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Hairpin- and cruciform-mediated chromosome breakage: causes and consequences in eukaryotic cells

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1. ABSTRACT

Chromosomes of many eukaryotic organisms including humans contain a large number of repetitive sequences. Several types of commonly present DNA repeats have the capacity to adopt hairpin and cruciform secondary structures. Inverted repeats, AT- and GC-rich micro- and minisatellites, comprising this class of sequence motifs, are frequently found in chromosomal regions that are prone for gross rearrangements in somatic and germ cells. Recent studies in yeast and mammals indicate that a double-strand break occurring at the sites of unstable repeats can be an initial event in the generation of chromosome rearrangements. The repeat-induced chromosomal instability is responsible for a number of human diseases and has been implicated in carcinogenesis. In this review, we discuss the molecular mechanisms by which hairpins and cruciforms can trigger chromosomal fragility and subsequent aberrations in eukaryotic cells. We also address the relationship between secondary structure-mediated genetic instability and human pathology.

2. INTRODUCTION

Chromosomal double strand breaks (DSBs) compromise the integrity of eukaryotic genomes. One of the profound outcomes of the breakage is gross chromosomal rearrangements (GCR) that can have either deleterious or advantageous consequences. Karyotypic abnormalities, including GCRs, are a hallmark of many tumors (1-4). There are numerous reported cases of oncogene activation occurring as a result of translocation or gene amplification events (5-7). Chromosome aberrations can also lead to hereditary diseases and are frequently observed in human syndromes that arise due to defects in DNA repair genes (8, 9). At the same time, chromosome rearrangements can be a part of the programmed genetic modifications during cellular differentiation and development (10-12). GCRs also play a major role in chromosome evolution of eukaryotic organisms (13-16). In recent years it has become evident that genomic regions containing repetitive sequences capable of adopting noncanonical DNA conformations have an increased

susceptibility for breakage and rearrangements. In this review, we focus on chromosomal fragility and aberrations triggered by DNA repeats that can form hairpin and/or cruciform secondary structures in eukaryotic cells. Other types of genetic instability induced by these structures, fragility in prokaryotes as well as breakage and aberrations induced by other secondary structures are addressed elsewhere (17-24).

3. HAIRPIN AND CRUCIFORM STRUCTURES

Hairpin- and cruciform-forming repetitive sequences include inverted repeats (IRs), AT- and GC-rich micro- and minisatellites. They can be found in higher eukaryotic genomes including the human genome (25-33). These sequence motifs are characterized by internal symmetry that allows transition from interstrand to intrastrand base pairing. If complementary interaction between symmetric regions happens only in one strand it leads to a hairpin formation. Cruciform structure can occur if both strands are engaged in the extrusion of hairpins. In AT- and GC-rich micro- and minisatellites more than one hairpin can form, often on opposite arms of a duplex resulting in slip-stranded DNA (Figure 1). *In vitro* and *in vivo* studies done in prokaryotes and eukaryotes have identified several key factors that govern the formation and the stability of hairpin stems. These include length of the repeats, sequence composition of the repeat and/or the spacer (a unique sequence separating the two repeat units) type and position of the repeat with respect to replication origin, location of repeats in the genome and genetic background (reviewed in 19, 21, 34, 35). Overall, long (> 150 bp) perfect palindromes (IRs that lack a spacer) with G+C clamps in the stem region and an AT-rich region at the center of symmetry, are expected to be the most efficient at forming stable hairpins or cruciforms. Formation of hairpins in single-stranded DNA is thermodynamically favored; whereas cruciform extrusion from double-stranded DNA requires energy which can come from negative supercoiling (the process of extrusion is complex and is discussed in detail in 35, 36). *In vivo*, optimal conditions for the hairpins and cruciforms might be provided by processes that require separation of the two strands of the duplex such as transcription and replication. As described below, the stable secondary structures in turn can hamper progression of replication fork and lead to breakage.

4. MECHANISMS OF DOUBLE-STAND BREAK FORMATION AND CONSEQUENCES FOR THE GENOME STABILITY

The formation of stable secondary structures predisposes the chromosomal regions where they reside to instability. Direct physical evidence was obtained in both meiotically and mitotically dividing cells that DSBs occur at the location of long inverted repeats or expanded tracts of triplet repeats (37-49). These findings have provided an important link between secondary structure-forming repeats and their potential to trigger genome instability. It is not well understood how the changes in DNA architecture can lead to DSBs. However, several models which are not mutually exclusive have been proposed that fall into two broad themes: 1) extruded hairpin or cruciform structures can block replication fork progression resulting in breakage, and 2) secondary structures are targets for cleavage by structure-specific nucleases. Below we discuss how these two ideas are entertained to explain the mechanisms of secondary-structure associated DSBs and instability in cells undergoing mitotic and meiotic divisions.

4.1. Mitotic instability

4.1.1. Hotspots for chromosomal fragility in higher and lower eukaryotes: a connection to replication

One of the first indications that secondary structure-forming sequences can perturb DNA replication and lead to chromosomal breakage in somatic cells came from *in situ* studies of rare and common fragile sites in humans and other mammals (reviewed in 50-52). Fragile sites are chromosomal regions that exhibit gaps and breaks on metaphase spreads, either spontaneously or in response to cell culture conditions or treatment with certain chemicals. They are classified as rare and common based on their frequency of occurrence in the population. Molecular analysis of 8 rare human fragile sites revealed that they are enriched with repeats capable of adopting stable secondary structures. 6 rare sites (FRAXA, FRAXE, FRAXF, FRA10A, FRA11B and FRA16A) are composed of expanded (up to 2000 copies) CCG-rich microsatellite repeats while the two others (FRA10B and FRA16B) are comprised of massively amplified ~33bp and ~42bp AT-rich minisatellites. Recent studies have shown that at least two cloned common fragile sites (FRA7E and FRA7I) share a similar sequence composition and are rich in AT and TA dinucleotides which also have potential to adopt secondary structures (53). Although expanded AT- and GC-rich repeats can result in spontaneous chromosomal breakage, the fragility is greatly induced upon exposure of cells to the growth conditions or agents that either impede DNA synthesis (such as absence of folic acid or presence of aphidicolin, methotrexate, BrdU in the media) or that perturb chromatin organization (such as distamycin A, berenil, netropsin) (reviewed in 51). These findings supported the idea that fragility can result from inhibition of replication elongation by extruded structures. Although fragility was used to describe microscopic observations of metaphase spreads in cytogenetic studies, it was proposed that similar mechanisms might operate *in vivo*. Consistent with this, FRAXA, FRAXE, FRA16B and FRA10B sites were found to be replicated with a delay as compared to either the same loci lacking repeat expansions or proximal non-fragile regions (54-56). Evidence for *in vivo* breakage at secondary-structure forming fragile sites has come from studies analyzing associated chromosomal rearrangements. Cuillo *et al.* (57) have shown that in breast cancer cells, the chromosomal region containing common fragile site FRA7I was involved in intachromosomal amplification. FRA7I was localized to the boundary of the amplicon suggesting that a break at the fragile site triggered the rearrangement. Additional support comes from studies of sister chromatid exchange in carriers of FRAXA and FRA16B (58-60). Both fragile sites were hotspots for homologous recombination upon induction while FRA16B also induced sister chromatid exchange spontaneously.

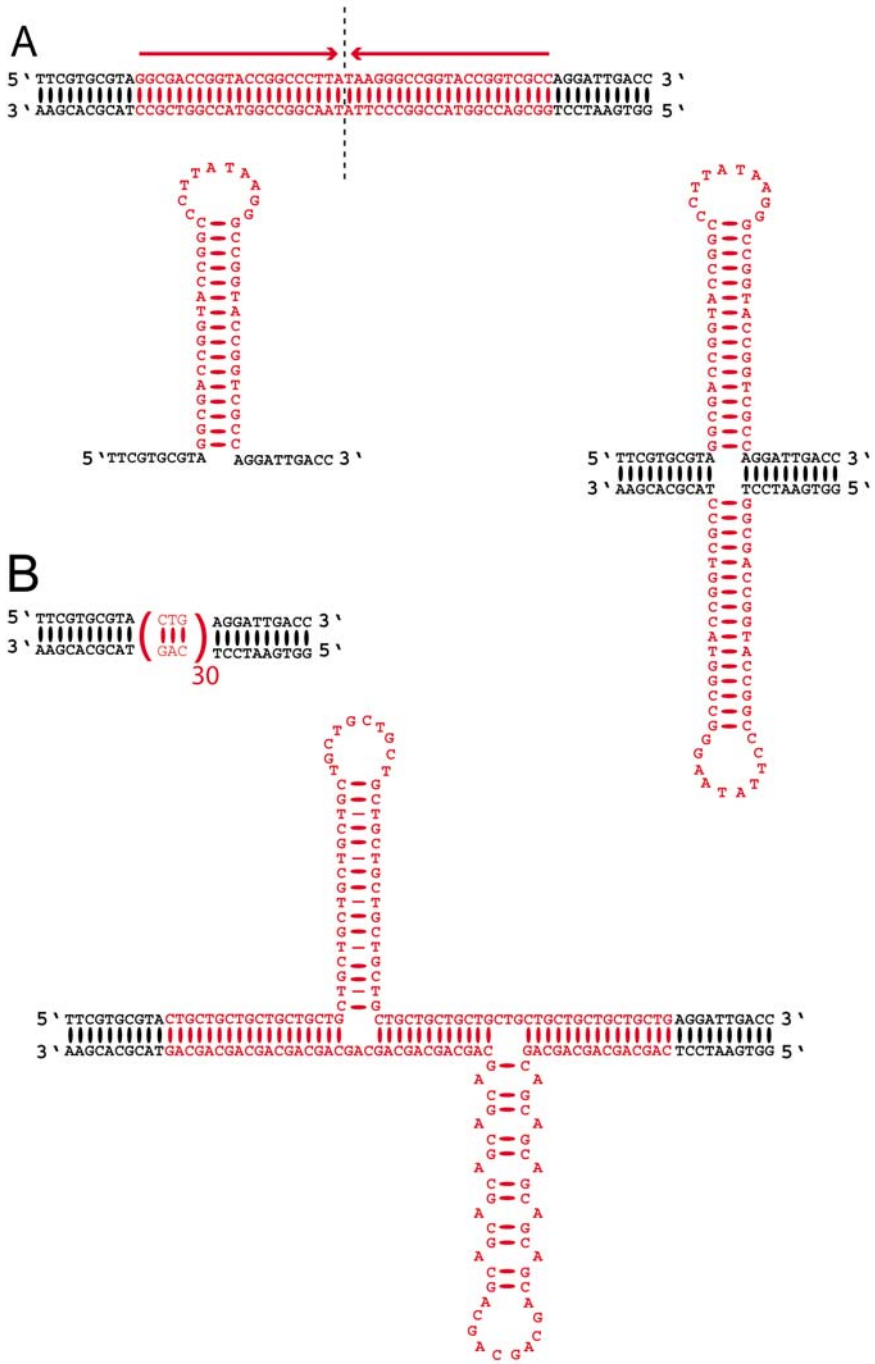

Figure 1. Secondary structures formed by inverted repeats and AT- and CG-rich micro and minisatellites. Red color indicates repetitive sequences. Flanking non-repetitive DNA is denoted in black. A. Inverted repeats. Perfect 20 bp palindrome is shown as an example. Vertical dashed line is the center of symmetry. Inverted orientation is indicated by solid red arrows. Hairpin and cruciform structures are depicted below the sequence motif in a duplex DNA. Even in a perfect palindrome, 3-4 bases at the hairpin tip are expected to be unpaired (35). This short inverted repeat is shown for ease of presentation. However, fragility is a feature of much longer repeats (see text for the details). B. AT- and CG-rich micro and minisatellites. Trinucleotide CTG/CAG track (30 repeats) is shown as an example of CG-rich microsatellite which can adopt secondary structures. In addition to hairpins (and possibly cruciforms) diagrammed in A, they can also adopt slipped-stranded DNA comprising of CTG and CAG hairpins on opposite strands (shown in the figure) (36). Although, both hairpins contain mismatches in every third nucleotide, they have different stabilities *in vitro* and *in vivo* (summarized in 117). Unlike the A-A mismatches in CAG hairpins, the T-T mismatches in CTG hairpin have two hydrogen bonds and are well stacked in the helix making the hairpin structure more stable. Depending on the nature of AT- and CG-rich micro and minisatellites, different structural constraints can govern the stability of hairpins.

In model experiments, when artificially constructed hairpin- and cruciform-forming sequences were introduced into mammalian cells, a high level of associated instability was observed and interpreted not only as a consequence of replication problems but also as a result of breakage at the site of the secondary structures initiated by nuclease attack. In rodent cells, transfected linear hairpin-ended fragments or a circular 15.3 kb palindromic DNA frequently gave rise to monomeric circular rearranged molecules containing insertions of varying sizes and/or asymmetric deletions at the center of inverted repeats (61, 62). These results suggested that hairpins are substrates for cleavage by a putative structure-specific nuclease which is followed by processing (resection and incorporation of nucleotides) and non-homologous end-joining (NHEJ). A similar conclusion implicating nuclease attack of potential secondary structures in somatic cells was drawn from several studies involving palindromic sequences integrated into the mouse genome. Damage occurring at the location of such transgenes resulted in high genetic instability. Asymmetric deletions within the palindromic repeats were frequently detected (63, 64). Such rearrangements eliminated the center of symmetry between inverted repeats thereby stabilizing the palindromes.

Chromosomal instability caused by secondary structure-forming repeats is not limited to mammalian cells but rather a general phenomenon of eukaryotic cells, which has also been observed in model organisms. Studies in yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have allowed to take advantage of the power of genetic analysis, the convenience of experimental assays and the relative simplicity in the detection of intermediates to better understand what is happening at the location of extruded secondary structures. The term "fragility" originally applied to describe cytogenetic observations in mammalian systems (see above), was later adopted to define the breakage and its consequences occurring *in vivo* in yeast cells. Different sequence motifs that can adopt hairpin and cruciform structures, such as inverted repeats, expanded tracks of CTG/CAG and CCG/CGG repeats in a number of experimental systems have been shown to be strong hot spots for allelic and ectopic homologous recombination, sister-chromatid exchange and gross chromosomal rearrangements (38-41, 43, 45-47, 49, 65-70). The ability of a repeated DNA sequence to adopt stable hairpin or cruciform structures strongly correlates with its propensity to induce genomic instability. The parameters that facilitate the probability of secondary structure formation and/or its stability, such as high level of homology, minimal spacer length (for the inverted repeats) and longer size of the repeats (for both inverted and triplet repeats), also increase the susceptibility for fragility (39, 45, 46, 49, 65). This supports the hypothesis that DSB formation at the location of secondary structures is responsible for repeat-induced chromosomal instability. In three independent studies, unstable repeats were shown to cause chromosomal DSBs *in vivo*. Freudenreich *et al.* (39) have detected breakage of chromosome containing 250 copies of CTG repeats. Chromosomal DSBs were also found at an *Alu* quasi-palindrome and at the site of two inverted Ty1 elements (43, 44). Lobachev *et al* (44) mapped DSBs to the site of an inverted *Alu* repeat insertion and based on the analyses of DSB intermediates concluded that the break was generated by a structure-specific nuclease. Several observations support the idea that DSBs are produced as a result of compromised replication. First, in two of the above listed studies, DSBs were readily detected under conditions when DNA replication was perturbed. The CTG-induced breakage and recombination was stimulated by growing the yeast cells in the presence of replication inhibitor, hydroxyurea (39), while inverted Ty1 formed a fragile site upon depletion of the replicative polymerase alpha (43). Second, mutations in Rad27, DNA ligase1 and primase proteins involved in lagging strand DNA replication, increase the frequency of mitotic recombination stimulated by CAG/CTG triplet repeats (66). Similarly, inverted repeat-stimulated homologous recombination is elevated in *pol3-t* mutants, defective in DNA polymerase delta (45, 46, 67). Third, chromosome integrity at the location of CAG/CTG triplet repeats is controlled by components of the checkpoint machinery including those that monitor progression of replication fork (40, 68). Fourth, using 2-D gel analysis it has been demonstrated that long tracks of CGG/CCG and CTG/CAG triplet repeats located on plasmid cause pausing of the replication fork (71). Likewise, hyper-recombinagenic 320 bp inverted *Alu* repeats also attenuate replication progression on plasmid and in chromosome (I. Vonegue, K.S. Lobachev and S. Mirkin, in preparation; V. Narayanan and K.S. Lobachev, unpublished data).

Although there is an evident connection to replication, the exact cause for DSB formation by unstable repeats in mitotically dividing eukaryotic cells is unknown. However, genetic analysis along with analysis of DSB intermediates and the resulting rearrangements in both yeast and mammals have led to three models explaining how secondary structures can induce DSBs in mitotic cells.

4.1.2. Models for secondary structure–dependent DSB formation

4.1.2.1. Hairpin-mediated replication arrest and breakage

The crux of this model is that when a hairpin forms in the template strand or in the displaced flap of an Okazaki fragment (applicable to triplet repeats) progression of the replication fork can be blocked, leading to fork collapse and DNA breakage (Figure 2, Hairpin-mediated breakage). A similar mechanism was proposed to explain the ability of long tracts of CAG/CTG triplet repeats to induce mitotic homologous recombination and chromosomal breaks in *S. cerevisiae,* and the recombination hotspot activity of a 160 bp palindrome in *S. pombe* (37, 39, 66, 68). This model is consistent with experimental data on secondary structure-induced fragility as well as with the general observation that replication arrest or defective replication machinery can lead to DSBs and subsequent activation of homologous recombinational repair (reviewed in 72).

The most enigmatic step in this model is how a hairpin-arrested replication fork is converted to DSB. It is possible that arrested replication fork, which is enriched

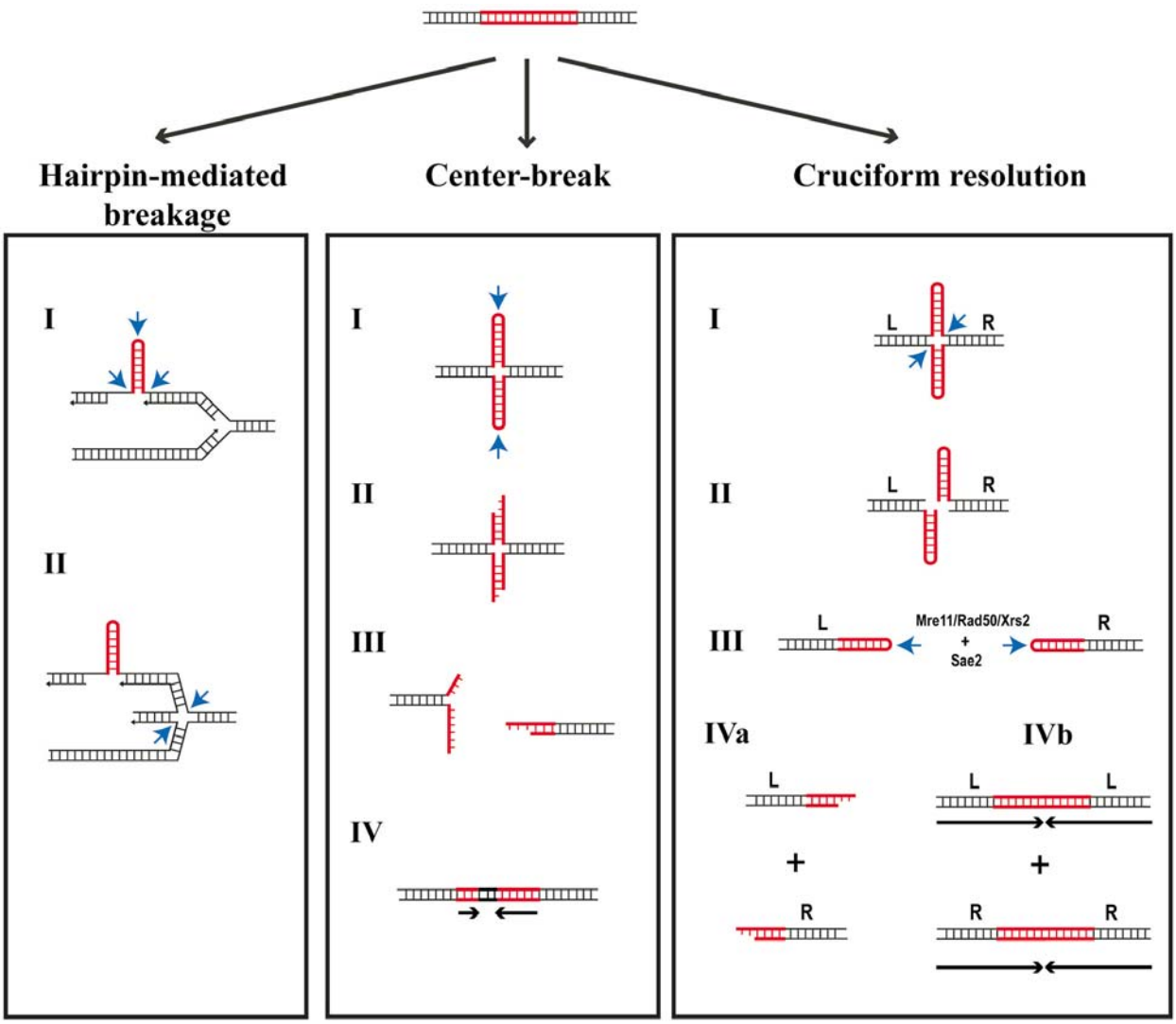

Figure 2. Models of hairpin or cruciform-stimulated DSBs. Blue arrows indicate putative or experimentally proven nuclease cleavage sites. Red color depicts hairpin/cruciform forming sequence motif. Horizontal arrows correspond to the repeats in rearranged molecules. L and R in cruciform resolution panel denote left and right regions flanking the secondary-structure forming sequence. A detailed description is presented in the text.

with single-stranded DNA, is innately unstable and can be subjected to the mechanical breakage. Alternatively, breakage can be mediated by nucleases which either attack singe-stranded DNA or the abnormal structures at the arrest site (Figure 2, Hairpin-mediated breakage, I). It is conceivable that the hairpin is the target and can be removed from the replication fork by a structure-specific nuclease leading to DSB formation. A similar mechanism is described in bacterial studies where structure-specific nuclease SbcCD can cleave the hairpins formed by inverted repeats during lagging-strand DNA synthesis (73). The possible candidate in eukaryotic cells that might have function analogous to bacterial SbcCD is the Mre11/Rad50/Xrs2 (Mre11/Rad50/Nbs1 in human) complex. Mre11 and Rad50 are eukaryotic homologs of bacterial SbcD and SbcC, respectively (74). Furthermore, yeast Mre11/Rad50/Xrs2 and human Mre11/Rad50/Nbs1 possess endonuclease and exonuclease activities that can cleave and process hairpin substrates *in vitro* (75-77). In *S. pombe*, induction of homologous recombination by the 160 bp palindrome depends on activity of Rad50/Rad32 complex (a Mre11 complex homolog of *S. cerevisiae*) (38). This observation led the authors to suggest that this complex is directly responsible for the cleavage of the hairpin extruded during DNA replication. The caveat with this conclusion is that genetic analysis in this work was not accompanied by the physical detection of the DSBs. In *S. cerevisiae*, inverted *Alu*-mediated homologous recombination was also found to be dependent on endonuclease activity of Mre11/Rad50/Xrs2 complex and function of the Sae2 protein (69). However, analysis of DSB formation in wild type, *mre11*, *rad50*, *xrs2* and *sae2* mutants demonstrated that the Mre11 complex is not responsible for initiating the break, but rather involved into processing the DSB intermediates at later repair steps (69) and Figure 2, Cruciform resolution model). Additional

experiments are required to determine if the role of Mre11 complex in two yeast species is indeed different or the same.

Another possible scenario is that hairpin-induced replication arrest might cause replication fork reversal, formation of a Holliday junction, and subsequent cleavage by resolvase (Figure 2, Hairpin-mediated breakage, II). Formation of a Holliday junction at a blocked replication fork, followed by resolution with RuvABC resolvase is a mechanism that seems to operate in *Escherichia coli* (78). There are no known homologs of RuvABC in eukaryotes, including yeast that functions in the nucleus (reviewed in 79). A Holliday junction resolvase, Cce1 of *S. cerevisiae*, (Ydc1 of *S. pombe*) has its activity limited to the mitochondria and does not play any role in a chromosomal metabolism (80). The Mus81/Mms4 complex has been proposed to function as a eukaryotic Holliday junction resolvase (81). However, disruption of *MUS81* or *MMS4* genes did not affect inverted *Alu*-stimulated recombination or DSB formation (44 and unpublished results). Genetic and biochemical results in yeast and mammalian systems indicate that besides Mus81/Mms4 there is at least one other protein that possess Holliday junction resolving activity (82-84). The identity of this putative protein and its role in fragility remain to be determined.

4.1.2.2. Center-break mechanism

The second and third models presented in Figure 2 (Center-break and Cruciform resolution models) both involve cruciform extrusion as the initiation step for nucleolytic attack. Cruciforms might arise in the regions with increased negative superhelical torsion that are created by a helicase moving ahead of the replication fork. In replication defective mutants, discoordination between DNA synthesis and unwinding would increase the length of negatively supercoiled DNA (85) thereby creating optimal conditions for the cruciform formation. Similarly, alterations in chromatin structure that favor cruciform extrusion can be generated by chromatin remodeling complexes during gene regulation (86). Stable secondary structures could block fork progression and lead to fragility by the mechanisms presented below.

In the "center-break mechanism", hairpin ends of the cruciform are the targets for nucleases (as yet unidentified). Introduction of single-stranded nicks into both hairpins (Figure 2, Center-break model, I and II), followed by destruction of the extruded cruciform (III) can lead to a DSB and subsequent repair either by NHEJ or homologous recombination (IV). As mentioned above, this model was proposed to explain asymmetry in the center of the recovered stabilized molecules after long palindromes were introduced into mouse or hamster tissue culture cells (64). Proteins that act on the hairpins in this mechanism remain to be identified. The candidate nucleases are the Mre11 complex (discussed above); RAG1/RAG2 and Artemis, mammalian proteins that nick hairpins formed during V(D)J recombination (87, 88); and ERCC1-XPF, structure specific nuclease that plays a role in the nucleotide excision pathway (89).

It should be noted that asymmetry in the center of rearranged palindromes in mammalian cells can be also explained by the third mechanism described below which has been demonstrated for yeast cells.

4.1.2.3. Cruciform resolution

In the third model, an extruded cruciform is a substrate for a putative nuclease which introduces symmetrical cuts on opposite sides of the four-way junction (Figure 2, Cruciform Resolution model, I and II). This mechanism was proposed to explain chromosomal fragility at the location of *Alu* quasi-palindrome in yeast (69). In this study, Lobachev *et al* have not only shown that long inverted repeats stimulate mitotic homologous recombination and cause chromosomal DSBs, but were also able to identify and analyze DSB intermediates. This provided insight into the mechanisms by which DSBs form and cause specific chromosomal rearrangements. It was found that both DSB ends are capped by covalently closed hairpins (III), supporting the cruciform resolution mechanism of DSB formation. The following sequence of events was deduced based on genetic and physical analyses. The endonuclease function of the Mre11/Rad50/Xrs2 complex and the activity of Sae2 protein are required to open the hairpins and initiate single-strand resection of the DSB molecules. Following resection (IVa), DSBs can be repaired by homologous recombination. Unprocessed hairpin-capped DSB molecules accumulate in mutants defective in the endonuclease function of the Mre11 complex, and frequently give rise to large chromosomal inverted duplications (IVb) that are highly unstable and can be directed to further rearrangements (69, 70). This mechanism thus far was described only for yeast. A similar pathway might also exist in mammalian cells providing an alternative explanation for the observed asymmetry in the recovered stabilized palindromes (see above). In this scenario, following cruciform resolution, opening and resection of the hairpin-capped molecules, DSBs can be repaired by non homologous end-joining machinery.

The proteins involved in cruciform resolution and generation of hairpin-capped breaks are unknown. It is puzzling why yeast (and perhaps mammals) would require such an activity. One possibility is that the cruciform structure might resemble a Holliday junction intermediate that occurs during late steps of homologous recombination. The cruciform structure that might arise during replication can be misrecognized and cleaved by the resolvase. Such a mistake at the wrong place at the wrong time can have dire consequences for genome integrity since it can lead to gross chromosomal rearrangements. As mentioned in the previous section, the identity of the eukaryotic resolvase is still an unsolved mystery and its role in cruciform resolution remains to be established.

It is important to note that in the Lobachev *et al* study (44), only covalently-closed hairpins were detected at the ends of DSB intermediates but nicked hairpins were not. One interpretation of this data is that cleavage of the cruciform structure might be coupled with efficient ligation of nicks that could be carried out by one of the yeast ligases, Lig1 or Lig4. Alternatively, both activities, cleavage and

rejoining, might reside in the same enzyme. Two distinct classes of such bifunctional proteins are known to exist in some prokaryotic and eukaryotic organisms. Enzymes belonging to the first class were found in organisms that replicate as linear genomes with covalently closed hairpin ends instead of telomeres (90). Sequence-specific breakage and hairpin formation is essential for such organisms, since it converts circular inverted dimers occurring after replication of such genomes back to unit-length molecules. This reaction, referred to as telomere resolution, is carried out by ResT in *Borrelia burgdorferi*, TelN in *Escherichia coli* phage N15, Tel PY54 of the *Yersinia enterocolitica* phage PY54, and TelK of the *Klebsiella* phage _KO2, enzymes related to tyrosine recombinases and type IB topoisomerases (91-94). The second group of enzymes, is comprised of prokaryotic and eukaryotic transposases, the V(D)J recombinase RAG1, and retroviral integrases. The formation of the hairpin is an intermediate step during DNA breakage and joining reaction carried out by these proteins (95). Conceivably, an analogous enzyme belonging to either of the above classes could have evolved in yeast (and possibly mammals) as a protein that acts on a cruciform and/or Holliday junction in a sequence-independent manner.

4.1.3. Implications for tumorigenesis

Gross chromosomal rearrangements such as deletions, duplications, translocations, amplifications and more complex rearrangements are frequently found in leukemias, lymphomas and sarcomas (1-4). Inactivation of tumor suppressor genes or activation of proto-oncogenes as result of deletion, translocation or amplification events are the mechanisms implicated to play an important role in cancer development (96). The observation that there are recurrent chromosomal aberrations in certain types of cancers has led to the hypothesis that DSB-triggering sequence motifs can predispose particular regions to rearrangements. The involvement of secondary structure-forming repeats in tumorigenic rearrangements is thus far circumstantial, and is based on a limited number of examples where sequence motifs capable of adopting non-B DNA conformation are a part of the rearranged regions or co-localize with the cancer breakpoints. Cuillo *et al.* (57) have shown that the common fragile site FRA7I sets the telomeric boundary of an inverted duplication containing the *PIP* gene which is frequently overexpressed in several solid tumors. Results from this study suggest that fragility at an AT-dinucleotide containing region can be a triggering event in the generation of a carcinogenic chromosomal abnormality. Additional indirect evidence linking fragile sites containing unstable repeats with chromosome rearrangements comes from studies on individuals with malignant diseases. The breakpoints of the genomic rearrangements of cancer cells from these patients were either at or in close proximity to rare fragile sites including those that have been characterized at the molecular level and have been shown to contain secondary-structure adopting repeats (summarized in 97). In addition, a more systematic analysis of DNA sequences close to rearrangement breakpoints in different neoplasms showed that repeats that can form hairpin and cruciform structures are overrepresented (17). Finally, microarray-based genome-wide analysis revealed that DNA palindromes are abundant in human cancer cells and often co-localize with the chromosomal regions that are predisposed to oncogene amplification (98, 99).

Several recent studies in yeast have demonstrated that hairpin- and cruciform-forming sequences can induce almost all types of gross chromosomal rearrangements observed in cancer cells (40, 43, 68, 70). Based on the striking similarity between chromosomal rearrangements detected in yeast and human tumors, it is tempting to speculate that underlying mechanisms of instability might be conserved among eukaryotic cells. Narayanan *et al* (70) demonstrated that palindrome-mediated hairpin-capped DSBs can result in chromosomal arm loss, extrachromosomal and intrachromosomal gene amplification events. Both arm loss and intrachromosomal gene amplification were frequently accompanied by nonreciprocal translocations. This spectrum of rearrangements was governed by 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. Interestingly, the majority of the breakpoints of the resulting GCRs did not co-localize with the initial hairpin-capped break site and the sequence that triggered GCR was still present at the center of the duplicated or amplified regions. This observation might explain why some recurrent aberrations in cancer cells do not have structure prone sequence motifs at the rearrangement breakpoints. The yeast experiments indicated that it was possible to model events leading to cancer associated abnormalities, identify rules dictating specific patterns of rearrangements, predict the location of the causative secondary structure-forming sequences and uncover susceptible phenotypes. The mechanism of palindrome-mediated instability defined in yeast makes specific predictions about the structural organization of the aberrations. If the mechanism is conserved among eukaryotes, the predictions can be used in cancer genomic studies to unveil the origin and nature of the tumorigenic rearrangements.

4.1. Meiotic instability

4.2.1. Hairpin- and cruciform-forming repeats are hotspots for rearrangements

Repeats that can adopt hairpin and cruciform structures also induce instability during meiosis. As observed in mitotically dividing cells, meiotic instability is attributed to formation of the secondary structures and subsequent chromosomal breakage. Meiotic fragility in humans and model organisms is assessed by analyzing breakpoints of chromosomal rearrangements, measuring frequency of recombination in a region containing the unstable repeats and monitoring the DSB formation at the location of secondary structures. However, the analysis of breakpoints of rearrangements negates definite conclusions as to whether the fragility and rearrangements occurred during premeiotic or meiotic divisions in germline development. Although we discuss rearrangement breakpoint studies in "meiotic instability" section, one cannot exclude a mitotic origin of fragility.

In humans, palindromic AT-rich repeats (PATRRs) are hotspots for recurrent constitutional

translocations (reviewed in 100). These translocations can result in reproductive problems in carriers or in an inherited syndrome, neurofibromatosis type I. Three such PATRRs (11, 17, and 22) that induce human genome instability have been identified as of date. Analysis of the translocation breakpoints revealed that interchromosomal exchange occurs between different PATRRs or frequently involves other palindromic sequences on partner chromosomes. *De novo* translocations are readily detected by a PCR-based approach in sperm but not in somatic cells indicating that the breakage and exchange reactions involving PATRRs happens with high frequency during or after meiosis (101). The mechanism of palindrome-mediated translocations proposed by the authors invokes the center-break or cruciform resolution models discussed above. Based on the fact that breakpoints co-localize with the center of the palindromic sequences, the authors suggested that hairpins of the extruded cruciform or hairpin-capped DSB intermediates that resulted from cruciform resolution can be targets for cleavage by a putative nuclease. Two opened hairpins on different chromosomes can provide substrates for NHEJ. To this point it is unknown which enzyme is responsible for the cleavage, but the high efficiency of breakage in meiosis strongly implicates the Spo11 nuclease (100, 102 and discussed below). Consistent with these observations in humans, an extremely high level of instability of long palindromes has been observed in germline cells of transgenic mice (63, 103-106). The palindromic transgenes were found to be rearranged in 15 to 56% of the progeny. Often, asymmetric deletions in the center of palindrome and complex chromosomal rearrangements within or adjacent to the palindromic insert were recovered (similar to stabilization events observed in mitotic cells). The center-break mechanism was implicated to be responsible for the observed instability.

Another indication that unstable repeats might induce chromosomal breakage during meiotic divisions comes from studies of Jacobsen syndrome, a chromosome deletion disorder (107). Jacobsen patients have a deletion of the long arm of chromosome 11 from band 11q23 to the telomere. Some of the affected patients had a mother who is the carrier of the rare fragile site FRA11B. The breakpoints analyzed at the molecular level were mapped to expanded tracks of CCG repeats at the FRA11B or to the other CCG trinucleotide repeats in a neighboring distal region implicating that secondary structure was responsible for the fragility (108, 109).

Similar to analysis of somatic DNA breakpoints, the systematic analysis of DNA sequences involved in chromosomal rearrangements underlying human inherited diseases, have demonstrated that non-B DNA- forming motifs (including inverted repeats) are overrepresented at the breakpoint junctions supporting their role in the induction of rearrangements during germline development (17).

4.2.2. Evidence from yeast: Spo11-dependent and independent breakage

Studies in yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have provided direct proof for the fragility at secondary structures during meiosis. In addition, genetic and physical analyses in these model organisms have yielded important insights into mechanisms of the breakage. Both palindromes longer than 50 bp and CAG repeats of 79 copies were found to be hotspots for meiotic gene conversion in heteroallelic recombination assays (37, 42, 48, 110). It has been demonstrated that the ability of these sequence motifs to induce meiotic recombination is due to DSB formation within the repeated sequences. In S. *cerevisiae*, DSBs formed at the location of the inverted and triplet repeats require the topoisomerase II-like Spo11 protein, the nuclease that is also responsible for generation of DSBs at other meiotic recombination hotspots. Spo11 preferentially makes breaks at regions of open chromatin (e.g. those that are actively transcribing) (reviewed in 111). The authors proposed that extruded hairpin or cruciform structures change the local DNA architecture making it more accessible for the Spo11 attack (110). Similarly, in S.*pombe*, it has been shown that DSBs at or near 160 bp palindrome require Rec12 (ortholog of Spo11) (37). This latter study also identified an alternative pathway for palindrome-mediated DSB formation. In the *rec12Δ* mutant background, DSBs were detected at the time of premeiotic replication and were dependent on the activity of Mre11 complex. This result suggests that in the second, Spo11-independent pathway, hairpins formed during meiotic DNA synthesis are susceptible to cleavage by Mre11 complex thereby forming recombinagenic DSBs. The two yeast species thus differ in the way by which secondary structure-associated DSBs are generated in meiosis. It would be interesting to explore what mode of breakage is operating during meiosis in mammalian cells.

5. CONCLUDING REMARKS

With the growing body of data indicating that fragility at the location of secondary structure forming repeats is a contributing factor to eukaryotic genome instability, this area of research is gaining increasing attention. Elucidation of the molecular mechanisms underlying chromosomal fragility mediated by unstable repeats is clearly important for studying the predisposition of different individuals to diseases, the origin of inherited disorders, the cancer diagnostics and treatments as well as for our understanding of the fundamental processes that determine the architecture and dynamics of eukaryotic genomes. Despite the rapid progress in this field, there is still a dearth of knowledge. Below are some problems and questions that we anticipate will be addressed in the near future.

1. We are still lacking accurate data on the content and the distribution of hairpin- and cruciform-adopting sequences in eukaryotic genomes, including the human genome. The problems come from the innate difficulty in maintaining and analyzing the unstable sequences (112, 113). The conventional methods of cloning chromosomal regions containing secondary structures in wild type *E.coli* strains and subsequent PCR-based analysis might have generated a number of sequence information artifacts. An example is the palindrome-containing human

NF-1 locus which is misrepresented in GenBank due to corruption presumably in the *E.coli* cloning step (112). In addition, there are at least a thousand gaps still remaining in the reference human genome, most likely corresponding to regions that are not clonable in *E.coli*. These gaps are due to the inability to isolate clones in *E. coli* and hence may well represent sequences with the propensity to form secondary structures (114). Special precautions in the propagation of repeats should be followed and more reliable robust techniques have to be developed for the analysis. A promising alternative comes from using yeast as a host for cloning as it provides an environment where secondary structures are more stable than in bacteria (112, 115).

2. Hairpins and cruciforms are the central intermediates in the proposed models for the instability. The transient nature of the secondary structures imposes difficulties for their detection *in vivo*. Possibly, the identification of genetic backgrounds that promote the probability of the extrusion and/or the stability of the intermediates will help overcome these hurdles similar to how it was achieved in *E. coli* (116). Strains with defects in DNA replication, checkpoint surveillance, chromatin organization and remodeling are possible candidates.

3. Is there more than one pathway for the initiation of DSBs at unstable repeats? The three models presented above are not necessarily mutually exclusive and could function in different scenarios of hairpin and cruciform formation in eukaryotic cells.

4. Although the connection between fragility and aberrant replication is now clearly established, the exact sequence of events at the replication fork is still unclear. Firstly, what component of the replication machinery is perturbed by the secondary structure? Is it DNA polymerization or unwinding ahead of the fork? Secondly, if fragility is indeed induced by structure-specific nucleases, the attack might be a consequence of the activation of nuclease in response to compromised replication. In this case, mediators that sense replication arrest and transmit the signal for the breakage should exist. Alternatively, the faulty replication might generate optimal conditions for the hairpin/cruciform extrusion which would lead to two independent events: replication block and subsequent nuclease cleavage of the secondary structure. Mutant analysis coupled with detection of structural intermediates can aid to answer these questions.

5. The proteins involved in the initiation of DSB formation by unstable repeats in mitotically dividing eukaryotic cells are unknown. The identification of the players can be achieved by using computational, biochemical and genetic approaches. Yeast is an ideal model organism where this can be accomplished. One of the intriguing questions is whether the function of the putative nuclease is only to remove barriers from blocked replication fork or has other roles in DNA metabolism.

6. Does the same mechanism lead to DSBs in long inverted repeats and in long tracks of CTG/CAG repeats? In yeast, inverted repeats induce a unique kind of DSB that has hairpin-capped termini. What is the nature of the DSB intermediates in the case of triplet repeats? Currently there are only two clear examples of DSB formation associated with unstable motifs. Hence, it will be also important to look for other hairpin and cruciform-forming sequences that cause DSBs and to identify common patterns in their instability. For example, expanded CCG/CGG tracks can adopt not only hairpin and cruciform structures but can also form quadruplexes (20). Therefore, CCG-mediated fragility could be under different or additional genetic control in comparison with CTG/CAG and/or inverted repeats.

Future studies will shed more light on the underlying mechanisms of repeat-induced fragility. The research in yeast systems is particularly powerful because of the great ease in manipulating the genome. This implies that unstable sequences can readily be inserted at different chromosomal loci, the fragility can be monitored, and most importantly, the impact of different mutant backgrounds can be assessed. Given the striking similarity of the GCRs generated by unstable repeats in both humans and yeast, it seems likely that any information gleaned from yeast will provide insights into the origins of human pathology.

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