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Sites of genetic instability in mitosis and cancer

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Abstract

Certain chromosomal regions called common fragile sites are prone to difficulty during replication. Many tumors have been shown to contain alterations at fragile sites. Several models have been proposed to explain why these sites are unstable. Here we describe work to investigate models of fragile site instability using a yeast artificial chromosome carrying human DNA from a common fragile site region. In addition, we describe a yeast system to investigate whether repair of breaks at a naturally-occurring fragile site in yeast, FS2, involves mitotic recombination between homologous chromosomes, leading to loss of heterozygosity (LOH). Our initial evidence is that repair of yeast fragile site breaks does lead to LOH, suggesting that human fragile site breaks may similarly contribute to LOH in cancer. This work is focused on gaining understanding that may enable us to predict and prevent the situations and environments that promote genetic changes that contribute to tumor progression.

Keywords

fragile site; FRA3B; flexibility peak; mitotic crossover; loss of heterozygosity; cancer

Introduction

Genetic changes that alter the expression of genes that regulate cell growth or genes that maintain genomic integrity drive tumorigenesis. Although each tumor has a unique set of changes to cellular DNA, these alterations are not wholly random. Loss of heterozygosity (LOH) at particular tumor suppressor genes such as p53 and amplification of certain oncogenes such as MET are frequently observed. The mechanisms that underlie these recurrent changes have long been a subject of focused research. One group of genomic loci that frequently are found to be unstable in cancer cells (i.e., associated with deletions, amplifications, and translocations), and which can drive tumorigenesis, is comprised of common fragile sites (CFS). These sites are chromosomal loci that form gaps or breaks on metaphase chromosomes under conditions that partially inhibit replication.¹ In particular, CFS are highly sensitive to inhibition of DNA polymerases (for more discussion of other particular genomic zones that are prone to mutation under stressful environments, the interested reader is directed to the review by Rosenberg *et al*, see Ref. 33)

In a recent meta-analysis of databases of tumor-associated genetic changes, Burrow *et al.*² found that more than half of the reported cancer translocations have at least one breakpoint in a chromosome band containing a CFS. In addition, there are many reports of deletions within tumor suppressor genes that harbor CFS,^{3,4} and of CFS mapping to the borders of

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Conflicts of interest

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oncogenic amplicons that appear to result from breakage–fusion–bridge cycles.^{5,6,7} Mitotic sister chromatid exchange is frequently observed at CFS,⁸ which suggests that breaks at CFS could potentially also drive loss of heterozygosity (LOH) in cancer cells if the homologous chromosome is chosen for repair by recombination.^{9,10} A variety of agents when combined with replication stress, including caffeine, cigarette smoke, pesticides, chemotherapeutic drugs, and hypoxic conditions, also have been reported to increase the frequency of gaps and breaks at CFS, which may further drive cancer initiation or progression.¹¹

Analyses and comparisons of CFS to determine the reason for their instability have demonstrated that CFS are slow to finish the process of replication.^{12–15} Therefore, these loci apparently are difficult to replicate and are particularly sensitive to replication delay.

Hypotheses proposed to explain why CFS are unstable

Inhibition of replication causes uncoupling between helicase and polymerase proteins, resulting in the excessive accumulation of single-stranded DNA (ssDNA) at the replication fork. This model proposes that DNA sequences at CFS are particularly prone to forming secondary structures in this *single-stranded DNA* (ssDNA).⁸ These secondary structures further stall replication, and may lead to breaks either directly, by cleavage of the structure, or indirectly as a result of broken anaphase bridges formed at these regions because replication does not complete prior to cell division.¹⁶

Fragile sites are located at the boundaries between early- and late-replicating zones of the DNA. Replication forks from earlier-replicating zones may pause in CFS regions.^{17, 18} When replication is delayed, these paused forks may be prone to collapse into a DNA break, or, the nearby late-replicating region may not complete replication prior to chromosome condensation, leading to a break at this site.

There is a relative lack of origin initiation events within CFS regions. Conditions that slow polymerase progress result in the cell dividing before replication of the CFS region is complete, leading to DNA breaks.¹⁹

Many CFS are located within genes that are very large and take a long time to transcribe. Collisions between the RNA transcription machinery and DNA polymerase lead to breaks.²⁰

These four models partially overlap, and are not necessarily mutually exclusive. However, they make different predictions about the location of breaks within CFS regions (the CFS that have been molecularly characterized are large, from 200 Kb to a Mb or more, with breaks throughout⁸).

In particular, the first and second models suggest there may be certain sequences within CFS regions that are hotspots for breaks, such as sequences with high potential to form secondary structures and replication pause sites. For example, several CFS have been reported to contain a greater density of *flexibility peaks* relative to non-CFS regions.^{21, 22} Flexibility peak is a term used to describe an AT-rich region of DNA that is characterized by the potential for high twist angle between bases^{21, 22} (for more discussion of DNA destabilization as it relates to conformation and flexibility, the interested reader is directed to the review by Zhurkin and Benham, see Ref. 34). In the third and fourth models above, no particular sequence within a CFS would be expected to be a hotspot, but the third model may predict that breaks would be more likely to occur in a region farthest from the site of replication initiation.

In the work described here, to investigate the mechanism of CFS-induced breaks, we ask whether the flexibility peaks that have been identified within human CFS FRA3B are hotspots of instability. Second, to explore the consequences of CFS breaks, we investigate whether repair of fragile site breaks drives LOH events due to mitotic homologous recombination.

To gather detailed data on exact break locations within CFS, we used a yeast artificial chromosome (YAC) containing the human common fragile site FRA3B. This YAC does not contain any sequences required for yeast survival, and thus there is no selective pressure to retain particular regions of it. We modified the yeast carrying this YAC so that repair of breaks by telomere capping close to the break site is favored. Data described below suggest that break sites are not randomly distributed, but rather are clustered at the centromere-distal end of the FRA3B sequence insert.

To investigate mitotic homologous recombination, we take advantage of a naturally occurring yeast fragile site known as FS2 (fragile site 2). Similar to human CFS, recurrent breaks occur at FS2 under stressful conditions where replication is impaired.²³ Our results described below suggest that inhibition of yeast DNA polymerase does stimulate mitotic recombination between homologous chromatids with reciprocal crossovers at FS2, resulting in LOH.

Mapping break locations in a YAC containing human FRA3B sequence

We chose to examine FRA3B, one of the CFS most frequently broken in human lymphocytes, which is located within *FHIT*, a tumor suppressor gene on human chromosome 3. *FHIT* is large, encompassing more than 1.5 Mb, and FRA3B is a ~200 Kb region within this gene from approximately intron 3 through intron 5. CEPH cloning library YAC 850a6 contains 1.3 Mb of human sequence, including *FHIT* exon 1 through part of intron 5, encompassing the entire FRA3B region (Fig. 1).^{24–27}

We chose to map break locations in YAC 850a6 following replication stress in yeast as this system has several features designed to simplify interpretation. First, since the fragile site FRA3B is carried on the extra, artificial chromosome rather than the yeast's own chromosome, there is no selection against loss of part of this chromosome to preserve essential yeast genes. In addition, because the chromosome is carried as a single copy in a haploid yeast cell, our analysis is not complicated by the presence of a homologous copy of the sequence. Also, because the genomic background of the yeast cell is easily modified, we can make modifications to prevent repair processes that obscure the YAC break site. We made two modifications to the yeast genome to favor repair of breaks by telomere capping close to the break site. First, we inserted the *pif1-m2* mutation, which increases the frequency of telomere-capping.²⁸ Second, we deleted *EXO1*. Exo1p normally resects the 5' end at double-strand breaks, therefore deletion of *EXO1* results in shorter resection tracts; these shorter tracts are expected to be preferable substrates for the telomere-capping mechanism.^{29–31}

Human fragile sites break when cells are exposed to aphidicolin, a drug that inhibits DNA polymerases.¹ Although yeast are insensitive to aphidicolin, we can induce the stress of decreased DNA polymerase activity by employing a construct designed by Lemoine *et al.*,²³ in which polymerase alpha expression is controlled by the level of galactose in the media. In this construct, the *GAL1/10* promoter drives expression of the *POL1* gene (encoding the catalytic subunit of polymerase alpha). When grown on medium containing low levels of galactose, cells with this *GAL-POL1* construct have low levels of polymerase alpha, and breaks are stimulated at a naturally-occurring fragile site in yeast, FS2 (fragile site 2).²³

Thus, we used this system to induce breaks in the YAC carrying human FRA3B fragile site sequence by growing yeast cells on medium with low levels of galactose.

Genetic markers at the centromere-distal ends of each arm of the YAC facilitate identification of broken YACs by a phenotypic change (Fig. 1). The left arm is short and carries the *TRP1* marker gene and a yeast origin of replication (ARS), and the right arm carries the human DNA insert and the *URA3* marker gene. We also inserted another marker gene for hygromycin-resistance gene (*HPH*) distal to *URA3*. If a break occurs within the human DNA insert of the YAC, the cell retains *TRP1* but loses both the *URA3* and *HPH* genes. Loss of *URA3* results in resistance to 5-fluororotic acid (5-FOA), so cells with a broken YAC become auxotrophic for tryptophan and uracil, resistant to 5-FOA, and sensitive to hygromycin. Genomic DNA from colonies with the phenotype indicating a broken YAC is evaluated by PCR using 16 primer sets spaced each 25–50 Kb across the FRA3B insert (Fig. 1), with the break assumed to occur between the last primer set that amplified a product and the first set with no product.

In our initial data, in 23 independently isolated colonies, we identified breaks in the YAC only in the most centromere-distal region of the FRA3B insert, within *FHIT* intron 5: ten with a break between primer sets 13 and 14; three broken between sets 14 and 15; seven broken between sets 15 and 16; and three broken distal to primer set 16 (Fig. 1). Although FRA3B has been reported to exhibit breaks and gaps throughout a 200 Kb region,^{24,25} the clustered location of breaks in *FHIT* intron 5 that we observed in the YAC insert suggests that the tendency for break formation is not evenly distributed throughout the region, or that the process by which these breaks are repaired favors telomere capping in only this subregion of the sequence. We note that there are several peaks of high flexibility within the FRA3B DNA carried on the YAC. Mishmar *et al.*²¹ created the FlexStab program for analysis of flexibility; this program evaluates local variation in DNA twist angle for dinucleotide pairs in sliding 100 bp windows, with values summed for the window and averaged for window length. Windows that are more than 4.5 standard deviations from the average flexibility are considered flexibility peaks. Mishmar²¹ first reported that the human FRA7H region has a higher density of flexibility peaks than chromosomal bands lacking fragile sites, and later publications identified a similar pattern in other fragile sites,⁸ leading to a shared hypothesis in the field that these flexibility peaks may instigate instability in fragile sites. However, the breakpoints we mapped are not clustered in regions with such peaks. Thus, our results to date suggest that flexibility peaks are not favored break sites, or that breaks do not occur at the site where polymerase is stalled. Because there are two flexibility peaks between primer sets 14 and 15, and two peaks between sets 15 and 16, we plan to design additional primers to further narrow the break locations between these primer sets to determine whether breaks in these regions occur at flexibility peaks, and/or at other DNA sequences with high secondary-structure forming potential. It also is possible that flexibility peaks between primer sets 14 and 16 do stimulate breaks, but that the breakpoints mapped between primer sets 13 and 14 result from more extensive processing of the broken end by an exonuclease prior to telomere capping. Although we tried to avoid that by using cells mutant for *exo1*, further work is needed to examine whether other exonucleases, such as Sgs1p, might compensate in its absence.^{29–31} Finally, it is important to note that the breakpoint locations we have mapped are all at the end of the human DNA insert that is farthest from the yeast origin on the YAC. This result may support the third model for fragile site instability (lack of origin activation). If there is a lack of origin activation within the FRA3B region carried on the YAC, then the centromere-distal end of the insert is more likely to have difficulty completing DNA replication prior to cell division than the proximal end of the insert. However, this result may also support the first model for fragile site instability (secondary structure formation in extended ssDNA) if further narrowing of the

break locations reveals that breaks have occurred at a specific sequence that has a strong potential for intra-strand secondary structure formation.

Investigating whether fragile site instability stimulates mitotic recombination causing LOH

LOH in genomic regions with tumor suppressor genes is an important driver of tumor initiation and progression.³² Both deletion and mitotic recombination can result in LOH. Mitotic recombination has been understudied as a cause of LOH, because it has been technically challenging to recover both cells following a recombination event and because in mitosis, recombination events between homologous chromosomes is rare relative to recombination between sister chromatids. To overcome these technical challenges we chose to use a system recently developed in yeast that allows recovery of both cells following mitotic recombination between homologous chromosomes^{9,10} (for highlights of a recently developed system to analyze all cell products following meiotic recombination events between homologs in a mouse model system, the interested reader is directed to the review by Cole *et al.*, Ref. 35)

The yeast model system developed for analysis of mitotic recombination between homologous chromosomes affords us the opportunity to study whether recombination events resulting in LOH are stimulated by instability at fragile sites. This system uses diploid yeast that have ~ 0.5% sequence divergence between homologous chromosomes and which are homozygous for the *ade2-1* mutation.¹⁰ This mutation results in cells that are Ade⁻ and red in color, because in the adenine biosynthesis pathway, the substrate bound by the Ade2p enzyme is a red molecule. The particular mutation in *ade2-1* is an ochre stop codon, which can be suppressed by the SUP4-o tRNA. By placing the *SUP4-o* gene on only one homolog of a chromosome of interest, the phenotype of the cell is then white and Ade⁺ (Fig. 2). When these cells are plated, mitotic recombination events that cause LOH at the *SUP4-o* locus will result in red/white sectorized colonies (Fig. 2).¹⁰ We have placed the *SUP4-o* gene on the distal end of the right arm of one homolog of chromosome III. This chromosome contains the naturally-occurring yeast fragile site, FS2, which mimics the instability at CFS in human cells because breaks at the site are stimulated by partial inhibition of replication.²³

To study whether breaks at FS2 stimulate mitotic recombination events resulting in LOH, we inserted the *GAL-POL1* construct into yeast with the red/white detection system. As described earlier, when cells are grown in medium with low levels of galactose, this construct causes replication stress by lowering the level of polymerase alpha.²³ Cells are grown for 6 h in low galactose media, and then plated for single colonies on medium with high galactose. Colonies that appear as red/white sectors are selected for further analysis. A single cell is purified from each half of the sector, and expanded for genomic DNA harvest. Since the starting diploid strain has ~0.5% sequence divergence, single nucleotide polymorphisms that differ between the two homologous copies of chromosome III are evaluated to identify the type of event resulting in LOH, and the location on the chromosome where that event was initiated. For example, as illustrated in Figure 2, a reciprocal crossover (RCO) will result in homozygosity of all SNPs distal to the crossover, in both the red and white cells. In contrast, a gene conversion event that is not associated with a crossover will result in one cell remaining heterozygous at all SNPs, while the other will become homozygous for SNPs in the region of gene conversion. A break-induced replication event will result in one cell remaining heterozygous at all SNPs, while the other becomes homozygous for SNPs distal to the event.

To date we have analyzed 15,085 colonies from cells exposed to replication stress due to decreased expression of *POL1*. Of these, 38 colonies appeared as red/white sectors. Seven of

the sectored colonies resulted from RCOs on the right arm of chromosome III, a frequency of 5.84×10^{-6} RCO/kb. This is a 10-fold increase over the spontaneous RCO frequency of 3.36×10^{-7} /kb observed by Barbara and Petes.⁹ on the left arm of yeast chromosome V (there are no fragile sites in this region). Six of the RCOs we obtained are located at fragile site FS2, and one is centromere-distal to FS2. Thus, our initial results support the hypothesis that instability at fragile sites stimulates mitotic recombination resulting in LOH. The remaining 31 sectored colonies all result from break-induced replication events. It is interesting that break-induced replication events are more frequently observed than RCOs. Because break-induced replication is thought to be favored when only one side of a double-strand break is available for repair, we hypothesize that one-ended breaks are frequent in our system as a result of the particular type of replication stress used. A low level of polymerase alpha is expected to cause replication fork stalling, and collapse of a stalled fork would produce a one-end double-strand break.

Discussion

CFS are a normal part of human chromosome structure, yet instability at these loci under conditions of replication stress may contribute to genomic changes that are involved in both tumor initiation and progression. Because CFS lack a defining linear base sequence motif that might point to a clear mechanism for how breaks form in these regions, extensive research has been undertaken to investigate multiple models that have been proposed to explain these sites of genomic instability. All of these models account for the fact that one characteristic common to all CFS studied at the molecular level is late completion of DNA replication. These models include replication fork stalling in regions of DNA sequence with a high tendency to form intrastrand secondary structures; replication dynamics at early/late replication transition zones; origin initiation paucity; and transcription-replication collisions.^{8,16–20} The first two models imply that certain regions within CFS loci are expected to be hotspots for breaks. Using as a model system yeast with a YAC carrying human fragile site DNA, our initial results reported here suggest that DNA flexibility peaks (defined as regions identified with a 100bp sliding window to have an average twist angle above 13.7°), do not appear to be favored break sites. We plan to conduct further fine-structure analysis to identify additional breakpoints, as well as to examine other DNA sequences that tend to form secondary structures. Further research will enable us to determine which of these four models is most appropriate, or whether CFS are not a unified group but instead can be subdivided based on the model that best explains the instability of CFS with distinct properties.

The connections between CFS and genomic changes in cancer have been studied extensively. Research in this area has been focused primarily on describing the changes present at CFS in cancer cells and on determining whether genes in or near CFS are tumor suppressors or oncogenes.^{3–11} Instability at CFS clearly results in deletion, amplification, and translocation in tumors. The possible contribution of CFS to loss of heterozygosity (LOH) by stimulating mitotic recombination, as suggested by the work reported here, calls for further investigation. Our yeast model system facilitates detailed study of mitotic recombination events between homologous chromosomes when subjected to replication stress. Cancer cells too are likely to be dividing under conditions of replication stress, and many cancer treatments cause replication stress. Thus, it is important to understand the extent of, and conditions that affect, fragile site stimulation of LOH in tumors, which contributes to tumor evolution and progression.

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Figure 1.

Structure and characteristics of YAC 850a6 and its human DNA insert. (A) YAC 850a6 carries a 1.3Mb insert of DNA from human chromosome 3. The YAC also has a *TRP1* marker gene and a yeast origin of replication (ARS) on the left arm, and *URA3* and *HPH* markers on the right arm centromere-distal to the human DNA insert. (B) The human *FHIT* gene has ten exons, which are numbered and represented by short vertical bars. The FRA3B common fragile site is located within *FHIT*. The boundaries of this fragile site are not well defined, but there is general agreement that it is ~200 Kb in size and spans from *FHIT* intron 4 through part of intron 5. Red dotted lines indicate the portion of the *FHIT*/FRA3B region that are carried on YAC 850a6. (C) DNA flexibility in the *FHIT* gene from exon 3 through the portion of intron 5 carried on the YAC was analyzed using a 100bp sliding window. Regions with a twist angle deviation over 13.7° (top dotted line) are considered flexibility peaks because they are more than 4.5 standard deviations from the average flexibility.²¹ The locations of primer sets 1–16 used in the analysis of broken YACs are shown as yellow arrows. The locations of breaks in the YAC are identified by red squares. Each red square

represents the last primer set to produce an amplified product from one of the 23 broken YACs analyzed.

Figure 2.

System for analysis of mitotic recombination between homologous chromosomes. (A) The starting yeast strain is diploid and is homozygous for the *ade2-1* mutation, which causes a block in the adenine biosynthesis pathway and accumulation of a red-pigmented intermediate molecule. Only yeast chromosome III from this diploid is shown. One copy of chromosome III contains the naturally occurring yeast fragile site FS2. The homologous copy of chromosome III does not contain this fragile site, but does have the *SUP4-o* tRNA gene inserted as shown. This tRNA suppresses the *ade2-1* mutation, thus the starting strain is Ade⁺ and white in color. (B) A reciprocal crossover at the FS2 locus during mitotic cell division is shown. (C) In half of all reciprocal crossover events, chromosome segregation

will result in the pattern shown, causing a red/white sector colony in which chromosome III in each half of the sector is homozygous for all SNPs centromere-distal to the crossover. The left cell is red because the *ade2-1* mutation is not suppressed (no *SUP4^o* gene is present). As a result of the crossover, both copies of the *SUP4^o* gene are in the right cell, making it white. Other mitotic recombination events that result in red/white sectoring, not shown but discussed in the text, include gene conversion and break-induced replication.