Identification of Unstable Sequences within the Common Fragile Site at 3p14.2:
Implications for the Mechanism of Deletions within Fragile Histidine Triad Gene/Common Fragile Site at 3p14.2 in Tumors

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ABSTRACT

The FRA3B, at 3p14.2, lies within the fragile histidine triad (FHIT) gene and is the most highly expressed of the common fragile sites observed when DNA replication is perturbed by aphidicolin. Common fragile sites are highly unstable regions of the genome. Large intragenic deletions within FHIT, localized within the FRA3B sequences, have been identified in a variety of tumor cells. To characterize the FRA3B deletions in tumor cells and identify FRA3B sequences that are required for fragile site induction, we used microcell-mediated chromosome transfer to isolate hybrid cell clones that retain chromosome 3 homologues with various deletions within FRA3B. Detailed molecular mapping of the FHIT/FRA3B locus in the resultant hybrid cells revealed a complex pattern of instability within FRA3B. Each tumor cell line contained multiple chromosome 3 homologues with variable deletion patterns, often with discontinuous deletions, suggesting that the process of breakage and repair within FRA3B is an ongoing one. By comparing the approximate location of the breakpoints in the hybrid clones, we identified 11 recurring breakpoint/rearrangement sites within the FRA3B. A comparison of the frequency of breaks/gaps within FRA3B in the hybrid clones with various deletions of FRA3B sequences revealed that the loss of FRA3B sequences does not reduce the overall rate of breakage and instability within the remaining FRA3B sequences. The majority of breaks occurred in the proximal portion of the FRA3B, in a 300-kb interval between exon 4 and the proximal 50 kb of intron 5. Our observations suggest that there is no single sequence within the FRA3B that influences breakage or recombination within this region; however, we cannot rule out the presence of multiple “hot spots” within the FHIT/FRA3B locus. Together, the results suggest that factors other than the DNA sequence per se are responsible for the formation of DNA breaks/gaps.

INTRODUCTION

Chromosomal fragile sites are specific loci that show gaps, breaks, or rearrangements in metaphase chromosomes when cells are cultured under conditions that inhibit DNA replication (low folate or thymidine levels or in the presence of chemicals, such as aphidicolin; Refs. 1–6). Common fragile sites exhibit several features characteristic of highly unstable or recombinogenic regions of the genome. In addition to forming breaks and gaps on metaphase chromosomes, they are preferred sites for sister chromatid exchanges (7, 8), chromosomal deletions and rearrangements (9, 10), and the integration of transfected plasmid DNA (11). Fragile sites have also been implicated in the initiation and perpetuation of breakage-fusion-bridge cycles, leading to gene amplification in Chinese hamster cells in vitro (12). On the basis of the concordance of their location with chromosomal breakpoints seen in tumor cells, it was hypothesized that fragile sites, particularly the common fragile sites, may play a mechanistic role in the recurring chromosomal rearrangements and genetic changes observed in tumor cells (5, 13).

The molecular cloning and sequencing of several aphidicolin-induced common fragile sites has facilitated a detailed analysis of their behavior in normal and malignant cells, as well as the opportunity to examine the mechanisms leading to their instability. FRA3B at 3p14.2, the most highly expressed common fragile site, was isolated using four different approaches. Wilke et al. (14) used a positional cloning approach and FISH to identify a yeast artificial chromosome (850A6) that crossed FRA3B, and they identified a large region of >100 kb that is prone to form chromosome breaks/gaps. This region contains an HPV16 viral integration site and is ~160 kb telomeric of the t(3;8) breakpoint seen in a family with hereditary renal cell carcinoma (Fig. 1A). Smith et al. (15, 16) mapped a series of aphidicolin-induced breakpoints in chromosome 3-containing somatic cell hybrids to the same yeast artificial chromosome and identified two clusters of breaks flanking the HPV16 viral integration site. By cloning sequences surrounding plasmid DNA (pSV2neo vector) integrated after being transfected into cells treated with aphidicolin, we identified fragile site sequences ~350 kb distal to the t(3;8) breakpoint (17).

Ohta et al. (18) cloned the FHIT gene from the same region, and it is now known that FHIT spans FRA3B. The FHIT gene spans 1.2 Mb but encodes only a 1.1 kb transcript; the FHIT protein is a hydrolase involved in the cleavage of diadenosine triphosphates (19). Large intragenic deletions within FHIT, localized within the FRA3B sequences, have been identified in a variety of tumor cells (1, 18, 20). More recent analysis of other common fragile sites, FRA7G and FRA16D, have revealed a similar pattern of deletions within tumor cells, suggesting that this is a common feature of these genetically unstable regions of the genome (21–25).

In the FHIT/FRA3B locus, genomic breakage and instability extends from the t(3;8) breakpoint in intron 3 to a region ≥500 kb telomeric in intron 5 (14, 15, 17). In this study, we refer to this region as the “FRA3B” (Fig. 1A) with the understanding that aphidicolin-induced chromosomal breakage can occur outside of this region in a low percentage of cells and that deletions of FHIT sequences 5’ or 3’ of this interval have been identified in rare tumors or tumor cell lines. Detailed characterization of the DNA sequences located at a number of deletion breakpoints in tumor cell lines has suggested that the breakpoints may cluster at a limited number of sites within FRA3B (20, 26–30). However, these breakpoints do not correlate with the location of the various fragile site landmarks, i.e., the aphidicolin breakpoint clusters, the HPV16 integration site, and the pSV2neo integration sites. Whether FRA3B is comprised of a single large
unstable region or multiple, smaller, “hot spots” for DNA recombination or breakage is unknown. Similarly, the identity of the sequences required for fragile site expression (breaks/gaps) has not been determined.

To identify the sequences within FRA3B that are required for fragile site induction, we have made use of the deletions occurring within the FHIT/FRA3B region in tumor cells. Our approach was to use microcell-mediated chromosome transfer to isolate hybrid cell clones that retain chromosome 3 homologues with various deletions within FRA3B, followed by an analysis of the breaks/gaps induced by aphidicolin treatment. Our results revealed a complex pattern of instability within FRA3B. Each tumor cell line yielded multiple classes of hybrids with different deletion patterns. Moreover, loss of FRA3B sequences does not detectably reduce the overall rate of breakage and instability within the remaining FRA3B sequences.

**MATERIALS AND METHODS**

**Cell Lines.** Four tumor cells lines were analyzed. Two cell lines (SiHa, CC19) were derived from cervical carcinomas; one cell line (H211) was derived from a small cell lung carcinoma, and one cell line was derived from a gastric carcinoma (Kato III). The deletions in 3 cell lines (SiHa, H211, and Kato III) have been described previously (27, 28, 31). Cells were maintained in 90% DMEM, 10% fetal bovine serum, 10 mM HEPES, and 100 µg/ml Streptomycin (pH 7.2–7.3; Invitrogen Life Technologies, Inc., Carlsbad, CA) in a humidified 95% air/5% CO2 atmosphere (37°C).

Urd-C is a CHO cell line that is a mutant for the UMP synthase gene located on the long arm of human chromosome 3 and was kindly provided by David Patterson (University of Colorado). Urd-C cells were maintained in 95% Ham’s F-12, 5% fetal bovine serum, 3 × 10^{-3} M uridine, 10 mM HEPES, 100 units/ml penicillin, and 100 µg/ml Streptomycin (pH 7.2–7.3; Invitrogen Life Technologies, Inc.). The CHO-human somatic cell hybrid, H3-4, was derived from Urd-C cells and contains a rearranged human chromosome 3, der(3)(q33)p2–>q21;q26.2–>qter, as the only human component (kindly provided by Harry Drabkin, University of Colorado). The short arm of the der(3) is normal, whereas the long arm is rearranged. H3-4 cells were maintained in 90% DMEM, 10% fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin, and 100 µg/ml Streptomycin (pH 7.2–7.3; Invitrogen Life Technologies, Inc.).

**Microcell-mediated Chromosome Transfer.** To isolate deleted chromosome 3 homologues, microcell-mediated chromosome transfer was performed using a modification of the method of Sanford and Stubblefield (32). Hybrid cells were grown in selective media without uridine [95% Ham’s F-12, 5% fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin, and 100 µg/ml Streptomycin (pH 7.2–7.3)] to select for the retention of human chromosome 3 containing the UMP synthase gene and in ouabain (1 µM for 48–72 h) to eliminate residual donor human cells.

**Induction of Fragile Sites.** Expression of FRA3B was induced by treating somatic cell hybrids in log phase growth with aphidicolin (0.4 µM for 26 h; Sigma, St. Louis, MO) or aphidicolin (0.4 µM) plus caffeine (1 mM for 26 h; Sigma) at 37°C. Metaphase cells were prepared using standard techniques, and the chromosomes were banding. Hybrid cell lines were identified by using a trypsin–Giemsa banding technique as described (33). Metaphase cells were scored for the presence of breaks/gaps by analyzing 25 cells/cell line in a blinded fashion.

**FISH.** FISH was performed as described previously (34). Labeled PAC or BAC probes were prepared by nick translation using Bio-16-dUTP (Enzo Diagnostics, New York, NY), or digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN), and detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA), or rhodamine-conjugated antidigoxigenin antibodies (Boehringer Mannheim), respectively. Chromosomes were identified by staining with 4,6-diamidino-2-phenylindole dihydrochloride. Centromere-specific and whole chromosome painting probes for human chromosome 3 were obtained from Vysis, Inc. (Downers Grove, IL; Cep3 Spectrum Green and WCP3 Spectrum Green).

**PCR Analysis.** A total of 35 STSs spanning 1.2 Mb of the FHIT/FRA3B locus was analyzed, including markers for exons 1–10 of the FHIT gene (Fig. 1A). Of these, 25 STSs were within the FRA3B. The complete list of STSs, primer sequences, and PCR conditions is available on the laboratory’s web site.5 PCR reactions were carried out in 20 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM deoxynucleotide triphosphates, 1.25 units of Taq polymerase, 0.5 pmol of each primer, and 20 ng of DNA. PCR conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at an optimized temperature between 58°C and 65°C for 30 s, and an extension at 72°C for 45 s, with a final extension at 72°C for 5 min. The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

**Identification of PAC/BAC Clones from the FHIT/FRA3B Region.** A human PAC library (Genome Systems, Inc., St. Louis, MO) was screened using primers for FHIT exons 1–6 as described previously (35). BAC RPC11-16B5 was obtained from the BACPAC Resources Center at the Roswell Park Cancer Institute.6 BAC 254E1 containing D3S4103 was described by Fang et al.  

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5 Internet address: http://lebeaulab.uchicago.edu.
6 Internet address: http://baccpac.med.buffalo.edu.
RESULTS

Mapping of Molecular Deletions within FHIT/FRA3B. To identify the sequences within FRA3B that are required for fragile site induction, we have made use of the naturally occurring deletions within the FHIT/FRA3B in tumor cells. Because of the differing genetic backgrounds of the tumor cell lines, which may affect FRA3B induction, our approach was to transfer chromosome 3 homologues with various deletions within FRA3B into CHO cells (Urd-C) by microcell-mediated chromosome transfer. Multiple individual hybrid clones were isolated from each cell line, retaining at least one copy of each marker within FHIT/FRA3B tested. This type of analysis detects the loss of FHIT/FRA3B sequences that are deleted on all chromosome 3 homologues present in the cell population; however, it does not provide information on the configuration of FRA3B sequences for individual chromosomes.

To map the deletions of FRA3B sequences and identify the FRA3B sequences required for genetic instability, we generated CHO X human somatic cell hybrids using microcell-mediated chromosome transfer. Somatic cell hybrids were generated using CHO cells (Urd-C) by transferring chromosome 3 homologues with various deletions within FRA3B into CHO cells (Urd-C) by microcell-mediated chromosome transfer. The Urd-C cell line is deficient in UMP synthase, encoded by a gene on human 3q, thereby allowing for selective retention of this chromosome.  

To facilitate mapping of the deleted segments within FHIT/FRA3B, we generated PCR primers for 35 STSs spanning the 1.2 Mb FHIT/FRA3B locus, as well as a physical map of the interval (Fig. 1). The STSs include FHIT exons 1-10 and 25 STSs within FRA3B. PCR analysis of 9 tumor cell lines revealed the presence of homozygous deletions within FHIT/FRA3B in 8 lines (Fig. 2). The Caski cell line retained at least one copy of each marker within FHIT/FRA3B tested. Eleven recurring breakpoint/repair regions observed in two or more hybrid clones are identified by arrowheads at the bottom of the figure (arrowheads A-K).

Fig. 2. Map of the deletions within FHIT/FRA3B in tumor cell lines and microcell-mediated somatic cell hybrids. Top bar, the 35 STS markers used to map the locus by PCR analysis of parental tumor cell lines and somatic cell hybrids. The deletions observed in tumor cell lines and hybrids are shown beneath the top bar; solid bars, the marker was retained, whereas dashed bars, the sequences were deleted. Five tumor cell lines are illustrated in the top portion of the figure. Three cell lines were derived from cervical carcinomas (Cassi, C33A, and MS751), 1 was derived from a non-small cell lung carcinoma (H1792), and 1 cell line was derived from a colon adenocarcinoma (LoVo). Grouped below are the results for 4 additional tumor cell lines (H211, SiHa, CC19, and Kato III) and the derivative somatic cell hybrids retaining a single chromosome 3 homologue isolated by microcell-mediated chromosome transfer. Multiple individual hybrid clones were isolated from each cell line, 10 from H211, 9 from SiHa, 8 from CC19, and 4 from Kato III cells. In many cases, the same chromosome 3 homologue was identified in multiple clones, e.g., five SiHa hybrid clones (SiHa-17,34,39,40,41) had the same deletion. Eleven recurring breakpoint/repair regions observed in two or more hybrid clones are identified by arrowheads at the bottom of the figure (arrowheads A-K).
some 3 was confirmed by FISH of a centromere-specific probe for chromosome 3. To identify translocations between human chromosome 3 and hamster chromosomes, we performed FISH of a chromosome-painting probe for chromosome 3; no translocations or rearrangements were found. Thirty-one hybrid clones retaining a single human chromosome 3 were selected for further analysis.

The deletions within the FRA3B sequences were mapped by PCR analysis (Fig. 2). In each cell line, we identified multiple configurations of chromosome 3. Because the number of FHTIFRA3B configurations exceeds the number of chromosome 3 homologues in each cell, this is likely to be attributable to clonal heterogeneity within the lines and raises the possibility that the process of breakage and repair within FRA3B is ongoing within tumor cell populations. In many cases, the same chromosome 3 homologue was identified in multiple clones, e.g., five clones isolated from the SiHa cell line (SiHa-17,34,39,40,41) had the same deletion within FRA3B extending from D4260 to D3S1234 (Fig. 2). In total, we characterized 18 recognizably different chromosome 3 homologues with various deletions within FRA3B.

The deleted segments extended from intron 3 to D3S1234 (this marker is 108 kb proximal to FHTI exon 6, which was typically included from the deleted sequences). In general, there was good agreement between the sequences that were homozygously deleted in the original parental tumor cell line and the configurations of the FRA3B sequences in the hybrid cells (see H211 and Kato III in Fig. 2). However, in a few cases, sequences were retained in the parental cell line that were predicted to be deleted, based on the analysis of the individual hybrids (marker D3S1300 in the SiHa clones). This observation suggests that there are additional chromosome 3 homologues in this tumor cell line with other configurations of FRA3B sequences that are not represented in the hybrid clones.

As described previously by a number of investigators, the deletions within the FHTIFRA3B region were discontinuous in many hybrid clones (26, 29, 31). This phenomenon may result from the repair of double-strand breaks within FRA3B by cellular repair processes, particularly nonhomologous recombination or single-strand annealing. By comparing the approximate location of the breakpoints in the hybrid clones, we identified 11 breakpoint regions (arrows A–K in Fig. 2) within the FRA3B that occurred in hybrid clones derived from ≥2 of the tumor cell lines.

Identification of Genetically Unstable Sequences within FHIT/FRA3B. To identify the FRA3B sequences that are required for manifestation of breakage and instability, we induced the expression of fragile sites in hybrid cells with aphidicolin or aphidicolin plus caffeine treatment and compared the number of breaks at FRA3B in selected hybrid cell lines with deletions of various segments of the FRA3B. Metaphase cells were examined (25/cell line for each culture condition) to determine the total number of breaks/gaps and the number of breaks at human 3p14.2 (Fig. 3). To evaluate the degree of fragile site expression, we used the aphidicolin-inducible fragile site at hamster 1q26 as a control. This site is the most frequently expressed common fragile site in the hamster genome (12); however, as we noted previously, the breakage rate at this fragile site is lower than that of the human FRA3B in somatic cell hybrids (11). The CHO-human somatic cell hybrid, H3-4, containing a rearranged human chromosome 3 with an intact p arm was cultured and processed in parallel with the hybrid cell lines to determine the frequency of breaks and gaps at the FRA3B. PCR analysis of H3-4 using the 35 STSs confirmed that the FHIT/FRA3B locus was intact with no deletions detected.

For each hybrid cell line, untreated cells were cultured and examined in parallel with cells exposed to aphidicolin (0.4 μM) or aphidicolin plus caffeine (1 mM) for 26 h before metaphase cell preparation. The incidence of spontaneous breakage was very low (range of 0–15 total breaks/gaps per cell line, mean 5.5 breaks/25 cells/line), as was the incidence of breakage at the FRA3B (3 of 375 cells, 0.8%). In contrast, the mean percentage of cells with FRA3B breaks in H3-4 cells exposed to aphidicolin was 22% (range 16–32%). This figure is comparable with the percentage of human metaphase cells that express FRA3B in healthy individuals (19%; Ref. 2).7

7 Le Beau et al., unpublished observations.
The results of the analysis of hybrid cells with deletions of FHIT/FRA3B sequences are given in Fig. 3. Our results revealed that the level of breakage within the FRA3B was relatively consistent, regardless of the size or location of the deleted sequences, e.g., chromosome 3 homologues with smaller deletions of the 3′ end of the FHIT/FRA3B locus, as well as those with larger deletions that encompass most of the FRA3B, are still capable of expressing breakage at a comparable frequency (compare H211 clones 7, 15, and 10 with H211-3 and 40 or with SiHa-39 and 40). We examined only a single cell line with a deletion of the 5′ FHIT/FRA3B sequences (CC19-4); thus, we cannot draw conclusions about the influence of loss of 5′ versus 3′ FHIT/FRA3B sequences.

Caffeine abolishes the G2-M DNA damage checkpoint by inhibiting ATM and ATR kinase activities and, hence, the ATM/CHK2 pathway. Cells cultured in the presence of aphidicolin plus caffeine show an increased number of cells expressing fragile sites and an increased number of breaks per cell. To enhance the induction of fragile sites, we treated the hybrid cells with aphidicolin plus caffeine. Similar to the hybrid cells cultured with aphidicolin alone, the breakage rate and pattern in the hybrid cells did not differ appreciably from that of the H3-4 control cells with intact FRA3B sequences.

Although the boundaries of the genetically unstable sequences comprising the FHIT/FRA3B have not been mapped precisely, the region of peak instability (referred to here as the “FRA3B”) is known to extend from the breakpoint of the t(3;8), ~65 kb centromeric of exon 4, to a region ~120 kb telomeric of exon 5 (14, 17, 37). Thus, even our hybrids with large deletions of most of the unstable region retain FRA3B sequences at the centromeric and/or telomeric portion (H211-3, H211-40, SiHa-39, SiHa-40, and KatoIII-H1). Our observation of comparable levels of aphidicolin-induced breakage at the FRA3B in these hybrid cells and in cells with an intact fragile site region suggests that deletion of part of the FRA3B simply results in a shift of the chromosome breaks to another portion of the FRA3B, without altering the overall breakage rate.

To evaluate this hypothesis, we used dual-color FISH of the PAC and BAC probes spanning the FHIT/FRA3B locus (Fig. 1B) to determine precisely where the breaks occur in hybrids with internal deletions of the FRA3B. Five hybrid cell lines were examined, including 1 with a large deletion leaving only a small amount of 5′ and 3′ FRA3B sequences (H211-3), 1 with loss of 5′ FRA3B sequences (CC19-4) and 1 with loss of 3′ FRA3B sequences (H211-7; Fig. 4).

In general, the results supported the hypothesis that the observed breaks/gaps occurred in residual FRA3B sequences; however, there were several interesting observations: (a) chromosome breaks occurred at a higher frequency in the proximal FRA3B sequences than in the distal sequences. An example is H211-3, which retained only a small amount of FRA3B sequences at the 5′ end (FHIT exon 4-D3S4260) and ~30 kb at the 3′ end (APO37-AFO14). Although the number of breaks was small, all occurred centromeric of BAC clone B254E1. Similarly, in hybrid CC19-11 with several small, discontinuous deletions, most breaks occurred proximal to BAC clone B254E1, i.e., proximal to exon 5 and D3S1300; and (b) chromosome breaks were observed both proximal and distal to the region of peak instability in the FRA3B sequences (note the breaks proximal to exon 1 and distal to exons 6 and 7 in CC19-11 and CC19-4). This finding raises the possibility that the FRA3B sequences may be larger than recognized previously. Alternatively, we cannot rule out the possibility that deletions within FRA3B may impact on the stability of surrounding sequences.

We evaluated the position of the breaks at 3p14.2 relative to the FRA3B sequences. When the segmental location of the break could not be determined precisely, we conservatively assigned it to the most central location of the interval. A total of 59 breaks/gaps were observed at 3p14.2. Of these, 10 (17%) occurred centromeric to FHIT exon 1 proximal to known FRA3B sequences, 36 (61%) occurred in the 5′ portion of the FRA3B between FHIT exon 3 and D3S1300, 8 (14%) occurred in the 3′ portion of the FRA3B (D3S1300 to exon 6), and 5 (8%) breaks were distal to exons 6 and 7 and the known FRA3B sequences. Together, our analysis of fragile site expression in chromosome 3 homologues with various deletions of FHIT/FRA3B sequences suggests that loss of part of the genetically unstable FRA3B sequences does not substantially alter the subsequent breakage rate and that chromosome breaks and gaps occur at the highest frequency in the 300-kb interval between exon 4 and D3S1300.

DISCUSSION

The FRA3B at 3p14.2 is the most frequently observed common fragile site in the human genome. Although fragile sites were viewed originally as a cytogenetic phenomenon, the molecular cloning and characterization of several aphidicolin-inducible fragile sites have facilitated a detailed analysis of their behavior in malignant cells. These studies have led to the recognition that rearrangements within the FHIT/FRA3B locus are common in a variety of tumors, including carcinomas of the breast, ovary, pancreas, esophagus, kidney, and colon (1, 20). Although most of the rearrangements reported to date are large deletions on the order of hundreds of kb, translocation breakpoints have also been identified within FHIT/FRA3B (30). Most of the previous studies have used PCR analysis to identify homozygously deleted sequences within the FRA3B. A limitation of this approach is that although it detects the loss of FHIT/FRA3B sequences that are deleted on all chromosome 3 homologues present in the cell population, it does not provide information on the configuration of FRA3B sequences on individual chromosomes.
To map the deletions within FRA3B in tumor cells and identify the FRA3B sequences that are required for fragile site induction, we used microcell-mediated chromosome transfer to isolate hybrid cell clones that retain a single chromosome 3 homologue with various deletions within the FRA3B. By detailed molecular mapping of the resultant hybrid clones using PCR analysis of STS markers spanning the FRA3B locus, we identified 18 chromosome 3 homologues with variable deletions within FHIT/ FRA3B. Our analysis revealed a complex pattern of instability within FRA3B. Each tumor cell line contained multiple chromosome 3 homologues with different deletion patterns, and discontinuous deletions were common. In a number of instances, the pattern of discontinuous deletions in hybrid clones from the same parental line was similar except that one chromosome 3 had lost additional sequences. The heterogeneity observed raises the possibility that breakage and repair within FHIT/FRA3B in tumor cells is an ongoing process. If so, this type of genetic instability has important ramifications in that it may lead to altered function of genes located within common fragile sites during tumor initiation or progression.

By using inverse-PCR, Inoue et al. and Mimori et al. have mapped the deletion end points in 10 cell lines, including the H211, SiHa, and Kato III cell lines examined in our study. These investigators defined deletion end points, representing the two chromosome 3 homologues in each cell line; however, our findings indicate that each tumor cell line and, by inference, primary tumors contain a spectrum of chromosome 3 homologues with varying deletions. Of note is that the deleted homologues we identified in the H211 and SiHa cell lines correspond closely to the deletion end points identified by these investigators (compare H211-3 and 40 with H211 allele a; H211-7,23,15,20,10,24,34,38 with H211 allele b; SiHa-11,18 with SiHa allele a; and SiHa-10 with SiHa allele B; Ref. 30). However, our results obtained for the Kato III cell line do not match the deletion end points identified by Inoue et al. (27), raising the possibility that we have not identified all of the deletion configurations for this cell line or that the cells examined in the two laboratories have evolved independently.

To identify the FHIT/FRA3B sequences that are required for manifestation of breakage and instability, we compared the number of aphidicolin-induced breaks at 3p14.2 in selected hybrid cell lines with deletions of various segments of the FRA3B. Because human tumor cells are deficient frequently in DNA repair processes and manifest genetic instability, an advantage of our approach is that each chromosome 3 homologue has been removed from the influence of the genetic background of the original tumor cells and introduced into the same CHO cell background. Our results suggest that partial loss of FRA3B sequences does not appreciably reduce the overall rate of breakage and instability within the remaining FRA3B sequences.

To determine where aphidicolin-induced breaks occurred in chromosome 3 homologues with loss of sequences within FRA3B, we used dual-color FISH of pairs of PAC/BAC probes from this region. These studies revealed that aphidicolin-induced breaks occurred in residual FRA3B sequences, as well as proximal and distal to known FRA3B sequences. Moreover, the majority of breaks occurred in the proximal portion of the FRA3B in the 295-kb interval between exon 4 and AFO101 (AFO101 is within intron 5, 49 kb telomeric to exon 5). This observation is consistent with those of several other studies, which examined the distribution of aphidicolin-induced breaks within FRA3B. By using probes spanning the FHIT/FRA3B locus and extending >500 kb proximal for FISH analysis, investigators have demonstrated that aphidicolin-induced breaks occur over a large region, perhaps as large as 2 Mb. However, most of the breaks (~60%) occur over an ~300-kb region between exon 4 and proximal intron 5 (the interval between exon 4 and AFO101 cited above; Refs. 36 and 38).

These studies bring into question the precise boundaries of fragility for the FRA3B. Indeed, Becker et al. (36) have proposed that the FRA3B is >2 Mb in size, extending >800 kb proximal to FHIT exon 1 (including the PTPRG gene) and distal to FHIT exon 10. Our molecular mapping of the chromosome 3 homologues from tumor cells revealed that the deletions occur in a more limited 500-kb interval, between intron 3 (near exon 4) and intron 5 (~150 kb distal to exon 5). A caveat is that by analyzing tumor cells, we cannot distinguish between two potential phenomena, namely that deletions occur preferentially in this interval, or that they occur throughout the region, but that deletions in this interval are selected in tumor cells. In other studies, we demonstrated that the FRA3B is contained within a late-replicating domain (containing FHIT exons 4 and 5 and extending ~150 into intron 5) that is flanked by early replicating domains.
(containing and extending beyond FHIT exons 1–3 and 6–10). Late replication has been proposed as a mechanism for the genetic instability associated with common fragile sites. The late-replicating domain corresponds closely to this 500-kb interval spanning the deleted regions in tumor cells and containing the peak of aphidicolin-induced breaks in metaphase cells.

A number of fragile site landmarks has been identified within FRA3B by a variety of experimental approaches, including aphidicolin-induced breakpoints, viral/plasmid integration sites, and deletion or translocation breakpoints in tumor cells (Fig. 5). Although the genetically unstable region identified by the various experimental approaches is consistent, there is no correlation among the various landmarks. There is substantial evidence that genetic instability occurs over a large region; however, we still cannot rule out the possibility that there are multiple hot spots within this region for DNA breakage or recombination. Similarly, we cannot address whether various experimental approaches introduce a selection bias impacting on the location of the resultant (detected) breakage within FRA3B.

To provide clues to the mechanism(s) underlying the fragility at FRA3B and the contribution of FRA3B instability to cancer susceptibility, several groups of investigators have performed extensive DNA sequence (26–28) and structural analyses (27, 28) of this genomic region. A comparison of the DNA sequences around plasmid and viral integration sites and aphidicolin-induced breaks in hybrid cells did not yield clues as to the mechanism(s) of fragility. The notable findings were that the aphidicolin-induced breakpoint clusters are located close to high-flexibility DNA sequences, whereas the deletion breakpoint ends identified in tumor cells are not positioned near flexibility peaks. Rather, the majority of deletion end points were located near LINE1 or long terminal repeat elements (27, 28). Mimori et al. (28) proposed a model in which breaks may initially occur at regions of high flexibility, followed by DNA repair of the break by homologous pairing of flanking LINE1 elements with concomitant deletion of the intervening FRA3B sequences.

Together, the results suggest that factors other than the DNA sequence per se are responsible for the formation of DNA breaks/gaps. Moreover, the deletions within the FHIT/FRA3B locus are likely to be a direct result of this genetic instability. The agents that induce fragile sites, such as aphidicolin, interfere with replication fork progression. In replicating S phase cells, stalling of replication forks by aphidicolin leads to double-strand breaks, which may be processed initially by nonhomologous end joining, and later by homologous recombination (39). Both of these repair pathways can lead to molecular deletions, e.g., if tandem repeats, such as LINE1 elements, are present in the region of the breakpoint, and repair is initiated out of register, homologous recombination can lead to deletions of sequences homologous to the repeats. Depending on where stalling of the replication fork occurs within the FRA3B sequences, and the location of the recombination events, variable-sized deletions may ensue. The deletion end points may not necessarily represent the actual site of the double-strand break but, rather, the sequences involved in the repair process. Fragility within the FRA3B sequences is likely to be an ongoing process with repeated breakage and repair events resulting in the discontinuous deletions and in the multiple deletion patterns observed in the chromosome 3 homologues derived from each tumor cell line and, presumably, each primary tumor.

Our studies have extended the characterization of the FRA3B by defining the pattern of deletions in human tumors and the boundaries of the unstable region. Nevertheless, our understanding of the mechanisms that give rise to fragile sites is incomplete. The mechanisms involved in rearrangements at fragile sites are likely to apply to other sites of rearrangements in tumor cells; thus, the elucidation of the processes leading to genetic instability at fragile sites may lead to important insights into the pathogenesis of human tumors.

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Identification of Unstable Sequences within the Common Fragile Site at 3p14.2: Implications for the Mechanism of Deletions within Fragile Histidine Triad Gene/Common Fragile Site at 3p14.2 in Tumors

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