the frequency of palindromic sequences (detected by their ability to "snap-back" after denaturation) that are sometimes associated with the ends of amplified regions as inferred from array CGH [47,48]. While the authors did not distinguish between chromosomal and extrachromosomal palindromes, it is possible that they were detecting the same type of circular inverted dimeric molecules studied by Lavi and colleagues [45,46].

Conclusions and Future Directions

There are many pathways—including repair, recombination, and replication—that contribute to genome rearrangements. Our model of Origin-Dependent Inverted-Repeat Amplification provides a simple way to generate a specific class of inverted amplicons. To determine just how frequent amplification might occur by our proposed mechanism requires a better cataloging of the structure of the amplified DNA. Array CGH and deep sequencing can pinpoint regions of the genome that are amplified with respect to a reference genome, but they do not distinguish between extrachromosomal and integrated copies—nor do they determine the chromosomal location or orientation of the additional, integrated copies unless the novel junctions are specifically looked for among the non-aligning sequencing reads. More complete analysis of both yeast and human amplicons is definitely needed. In the clinical literature, there is a recurring comment that triplications with inverted central copies are vastly underreported and therefore underappreciated (for example, [8]). While there are striking structural similarities between events in yeast and human cells, the scale of the chromosomal rearrangements is vastly different. For example, the sizes of the regions in the triplication disorders can be several megabases. Is it reasonable to expect the displacing forks to be able to travel such long distances? Clearly the answer is not known, but as a point of comparison, when BIR was first described in yeast it seemed amazing that a single fork could traverse the entire length of a yeast chromosome arm [49,50]. A second way in which yeast and mammalian cells differ is in their propensity to undergo homologous recombination in response to double-stranded breaks: yeast is extremely proficient at mitotic homologous recombination, while in human cells non-homologous events predominate [51]. To generate the inverted triplication disorders, the dimeric circle must undergo homologous recombination with the chromosome. However, because the human inverted triplication disorders occur in meiosis, homologous recombination might in fact be favored.

Our pathway, in addition to being rather simple, is novel and noteworthy for several reasons. First, it suggests a unifying mechanism for a diverse set of gene amplification outcomes (tandem inverted repeats, inverted double minutes, terminal inverted duplication/deletions, and isochromosomes; Figure 3). Second, the causative event is not a double-stranded break but is an error in replication—transfer of the 3′ end of the leading strand to the lagging strand template at the same fork. Third, the 3′ end of the leading strand does not need to cover much territory in search of homology as the complementary lagging strand template is just angstroms away. Fourth, there is no obvious need to relocate polymerases or helicases at the fork to restart replication from the 3′ end of the displaced strand, as the lagging strand machinery would be available for this purpose. Fifth, the "closed" fork should be displaceable from the parental strands by branch migration brought about by the combination of the enzymatic activities of the helicases and topoisomerases that travel with the fork that approaches from the neighboring origin and the positive supercoils that accumulate ahead of it. Sixth, the displaced circle is an autonomously replicating entity, so the creation of the intermediate and its reintegration into the chromosome need not be temporally coupled. Seventh, the model supplies an alternate method to McClintock's BFB for generating "inv dup del." And finally, many of the steps in the model we have presented are experimentally testable—perhaps most easily in yeast where it is possible to select directly for desired amplification events and where the starting constructs and genetic backgrounds can be manipulated, but also during clonal expansion of transformed mammalian cells in culture.

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