

# Molecular Characterization of FRAXB and Comparative Common Fragile Site Instability in Cancer Cells

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The common fragile site, FRA3B, has been shown to be a site of frequent homozygous deletions in some cancers, resulting in loss of expression of the associated *FHIT* gene. It has been proposed that *FHIT* is a tumor suppressor gene that is inactivated as a result of the instability of FRA3B in tumorigenesis. More recently, deletions at other common fragile sites, FRA7G and FRA16D, have been identified in a small number of cancer cell lines. Here, we have mapped and molecularly characterized the frequently observed common fragile site FRAXB, located at Xp22.3. Like other common fragile sites, it spans a large genomic region of approximately 500 kb. Three known genes, including the microsomal steroid sulfatase locus (*STS*), map within the fragile site region. We examined FRAXB and four other fragile sites (FRA3B, FRA7G, FRA7H, FRA16D), and several associated genes, for deletions and aberrant transcripts in a panel of cancer cell lines and primary tumors. Deletions within FRAXB were seen in 4/27 (14.8%) of the primary tumors and cell lines examined. Three of the 21 (14.3%) cell lines examined were characterized by loss of expression of one or more FRAXB-associated genes. Moreover, all of the fragile sites examined were characterized by genomic deletions within the fragile site regions in one or more tumors or cell lines, including FRAXB, which is not associated with any known tumor suppressor genes or activity. Our results further support the hypothesis that common fragile sites and their associated genes are, in general, unstable in some cancer cells. © 2002 Wiley-Liss, Inc.

## INTRODUCTION

Chromosome fragile sites are loci that are “hot spots” for gaps and breaks on metaphase chromosomes when DNA synthesis is perturbed (reviewed in Glover, 1998). There are two classes of fragile sites, rare and common. Common fragile sites are expressed when cells are grown under conditions that inhibit DNA synthesis, such as folate deficiency or with aphidicolin, a specific inhibitor of DNA polymerases. They are seen in all individuals and represent a basic component of chromosome structure that is poorly understood. At least 80 common fragile sites exist, with at least one found on most human chromosomes.

Common fragile sites have been proposed to play a mechanistic role in chromosome translocations and other rearrangements in cancer cells in vivo, based on their behavior in vitro and their colocalization with chromosome breakpoints in vivo. In vitro, they have been shown to display characteristics of unstable and highly recombinogenic DNA, consistent with the hypothesis that fragile site gaps can lead to DNA double-strand breaks. After induction, these sites show increased sister-chromatid exchange on metaphase chromosomes and a high rate of deletions and translocations in somatic cell hybrid systems (reviewed in

Glover, 1998). In addition, it has been shown that breaks at fragile sites can trigger and set boundaries to gene amplification events in CHO cells (Coquelle et al., 1997).

Most information about the biological behavior of common fragile sites in vivo comes from the study of FRA3B, the most frequently seen fragile site on human metaphase chromosomes. It maps to a region of 3p known to be associated with deletions in a number of solid tumors (Glover et al., 1988; Hibi et al., 1992; Negrini et al., 1996) and spans the t(3;8) breakpoint segregating in a family with hereditary renal cell carcinoma (Glover et al., 1988; Huebner et al., 1997). FRA3B maps to the same region as the *FHIT* gene (Ohta et al., 1996; Wilke et al., 1996; Huebner et al., 1997). The *FHIT* gene spans an estimated 900–1,000 kb of 3p14.2. In contrast to its large genomic size, it encodes only a 1.1 kb transcript, the product of which is a hy-

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drolase involved in the cleavage of diadenosine triphosphates (Barnes et al., 1996). FRA3B maps in a region extending at least from *FHIT* intron 4 through part of the very large intron 5, a distance of about 500 kb (Ohta et al., 1996).

The *FHIT* gene has been shown to be frequently deleted and to produce aberrant transcripts in a large number of tumor types (Negrini et al., 1996; Ohta et al., 1996; Sozzi et al., 1996; Druck et al., 1997; Michael et al., 1997). The *FHIT* protein was shown to be absent or reduced in cell lines harboring homozygous or hemizygous deletions (Druck et al., 1997). The deletions frequently span hundreds of kilobases and largely occur within FRA3B. In addition, at least four translocations with breakpoints in *FHIT*/FRA3B have been reported (Ohta et al., 1996; Geurts et al., 1997; Fang et al., 2001). These findings are consistent with the hypothesis that FRA3B is unstable in some cancer cells and is mechanistically involved in the chromosome rearrangements. Based largely on the occurrence of frequent homozygous deletions, it was suggested that *FHIT* is a frequently inactivated tumor suppressor gene (Ohta et al., 1996). Whereas subsequent studies have left some doubt about the role of *FHIT* as a tumor suppressor gene (Otterson et al., 1998; Wu et al., 2000), recent functional studies have provided support for this view (Siprashvili et al., 1997; Fong et al., 2000; Dumon et al., 2001).

A number of possibilities exist to account for the frequent deletions of the *FHIT* gene in tumors. First, the frequent *FHIT* deletions may be independent of FRA3B and are selected for during tumorigenesis. Second, the deletions may be caused by instability at FRA3B and, again, selected for due to the tumor suppressor function of *FHIT*. Third, the deletions may be caused by fragile site instability in certain tumor cells and are maintained without selection. The latter two possibilities invoke a mechanism of fragile site instability in certain cancer cells. If this is true, it would suggest that additional common fragile sites are also unstable in cancer. Limited data exist that directly address this question. Homozygous deletions have been observed in a small number of tumors at other fragile sites, including FRA7G in ovarian cancer and FRA16D in gastric adenocarcinoma and multiple myeloma, suggesting that common fragile sites are generally unstable and may lead to gene inactivation in cancer cells (Huang et al., 1999; Mangelsdorf et al., 2000). It is unclear whether these deletions represent generalized fragile site

instability in cancer or simply a selective event that coincidentally includes fragile site loci.

Examining tumors for deletions and gene inactivation at other fragile sites is one way to provide insight into the mechanisms of fragile site instability and the role of *FHIT* as a tumor suppressor gene. In this article, we define the location, boundaries, and known genes associated with FRAXB, a frequently observed common fragile site that has not been associated with any known tumor suppressor gene locus. We examined the stability of FRAXB, its associated genes, and additional common fragile sites FRA7G, FRA7H, and FRA16D in a panel of cancer cell lines. Our results support the hypothesis that fragile sites, in general, are sites of frequent deletion in some cancer cells.

## MATERIALS AND METHODS

### FISH

Fluorescence in situ hybridization (FISH) was performed on metaphase cells from peripheral blood lymphocytes treated with 0.3  $\mu$ M aphidicolin to induce common fragile sites. As previously described (Dagenais et al., 1999), metaphase chromosome slides were denatured and then hybridized overnight with biotin-14-dATP-labeled probes. A BioNick Translation Kit (Gibco BRL, Gaithersburg, MD) was used to label probes from yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and cosmid clones. Prior to hybridization, probes were denatured for 8 min at 70°C and preannealed for 30–60 min at 37°C with human Cot1 DNA (Gibco BRL). FISH signals were visualized by incubation with two layers of fluorescein isothiocyanate (FITC)-conjugated avidin-DCS (Vector Laboratories, Burlingame, CA) and fluorescein-conjugated anti-avidin IgG. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). FISH results were analyzed with a Zeiss Axioscop epifluorescence microscope.

### Cell Lines and Tissue Specimens

Esophageal adenocarcinoma tissue samples were obtained from patients undergoing surgical resection at the University of Michigan hospital. Consent was received from all patients, and the project was approved by the Institutional Review Board (Medicine). All patient identifiers were removed. Tumor specimens were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Primary tumor samples analyzed were M60, P16, F93, S45, D01, and L86. A panel of 21 cancer cell lines was also used in these

experiments. These cell lines were derived from breast (HCC1937, SUM149, SUM159, SUM44, SUM102, SUM185, SUM190, SUM225, SUM229, SUM1315M02), cervical (HeLa, SiHa, CASKI, and CC-19), colon (HT-29, SW-480, LS180), gastric (KATOIII), and esophageal (BIC-1, FLO-1, SEG-1) carcinomas.

#### DNA/RNA Extractions

DNA was isolated from fresh tissue by standard proteinase K digestion and phenol extraction as previously described (Sambrook et al., 1989). Cell line DNA was isolated from confluent T75 flasks using the QIAamp Tissue Kit (Qiagen, Valencia, CA). Cell line total RNA was extracted from sub-confluent T75 flasks using Trizol according to the manufacturer's instructions (Gibco BRL).

#### Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Five micrograms of total RNA was reverse transcribed in a total volume of 20  $\mu$ l using an oligo-(dT) primer and the Superscript II Reverse Transcriptase (Gibco BRL) according to the manufacturer's instructions. Four microliters of the RT reaction was used for one round of PCR amplification consisting of 30 cycles at 94°C for 1 min, annealing temperature for 1 min, and 72°C for 3 min. The primers used to amplify *FHIT* transcripts were an exon 1 specific primer, 6159E (5'-CATCCTGGAAGCTTTGAAGCTCA-3'), and an exon 10 specific primer, 8243I (5'-CATGCTGATTCAGTTCTCTTTGG-3'). An annealing temperature of 56°C was used. The primers used to amplify the *ATP7A* transcript were an exon 3 specific primer, 64C (5'-GTCAGGAGCTTGTGAA-GATCA-3'), and an exon 20 specific primer, 75C (5'-CCCACATTAGCCATTGCCAGA-3'). An annealing temperature of 60°C was used. The *STS* transcript was amplified at an annealing temperature of 63°C using an exon 2 specific primer, 5691I (5'-TTTCTGTGGGAAGCCGAGAG-3'), and an exon 10 specific primer, 3875I (5'-TGCTGAGGGGT-GAGTTAAGG-3'). *GSI* was amplified at an annealing temperature of 58°C using an exon 1 specific primer, 2707J (5'-CTCATCTTTGACATGGAC-GG-3'), and an exon 4 specific primer, 2706J (5'-CAGACATATATTCAGGCCATCAAG-3'). *TLR5a* was amplified using an annealing temperature of 55°C with an exon 1 specific primer, 2705J (5'-CTCGCGGAGAGCGTAGC-3'), and an exon 7 specific primer, 2704J (5'-CAATTTAAGGGTACAT-CAACAAGG-3'). With the exception of the *FHIT* RT-PCR reactions, all reactions were carried out using the Expand Long Template PCR System

(Roche, Indianapolis, IN). *FHIT* was amplified using standard *Taq* polymerase (Gibco BRL).

PCR products were analyzed on 1% agarose gels and visualized by staining with ethidium bromide. The products in the gel were then transferred to nylon membranes and hybridized with <sup>32</sup>P-labeled cDNA probes specific to each reaction. Probes were labeled using the Random Primers Labeling Kit (Gibco BRL). Membranes were exposed to BioMax MS film (Eastman Kodak, Rochester, NY).

#### Genomic PCR

Genomic DNA was used as template for PCR with 25 markers from several common fragile sites. Markers from FRA3B were D3S2977, D3S4103, D3S1481, and *FHIT* exon 5 (forward 5'-TCATTTGGCTGGTTAGGCTC-3'; reverse 5'-AATGGC-ATCCTCTCTGCAAC-3'). Markers from FRA7G were D7S486, 7G2, and 7G14 (Huang et al., 1999). Markers from FRA7H were WI-16323 and D7S649. Markers from FRAXB were DXS6701, DXS1130, DXS1133, DXS1407, *STS* exon 2 (forward 5'-TTTC-TGTGGGAAGCCGAGAG-3'; reverse 5'-GGTTTGGAGGGTTTTGAACG-3'), and *STS* exon 10 (forward 5'-CCAACACGCCTGAGAGTGGC-3'; reverse 5'-TGCTGAGGGGTGAGTTAAGG-3'). Control markers were D3S3577, D3S4483, D7S500, DXS8079, *KAL* intron 13 (forward 5'-GACCCAAG-TCCACCTACTTC-3'; reverse 5'-TATCAAGCT-CTCCATTGTG-3'), and *ATP7A* exon 4 (forward 5'-TTCATCTTATCTCAGTAGAGG-3'; reverse 5'-TACTACCAACGGCTCATTTCGT-3').

Markers were amplified with *Taq* DNA polymerase (Gibco BRL) in reactions containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.1  $\mu$ M each primer, and 100 ng of template DNA. When a marker failed to amplify in a sample in two consecutive PCR reactions, multiplex PCR was performed using the marker in question and a proven control marker. Reaction conditions were essentially the same as above, but we used 1.4 mM dNTPs and 4 mM MgCl<sub>2</sub>. PCR products were analyzed on 1% agarose gels and visualized by staining with ethidium bromide.

## RESULTS

#### Mapping FRAXB

To determine precisely the location and boundaries of FRAXB, we performed FISH with ordered YAC clones from Xp22. One YAC, 933D4, was found to cross the fragile site on metaphase chromosomes from peripheral-blood lymphocytes treated with aphidicolin. These results indicate

TABLE 1. Identification of Cosmid, BAC, and YAC Clones at FRAXB

Clone	Distal <sup>a</sup>	FISH signal		Position relative to FRAXB
		crossing <sup>b</sup>	Proximal <sup>c</sup>	
3G1	15	0	0	Distal
19C7	7	0	0	Distal
87B11	12	0	0	Distal
74A4	8	5	1	Crossing
YAC933D4	13	6	4	Crossing
YAC29C11	4	2	4	Crossing
37D9	5	6	2	Crossing
YAC29G1	4	3	4	Crossing
BAC146L1	9	3	5	Crossing
BAC388M15	0	0	15	Proximal
BAC542P7	0	1	16	Proximal

<sup>a</sup>Number of metaphase cells in which FISH signal was located distal to the fragile-site break.

<sup>b</sup>Number of metaphase cells in which FISH signal was divided across the fragile-site break.

<sup>c</sup>Number of metaphase cells in which FISH signal was located proximal to the fragile-site break.

that FRAXB colocalizes with this clone. However, on some metaphase chromosomes, 933D4 hybridized either proximal or distal to the fragile site break, indicating that FRAXB extends beyond the boundaries of this YAC clone.

To determine the size and boundaries of FRAXB more accurately, we performed additional FISH experiments with a panel of YAC, BAC, and cosmid clones that map distal or proximal to YAC 933D4. We quantitated the number of times each clone hybridized proximal to, crossing, or distal to the fragile-site break. These data are summarized in Table 1. Three cosmid clones, 3G1, 19C7, and 87B11, were found to map exclusively distal to FRAXB, whereas an overlapping cosmid, 74A4, frequently crossed the break in addition to mapping distal to it. These data indicate that the distal boundary of FRAXB is near DXS1130, a marker shared by 87B11 and 74A4 (Fig. 1A).

In addition to YAC 933D4 and cosmid 74A4, several clones were found to cross FRAXB. These clones include cosmid 37D9, YACs 29C11 and 29G1, and BAC 146L1. These clones represent the core of FRAXB and span from DXS1130 proximally past DXS1131.

Two overlapping BAC clones, 388M15 and 542P7, hybridized proximal to the fragile site on metaphase chromosomes. These clones contain DXS237 and DXS1132, respectively, and represent the proximal boundary of FRAXB. Based on these data the proximal boundary is positioned between DXS1133 and DXS237. Like FRA3B, FRAXB is a

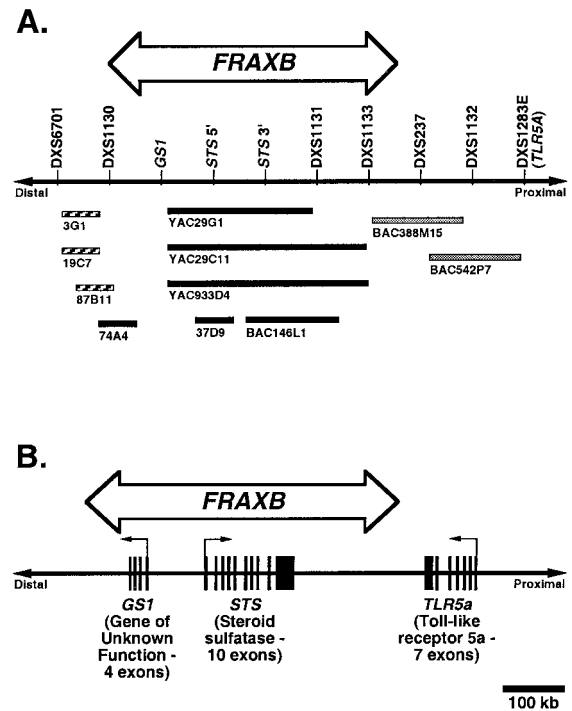


Figure 1. Genomic organization of FRAXB at Xp22.3. **A:** Determination of extent of FRAXB by FISH analysis. Markers from Xp22.3 are arranged from distal (left) to proximal (right). The position of the fragile site is indicated at the top. Horizontal lines represent cosmid, BAC, and YAC clones used as FISH probes. Striped lines indicate clones that hybridized distal to the fragile site. Solid black lines indicate clones that hybridized across the fragile site break. Gray lines indicate clones that hybridized proximal to the fragile site. **B:** Positions of genes in the FRAXB region. The position of the fragile site is indicated at the top. *GS1* contains four exons near the distal boundary of FRAXB. *STS* contains 10 exons near the center of FRAXB. A third gene, *TLR5a*, contains seven exons positioned just proximal to FRAXB. Arrows indicate the direction of transcription.

very large fragile site, spanning approximately 500 kb of genomic sequence as defined by the location of gaps and breaks on metaphase chromosomes.

Approximately 235 kb of FRAXB have been sequenced from overlapping BACs GSHB-227L7 (GenBank accession no. AC005704) and RP11-483M24 (GenBank accession no. AC073583). This sequence is composed of 57.7% A and T. Much of the sequence contains repeat elements, including Alu (9.2%), Mir (0.6%), L1 (8.3%), L2 (1.1%), and long terminal repeat (LTR) (10.2%) elements. The LTRs in this sequence include six human endogenous retrovirus-like (HERV-L)-derived sequences. In addition, the sequence contains simple di- and tetranucleotide repeats such as (CA)<sub>n</sub>, (TAGA)<sub>n</sub>, and (GGAA)<sub>n</sub>. Approximately 301 kb of sequence from overlapping BACs GSHB-214D18 (GenBank accession no. AC005296) and RP11-143E20 (GenBank accession no. AC074034) is also available just proximal to FRAXB. BAC clone



214D18 overlaps the BAC 542P7 used in our mapping studies. This adjacent sequence contains low to moderate amounts of Alu (4.5%), Mir (1.9%), L1 (7.5%), and L2 (2.4%) sequences. It is also characterized by a high proportion of LTRs (11.8%), the majority of which are of retroviral origin, including two HERV-L-derived sequences.

#### Identification of Genes in the FRAXB Region

Several genes have been identified that map within FRAXB. A gene of unknown function, *GSI*, was identified in the distal third of FRAXB. It consists of four exons spanning approximately 105 kb with a predicted transcript of 2.1 kb (Yen et al., 1992). The microsomal steroid sulfatase (*STS*) gene had been previously cloned and mapped to Xp22.3 (Ballabio et al., 1987; Mohandas et al., 1987; Yen et al., 1987). The *STS* gene contains 10 exons spanning 146 kb. Alternative polyadenylation signals in exon 10 generate transcripts of 2.7 kb, 5.2 kb, and 7.0 kb in a tissue-specific manner (Yen et al., 1988). The *STS* gene is inactivated in X-linked ichthyosis, primarily through large deletions. Both *STS* and *GSI* were localized in a contig that maps to the FRAXB region (Lee et al., 1993). A third gene encoding the Toll-like receptor 5a (*TLR5a*) was identified at a CpG island just proximal to the fragile site (Lee et al., 1994). It contains seven exons spanning 28.6 kb that encode a 2.8 kb transcript. The positions of these genes relative to FRAXB are depicted in Figure 1B.

#### Genes at FRAXB Are Inactivated in Cancer Cells

As one measure of gene deletion and aberrant transcription, expression of the genes that colocalize with FRAXB (*STS*, *GSI*, *TLR5a*) was examined in a panel of 21 cancer cell lines and one normal fibroblast control by RT-PCR and subsequent Southern blot hybridization of products. Because of the high frequency of deletion at FRA3B, expression of the *FHIT* gene was examined as a positive control for loss of expression and production of aberrant transcripts. The Menkes disease gene (*ATP7A*) at Xq13, which produces an RT-PCR product comparable in size to the FRAXB-associated genes, was used as a negative control.

*STS* was amplified using primers in exon 2 and in exon 10 upstream of the various polyadenylation signals to ensure that all of the possible transcripts could be detected. No aberrant transcripts were observed in any FRAXB-associated genes. However, these experiments revealed that *STS* and *GSI* transcripts were absent in two cell lines, BIC-1 (esophageal) and SW-480 (colon). *TLR5a* was no

TABLE 2. Loss of Expression of Genes Associated With Fragile Sites

Gene	Associated fragile site	Map position	Loss of normal transcript	% Loss
<i>ATP7A</i>	None	Xq13	0/21	0.0
<i>FHIT</i>	FRA3B	3p14	9/21	42.9
<i>GSI</i>	FRAXB	Xp22	2/21	9.5
<i>STS</i>	FRAXB	Xp22	2/21	9.5
<i>TLR5a</i>	FRAXB	Xp22	1/21	4.8
Xp22 genes	FRAXB	Xp22	3/21	14.3

longer expressed in one cell line, 1315M02 (breast). Overall, 14.3% (3/21) of the cell lines had lost expression of one or more genes in the FRAXB region (Table 2). The BIC-1 cell line had previously been karyotyped and found to be disomic for the X chromosome (Fang et al., 2001). The 1315M02 breast cancer cell line was karyotyped and found to have the expected two copies of the X chromosome. SW-480 is a colon cancer cell line with a chromosome modal number of 55 derived from a male patient (Leibovitz et al., 1976).

No *FHIT* transcript was detected in 19.0% (4/21) of the cell lines. Five cell lines (23.8%) had only aberrant *FHIT* transcript. An aberrant *FHIT* transcript in the presence of a normal *FHIT* transcript was seen in one cell line (4.8%). In total, 10/21 (47.6%) of the tumor cell lines were characterized by an alteration of *FHIT* expression.

An *ATP7A* PCR product of the expected size was seen in all cell lines examined. A summary of the gene expression data is shown in Table 3. Representative examples of RT-PCR data are shown in Figure 2A.

#### Homozygous and Hemizygous Deletions at Common Fragile Sites

The occurrence of homozygous or hemizygous deletions was examined in a panel of primary tumors and cancer cell lines to determine if sequences at common fragile sites are, in general, especially prone to deletions in cancer cells. Genomic PCR was performed on 21 cancer cell lines and six primary esophageal adenocarcinomas using markers from five common fragile sites: FRA3B (four markers), FRA7G (four markers), FRA7H (two markers), FRA16D (four markers), and FRAXB (six markers). Six markers from non-fragile site regions were also included as controls. These controls included two markers at 3p14, one marker at 7q31, one marker at Xp22.3, and two markers at Xq13. Whenever a marker failed to

TABLE 3. Summary of Gene Expression and Homozygous Deletion Data

Cell line	Sex <sup>a</sup>	Type <sup>b</sup>	Gene expression <sup>c</sup>					Homozygous deletion <sup>d</sup>				
			<i>FHIT</i>	<i>GSI</i>	<i>STS</i>	<i>TLR5<math>\alpha</math></i>	<i>ATP7a</i>	FRAXB	FRA3B	FRA7G	FRA7H	FRA16D
BIC-1	M	Esophagus	●	●	●	+	+	●	●	+	+	●
FLO-1	M	Esophagus	●	+	+	+	+	+	+	+	+	●
SEG-1	M	Esophagus	+	+	+	+	+	+	●	+	+	+
KATOIII	M	Gastric	●	+	+	+	+	+	●	+	+	+
HT-29	F	Colon	●	+	+	+	+	+	●	+	+	+
SW480	M	Colon	+	●	●	+	+	●	●	+	+	+
LS180	F	Colon	+	+	+	+	+	+	+	+	+	+
HeLa	F	Cervix	●	+	+	+	+	+	+	+	+	+
SIHA	F	Cervix	●	+	+	+	+	+	+	+	+	+
CASKI	F	Cervix	+	+	+	+	+	+	+	+	+	+
CC-19	F	Cervix	●	+	+	+	+	+	●	+	+	+
HCC1937	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM149	F	Breast	●	+	+	+	+	+	+	+	+	+
SUM159	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM44	F	Breast	●	+	+	+	+	+	+	+	+	+
SUM102	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM185	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM190	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM225	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM229	F	Breast	+	+	+	+	+	+	+	+	+	+
SUM1513M02	F	Breast	+	+	+	●	+	+	+	+	+	+
M60 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	+	+	+	+	+
PI6 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	+	+	+	+	+
F93 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	+	+	+	+	+
S45 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	●	+	●	●	●
D01 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	+	+	+	+	+
L86 <sup>e</sup>	M	Esophagus	ND	ND	ND	ND	ND	●	+	+	+	+

<sup>a</sup>M, male; F, female.

<sup>b</sup>Tumor origin.

<sup>c</sup>Gene expression determined by RT-PCR. "+" indicates normal expression, "●" indicates loss of normal transcript, "ND" indicates no data due to unavailable RNA from primary tumors.

<sup>d</sup>Homozygous deletion determined by genomic PCR. "+" indicates no deletion, "●" indicates homozygous deletion.

<sup>e</sup>Primary tumor.

amplify through two separate rounds of PCR, multiplex PCR was performed with a second marker to confirm the reliability of the DNA, PCR reagents, and conditions. Representative examples of multiplex PCR showing deletions are illustrated in Figure 2B.

Every fragile site was characterized by homozygous deletion in at least one tumor or cell line. The most frequent deletions were observed at markers from FRA3B, with 6/27 (22.2%) samples demonstrating homozygous deletions in at least one marker in this fragile site. FRAXB was characterized by deletions in 4/27 (14.8%) of samples. FRA16D was homozygously deleted in 3/27 (11.1%) specimens. FRA7G and FRA7H were each homozygously deleted in 1/27 (3.7%) of the samples (Tables 3, 4). None of the samples had homozygous deletions at any of the control markers or at loci flanking FRA3B and FRAXB. Overall, 33.3% (9/27) of the tumors and cell lines were characterized by homozygous dele-

tions of sequences from at least one fragile site. Three of the specimens (11.1%) had deletions at more than one fragile site. One cell line, BIC-1, had homozygous deletions at FRA3B, FRA16D, and FRAXB. A second cell line, SW-480, had deletions at FRA3B and FRAXB. One of the primary esophageal adenocarcinomas, S45, had deletions at FRA7G, FRA7H, FRA16D, and FRAXB. Two of the deletions at FRAXB occurred in cell lines that were included in the gene expression analyses, BIC-1 and SW-480. In both cases, deletion of FRAXB sequences correlated with loss of expression of *STS* and *GSI*. A summary of the homozygous deletion data is shown in Table 3.

#### Deletion Breakpoints Colocalize With Fragile-Site Boundaries

In several cases, deletion breakpoints colocalized with the established boundaries of the fragile sites. As mentioned previously, the distal boundary of FRAXB is at DXS1130 and the proximal boundary

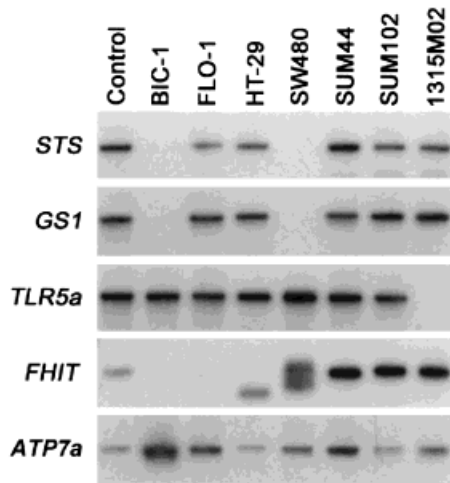
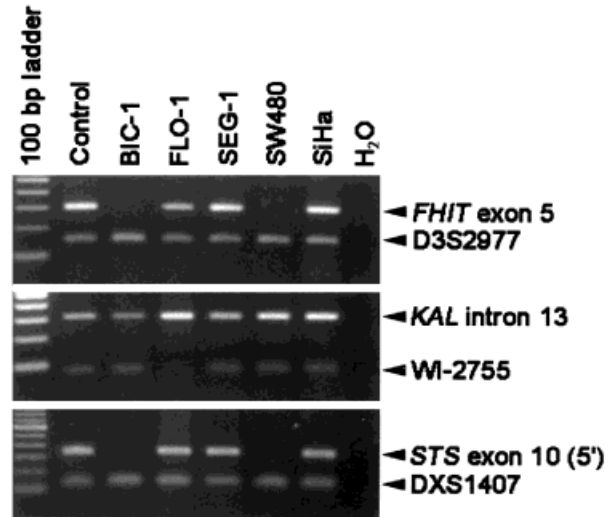
**A.**

Figure 2. Homozygous deletions and loss of gene expression at common fragile sites in cancer cells. **A:** Examples of loss of gene expression at FRAXB detected by RT-PCR. A control fibroblast and seven cancer cell lines are indicated above each lane. The RT-PCR products corresponding to *STS*, *GS1*, and *TLR5a* are shown in the first three panels, respectively. The fourth panel shows RT-PCR results for *FHIT*, containing both loss of gene product and aberrant transcripts. The

**B.**

fifth panel contains products from *ATP7A*, a control. **B:** Examples of homozygous deletions at common fragile sites detected by multiplex PCR. A control fibroblast and seven cancer cell lines are indicated above each lane. Representative PCR results revealing homozygous deletions are shown for *FHIT* exon 5 (FRA3B), *WI-2755* (FRA16D), and *STS* exon 10 (FRAXB).

is between DXS1133 and DXS237. During the course of the homozygous deletion studies, we discovered that two tumor cell lines, BIC-1 and SW-480, appear to have deletions of approximately the same size at FRAXB. PCR with additional markers revealed that these two cell lines indeed shared similar deletion breakpoints. Furthermore, these breakpoints colocalize with the boundaries of the FRAXB fragile site, as determined by our FISH studies (Fig. 3). Both cell lines have deletions at DXS1130 but retain the next most distal marker tested, DXS6701, indicating that the distal deletion breakpoint is between these two markers. Similarly, both cell lines have deletions at DXS1133 but retain the next most proximal marker, DXS237. These data indicate that the proximal deletion breakpoint is between these two markers in these cell lines. These deletions appear to remove sequences necessary for expression of FRAXB. When BIC-1 cells were treated with aphidicolin to induce fragile sites, no gaps or breaks were observed at FRAXB in 100 metaphase cells, indicating that the deletion eliminated the site's fragility.

Further analyses with the two primary esophageal adenocarcinomas characterized by FRAXB deletions revealed that they share the same proximal deletion boundary between DXS1133 and DXS237. The proximal breakpoint of tumor L86 is between DXS1133 and DXS1131, within the frag-

TABLE 4. Homozygous Deletions at Common Fragile Sites

Fragile site	Marker	Homozygous deletions	Total deletions in region
FRA3B	D3S2977	0/27 (0.0%)	6/27 (22.2%)
	D3S4103	3/27 (11.1%)	
	<i>FHIT</i> exon 5	3/27 (11.1%)	
FRA7G	D3S1481	2/27 (7.4%)	1/27 (3.7%)
	D7S486	0/27 (0.0%)	
	7G2	1/27 (3.7%)	
FRA7H	7G14	1/27 (3.7%)	1/27 (3.7%)
	WI-16323	1/27 (3.7%)	
	D7S649	0/27 (0.0%)	
FRA16D	D16S518	1/27 (3.7%)	3/27 (11.1%)
	WI-2755	2/27 (7.4%)	
	AA398024	2/27 (7.4%)	
FRAXB*	D16S3029	1/27 (3.7%)	4/27 (14.8%)
	DXS6701	1/27 (3.7%)	
	DXS1130	3/27 (11.1%)	
	<i>STS</i> exon 2	3/27 (11.1%)	
	<i>STS</i> exon 10	3/27 (11.1%)	
Controls	DXS1133	4/27 (14.8%)	0/27 (0.0%)
	DXS1407	0/27 (0.0%)	
	D7S500 (7q31)	0/27 (0.0%)	
	KAL intron 13 (Xp22.3)	0/27 (0.0%)	
	DXS8079 (Xq13)	0/27 (0.0%)	
	<i>ATP7A</i> (Xq13)	0/27 (0.0%)	
	D3S3577 (3p14)	0/27 (0.0%)	
D3S4483 (3p14.2)	0/27 (0.0%)		

\*Homozygous deletions or hemizygous deletions in cell lines or tumors with one X chromosome.

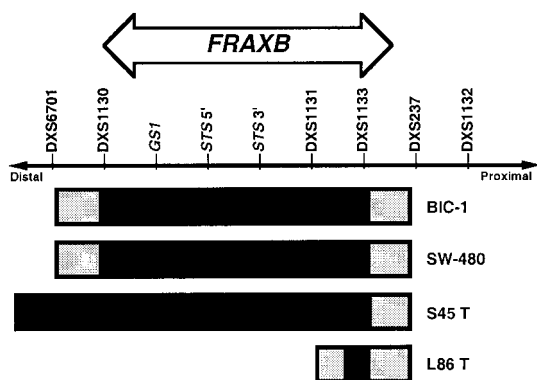


Figure 3. Colocalization of FRAXB boundaries and deletion breakpoints in cancer cells. Markers from Xp22.3 are arranged from distal (left) to proximal (right) with the position of FRAXB indicated at the top. Deleted regions in two cancer cell lines (BIC-1 and SW-480) and two primary esophageal adenocarcinomas (S45 T and L86 T) are indicated with black bars underneath the deleted markers. Shaded areas indicate the regions where the deletion breaks occur.

ile site, whereas tumor S45 appears to have a terminal deletion.

## DISCUSSION

### Structure and Composition of FRAXB

We have defined the boundaries of the common fragile site FRAXB at Xp22.3, localizing this fragile site to approximately 500 kb between DXS1130 and DXS237. The FRAXB region contains three previously identified genes, *STS*, *GS1*, and *TLR5a*. This region is currently being sequenced by the Human Genome Sequencing Center at the Baylor College of Medicine, and about 235 kb of sequence data are available from within FRAXB (GenBank Accession nos. AC005704 and AC073583). Additional sequence is available flanking the fragile site boundaries. From the available data, FRAXB appears to be moderately A-T rich (57.7%) and consists of approximately 31% interspersed repeat elements. This repeat element content is slightly lower than the 38.5% expected for DNA of this A-T content (Smit et al., 1996). Sequence analysis revealed that about 301 kb of sequence immediately proximal to FRAXB had a slightly higher A-T content (61%) and a similar frequency of interspersed repeats (32%). The proportions of long terminal repeats, transposable elements, L2s, and MIRS in the FRAXB sequence are similar to those previously reported for FRA3B, FRA7H, and FRA16D (Ried et al., 2000). There are differences in the proportion of Alu and L1 elements seen in FRAXB and other fragile sites. FRA3B and FRA7H have a relatively high proportion of L1 sequences, 11.7% and 13.2%, respectively (Ried

et al., 2000). The FRAXB sequence contains a slightly lower proportion of L1 sequences (8.3%). FRA3B also has a fairly low Alu content of 6.3%. The available sequence from FRAXB has a moderate Alu content of approximately 9.2%. FRA16D sequence contains 2.8% L1 and 13.8% Alu (Ried et al., 2000). In these respects, from the sequence currently available, FRAXB appears to fall between FRA3B and FRA16D in terms of repeat content. It is noteworthy that the sequence immediately proximal to FRAXB has a lower Alu content of 4.5% and an L1 content of 7.5%.

Viral integration sites have been previously reported at several common fragile sites. FRA3B is associated with an HPV16 integration site that was described in a primary cervical carcinoma (Wilke et al., 1996). More recently, an endogenous HERV-H sequence was identified at FRA7G (Huang et al., 1998) and an SV40 integration site was found in FRA7H (Mishmar et al., 1998). Our finding that sequences from within FRAXB and just proximal to the FRAXB proximal boundary as defined here contain a high proportion of retroviral sequences, including multiple HERV-L sequences, appears to support the hypothesis that common fragile sites are preferential sites of viral integration. Additional sequence from within the fragile site is needed to confirm this suggestion.

### Deletions and Loss of Gene Expression at Common Fragile Sites

Previous studies investigating fragile site stability have shown frequent deletions at FRA3B in a variety of cancers and cancer cell lines, including lung, digestive tract, and breast cancer (Negrini et al., 1996; Ohta et al., 1996; Sozzi et al., 1996; Druck et al., 1997; Michael et al., 1997). Recently, homozygous deletions have been observed in tumors at other fragile sites as well, including FRA7G in ovarian cancer and FRA16D in gastric adenocarcinoma and multiple myeloma, suggesting that common fragile sites are generally unstable and may lead to gene inactivation in cancer cells (Huang et al., 1999; Mangelsdorf et al., 2000). In addition, translocations within fragile sites have been identified at FRA3B and FRA16D (Ried et al., 2000; Fang et al., 2001). All of these alterations occur in regions that have been previously implicated in tumorigenesis, suggesting that increased genomic instability during tumor development may lead to preferential inactivation of genes at fragile sites and a selective growth advantage when the inactivated genes have tumor suppressor activity. FRAXB is not associated with any known tumor suppressor



activity, based on LOH studies and locations of known tumor suppressor genes. In addition, 85% of X-linked ichthyosis cases are due to inactivation of *STS* through large deletions at Xp22 that include FRAXB and large amounts of flanking sequence (Ballabio et al., 1987; Gillard et al., 1987; Yen et al., 1987). Patients with X-linked ichthyosis are not characterized by tumor development, providing further support that Xp22.3 is devoid of tumor suppressor activity. While we cannot completely rule out the possibility that an unknown tumor suppressor gene may map to this region, or that a selective advantage is conferred to cancer cells with Xp22.3 deletions, it is likely that fragile site instability at FRAXB in cancer cells can be examined without selection for deletion events.

As expected, a high frequency of deletions was observed at FRA3B in our panel of cancer cell lines and primary tumors. FRA3B is the most highly expressed common fragile site under a variety of conditions (Glover, 1998). Deletions were also seen, at a somewhat lower frequency, at FRA7G, FRA7H, FRA16D, and FRAXB. The relative frequency of deletions at these fragile sites correlates with the frequency at which these fragile sites are generally expressed on metaphase chromosomes (Glover et al., 1984). Homozygous deletions were primarily seen in cells derived from esophageal and colon tumors, with deletions rarely observed in cervical cancer cell lines and never observed in breast cancer lines. The fact that none of the non-fragile site control markers and few of the sequences immediately flanking the fragile sites were deleted suggests that these deletions are specific to the fragile sites, and not simply the result of a generalized genomic instability resulting in random deletions. This hypothesis is also supported by the three samples that were characterized by homozygous deletions at multiple fragile sites. It should be noted that additional small interstitial deletions might have occurred in the fragile site in the regions between the markers examined, as has been found at FRA3B in various tumors and tumor cell lines. As such, the deletion frequencies reported here are likely an underestimate of the actual deletions occurring within fragile sites.

Aberrant *FHIT* transcripts, indicating deletions and aberrant splicing, have been identified by RT-PCR in numerous cancers, including lung, digestive tract, and breast cancer (Negrini et al., 1996; Ohta et al., 1996; Sozzi et al., 1996; Druck et al., 1997; Michael et al., 1997). For example, our group performed studies to investigate deletions in *FHIT* in primary esophageal tumors and the relationship

of *FHIT* deletions to FRA3B. We concluded from these studies that both aberrant splicing and genomic deletions of *FHIT* are indeed frequently seen in primary tumors and occur at an early stage, consistent with a role for *FHIT* as a tumor suppressor gene (Michael et al., 1997). We and others have hypothesized that *FHIT* inactivation occurs as a consequence of fragile site instability during tumorigenesis. One result of the deletions at FRAXB in our cell lines is the loss of expression of associated genes, *GSI* and *STS*. It is of note that, whereas aberrant transcripts were frequently seen in *FHIT*, they were never observed at any of the genes at FRAXB. This is likely due to the sizes of these genes and their positions relative to the fragile sites. *FHIT* is a very large gene, spanning approximately 1,000 kb of genomic DNA. FRA3B is completely contained within the gene, centered around exon 5. Deletions at this fragile site can eliminate exon 5 and surrounding sequences without abolishing expression of the gene, resulting in aberrant transcripts. Aberrant transcripts could also arise during RNA processing of this large and complex region. In contrast, the genes at FRAXB are relatively small and are completely contained within the fragile site boundaries. As a result, deletions at FRAXB are likely completely to eliminate *GSI* and/or *STS* expression rather than cause aberrant transcripts. These data support the hypothesis that common fragile site instability in cancer cells results in a loss of associated gene function due to deletion events. If genes associated with specific fragile sites, such as *FHIT* at FRA3B, have important tumor suppressor functions, this generalized fragile site instability can confer a growth advantage on cells that have undergone fragile site deletions. In the case of FRAXB, no known tumor suppressor activity has been associated with the region. Thus, a growth advantage conferred by deletions in FRAXB is not known, but cannot be ruled out completely.

#### **Colocalization of Fragile Site and Deletion Boundaries in Cancer Cells**

Of the four samples that had homozygous deletions at FRAXB, two had deletion breakpoints corresponding to the fragile site boundaries and the other two had proximal deletion breakpoints at the proximal boundary of FRAXB (Fig. 3). These coincident deletion/fragile site boundaries provide further evidence for the preferential instability of common fragile sites in cancer. The deletion breakpoints seen in the BIC-1 esophageal cancer cell line also provide insight into the mechanism of

FRAXB fragility. The fragile site region appears to be precisely deleted in this cell line, with little or no flanking DNA deleted. This cell line no longer displays fragility at Xp22.3, indicating that sequence is important for fragility, and that this necessary sequence is found within the fragile site, rather than being located proximal or distal to the fragile site.

While the FRAXB region has not previously been shown to be deleted in cancers, deletions in another type of disorder have been reported. The involvement of *STS* in X-linked ichthyosis is unusual in that it is primarily inactivated through large genomic deletions, rather than by point mutations or small deletions (Yen et al., 1987). These deletions are thought to arise through unequal meiotic recombination events between a family of genes known as *VCX* (variably charged, X-chromosome mRNA on CRI-232) on Xp that is highly conserved (Knowlton et al., 1989; Yen et al., 1990; Fukami et al., 2000; Lahn et al., 2000). It is unlikely that the conserved FRAXB deletions observed in our primary tumors and cancer cell lines are due to somatic recombination between members of this family. Several published reports describe deletions in X-linked ichthyosis caused by *VCX* recombination. The deletions attributed to these recombination events have breakpoints distal to DXS6701 and proximal to DXS237, two markers retained in our specimens (Yen et al., 1990; Fukami et al., 2000). In addition, none of the *VCX* genes mapped to Xp22.3 are localized within FRAXB.

In summary, we have localized and defined the boundaries and genes associated with the common fragile site FRAXB. This fragile site spans approximately 500 kb of A-T-rich DNA. The repeat element content determined by analysis of available sequence reveals that FRAXB more closely resembles FRA16D than either FRA3B or FRA7H. We have mapped three known genes to the FRAXB region, *STS*, *GSI*, and *TLR5a*. We have examined FRAXB and four other fragile sites for instability at both the DNA and RNA levels as a test of the hypothesis that common fragile sites are generally unstable in cancer cells. Our results are consistent with the conclusion that common fragile sites, as a class, are unstable in cancer cells, resulting in deletions and loss of associated gene expression.

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