Chromosomal Fragile Site FRA16D and DNA Instability in Cancer

Marie Mangelsdorf,² Karin Ried,² Erica Woollatt,² Sonia Dayan, Helen Eyre, Merran Finnis, Lynne Hobson, Julie Nancarrow, Deon Venter, Elizabeth Baker, and Robert I. Richards³

Department of Cytogenetics and Molecular Genetics, Women’s and Children’s Hospital, Adelaide, South Australia 5006, Australia [M. M., K. R., E. W., S. D., H. E., M. F., L. H., J. N., E. B., R. I. R.]; Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia [D. V.]; and Department of Genetics, The University of Adelaide, South Australia 5000, Australia [R. I. R.]

ABSTRACT

It has been proposed that common aphidicolin-inducible fragile sites, in general, predispose to specific chromosomal breakage associated with deletion, amplification, and/or translocation in certain forms of cancer. Although this appears to be the case for the fragile site FRA3B and may be the case for FRA7G, it is not yet clear whether this association is a general property of this class of fragile site. The major aim of the present study was to determine whether the FRA16D chromosomal fragile site locus has a role to play in predisposing DNA sequences within and adjacent to the fragile site to DNA instability (such as deletion or translocation), which could lead to or be associated with neoplasia. We report the localization of FRA16D within a contig of cloned DNA and demonstrate that this fragile site coincides with a region of homozygous deletion in a gastric adenocarcinoma cell line and is bracketed by translocation breakpoints in multiple myeloma, as reported previously (Chesi, M., et al., Blood, 91: 4457–4463, 1998). Therefore, given similar findings at the FRA3B and FRA7G fragile sites, it is likely that common aphidicolin-inducible fragile sites exhibit the general property of localized DNA instability in cancer cells.

INTRODUCTION

The idea that fragile site loci might have a role in the chromosomal instability observed in cancer was originally proposed by Yunis and Soreng (1) on the basis of a concordance between the physical locations of chromosomal fragile sites and cancer breakpoints and/or cancer-associated genes. This proposal was initially supported by a statistical analysis (2). Examples were subsequently put forward to disprove the hypothesis (3, 4), whereas other studies revealed the lack of an association between the presence of a heritable fragile site within a family and any increased risk of cancer (5). Initially, no distinction was made between “rare” fragile sites, found in only a small proportion of the population, and “common” fragile sites, found in all individuals. Even with such a distinction, further analysis (6) failed to confirm a statistical association between common fragile sites and nonrandom chromosome breakpoints in cancer cells. Chromosome breakage is (like fragile sites themselves) a visible cytogenetic manifestation of chromosomal instability. Other forms of DNA instability have more recently become apparent as cryptic (cytogenetically invisible) deletions (7). Such deletions can be detected as LOH at polymorphic loci. Chromosomal regions that are deleted in cancer cells are thought, and in some instances found, to contain tumor suppressor genes (8, 9).

Recent detailed molecular analysis of the common fragile site FRA3B region revealed a predisposition to localized deletion that is frequently observed in certain forms of cancer (7, 10). The cancer-associated FRA3B deletions can result in inactivation of the FHIT gene, an event that is thought to have a role in tumor growth (11). Although the DNA sequence of the region containing FRA3B has been determined, neither the molecular basis for the cytogenetic expression of the fragile site nor the cancer-associated instability has been clearly resolved as yet (12). There has been considerable controversy and conflicting data regarding the role of FHIT in cancer (13–15). In one study using neoplastic cells that had FRA3B deletions and that therefore were deficient in FHIT protein, “replacement” with stable, overexpressed FHIT protein did not alter in vitro or in vivo properties of these cells (16). In another study (11), replacement of FHIT protein in cancer cells suppressed their tumorigenicity. It remains to be assessed whether dysregulation of the FHIT gene, and therefore, the inappropriate “cell cycle” appearance or cellular compartmentalization of FHIT protein is the mechanism for its contribution to neoplasia.

Another common fragile site, FRA7G, has also been shown to be located within a ~1-Mb region of frequent deletion in breast and prostate cancer (17, 18), as well as squamous cell carcinomas of the head and neck, renal cell carcinomas, ovarian adenocarcinomas, and colon carcinomas (19). The human caveolin-1 and -2 genes are located within the same commonly deleted region as FRA7G. Caveolin-1 has been shown to have a role in the anchorage dependant inhibition of growth in NIH 3T3 cells (20). The caveolins are therefore candidates for the tumor suppressor gene that is presumed to be located in the FRA7G region (19).

Fragile sites have been found to act as amplification boundaries (21, 22) in selection experiments; however, a role in cancer-associated amplification is yet to be demonstrated. It is not yet clear whether the reported instances of association are due to a predisposition conferred by the fragile site locus or, alternatively, the selection of an associated gain and/or loss of gene function.

The long arm of chromosome 16 is of particular interest in cancer genetics because it exhibits frequent loss in tumor cells (23, 24). It is also the location of the rare chromosomal fragile site FRA16B at 16q22.1 (25) and the common, aphidicolin-inducible fragile sites FRA16C at 16q22.1 and FRA16D at 16q23.2. FRA16D has been localized within a large overlapping region of chromosomal instability in breast and prostate cancer as defined by LOH (23, 24). In addition, a gene for Wilms’ tumor has been mapped to 16q (26), and a gene for familial leukemia has been mapped to 16q21–23.2 (27).

We have therefore physically mapped the FRA16D region in detail to provide markers for identifying any possible relationship between this fragile site and DNA instability in neoplasia. This analysis reveals the existence of an intimate relationship between the location of FRA16D and homozygous deletions in various tumors (this report and Ref. 28), culminating in the coincidence of two tumor cell DNA breakpoints with the most likely position of the fragile site.
Fig. 1. Positional cloning of FRA16D and location of LOH and translocation in cancer. A, the locations of LOH regions in breast and prostate cancer and the approximate location of the FRA16D fragile site are indicated with respect to genetic markers (downward arrows) in the 16q23.2 region. Markers in the vicinity of FRA16D are shaded. The approximate location as determined by Chesi et al. (45) of multiple myeloma breakpoints and the c-MAF gene (bar) are also shown by upward black arrows. Not to scale. B, map of the contig of YAC subclones across the FRA16D region with respect to genetic markers and FRA16D. Open boxes, YACs that map by FISH proximal to FRA16D; gray boxes, YACs that span FRA16D; black boxes, YACs that map distal to FRA16D. Not to scale.

MATERIALS AND METHODS

Isolation of DNA Probes and YACs in the FRA16D Region. Nine DNA probes, ACH202 (D16S114), c311F2, c302A6 (D16S1075), c301F10 (D16S373), 16-87 (D16S181), c306D2, 16-08 (D16S162), c307A12, and CRI-0119 (D16S590), which had been physically mapped into the 16q23 region (29), were chosen for FISH against FRA16D expressing chromosomes. Four of these markers mapped within the same somatic cell hybrid breakpoint interval defined by the cell lines CY113(P) and CY121 (29). One of these, c306D2 mapped proximal to FRA16D by FISH, whereas the others, c307A12, CRI-0119, and 16-08 mapped distal to FRA16D. These probes were therefore used as starting points to isolate a contiguous of cloned DNA spanning FRA16D. In the Los Alamos National Laboratory database, a STS sequence from c306D2 was found within the CEPH YACs My903D9, My912D2, and My933H2, whereas a STS in c307A12 was found in My891F3 and My972D3. These YACs were obtained from CEPH and the prepared DNA subjected to Pol digestion, Southern blotted, and probed with 16-08, 16-87, CRI-0119, and c306D2, and c307A12 in succession to confirm their content. In addition, a search of the Whitehead Institute database revealed that the two sets of YACs were joined into a contig by the YACs My801B6, My845D9, and My944D8. Each of these YACs was used as template DNA to assess STS content (D16S518, Afma336yg9, Wi2755, STSG-10102, and D16S3029) and subjected to FISH to assess position with respect to FRA16D (Fig. 1B).

Additional Probes, STSs, and BACs from the FRA16D Region. Additional probes were generated from the YAC 801B6 by subcloning PolI digests of YAC DNA and screening with total human DNA as probe. These subclones were digested with HincII to identify and isolate nonrepetitive DNA fragments as probes. This generated markers H13m, H23s, H23m, H29m, and H40m. Genome System Inc. BAC library filters were screened with the probes D16S3029, Afma336yg9, Wi2755, STSG-10102, H23s, H29m, and D16S3029 and nine BAC clones, including 379C2, 325M3, and 353B15, were identified. An additional STS, named 2AS, was established by “bubble” PCR from the end fragment of BAC 353B15 and was isolated as described by Gecz et al. (30). Briefly, the BAC DNA was digested with HindIII to identify and isolate nonrepetitive DNA fragments as probes. The final PCR was carried out with a combination of NotI-A bubble primer and Sp6-promoter primer as described except that an annealing temperature of 55°C was used for these STSs and hybridization probes were used to establish restriction maps of the YAC My801B6 and the BACs (Fig. 2A).

Subcloning and Contig Assembly. The YAC My801B6 and the BAC 325M3 were used as DNA templates for establishing a a subclone libraries in AGEM11 or AGEM12 vectors (Promega) according to the supplier’s protocol. My801B6 and 325M3 appeared to have intact human DNA inserts, based on comparative pulsed field gel mapping of the YACs and BACs across the region (data not shown).

FISH. FRA16D-expressing metaphases were obtained from peripheral blood lymphocytes by standard methods. Briefly, cultures were grown for 72 h in Eagle’s minimal essential medium, minus folic acid, supplemented with 5% FCS. Induction of FRA16D was with 0.5 μM aphidicolin (dissolved in 70% ethanol) added 24 h before harvest (31).

DNA clones were nick translated with biotin-14-dATP, preassociated with 6 μg/μl total human DNA, hybridized at 20 ng/μl to metaphase preparations, and detected with one or two amplification steps using biotinylated antiavidin and avidin-FITC as described previously (32). Hybridization signal was visualized using an Olympus AX70 microscope fitted with single pass filters for 4',6-diamidino-2-phenylindole (for chromosome identification), propidium iodide (as counterstain), and FITC.
Z53592, and D16S516 (GDB 200080). PCRs for GenBank accession number AA368108 (forward, 5'-TAATCCTACGCTCTGAGTAAGCCT-3'; reverse, 5'-GTATGATGATTTCAGGGGAC-3') were derived from partial sequence analysis of BAC353B15. Control PCRs for FRA3B deletions were D3S1234 (GenBank accession number 186387), D3S1300 (188420), and D3S1841 (254090).

Fig. 2. Positional cloning of FRA16D and the extent of heterozygous and homozygous deletion in the AGS tumor cell line. A, pulsed-field gel map of ~1 Mb of the right-hand side (RHS) of YAC My801B6 and the location of BACs, genetic markers, and STS markers (key markers are boxed). Restriction sites between Afma36yg9 and WI2755 are shown in B. The AGS stomach cancer cell line homozygous deletion is indicated: shaded circles, presence of PCR products for the STS markers; open circles, absence of PCR products for the STS markers. Maximal region of heterozygous deletion in AGS cell line is indicated by polymorphic D16S518 and D16S3029 PCR products, indicated as alleles A and B. The two AGS cell line chromosome 16s are indicated by shaded bars. B, restriction map of the critical FRA16D region (Afma36yg9 to WI2755) showing the location of key members of the λ subclone tile path used for FISH in Fig. 3. Clones designated A1-n are from 325M3; others are from 801B6. Open boxes, those subclones found to map proximal to the fragile site (on the basis that >85% of their FISH signals were proximal to FRA16D); gray boxes, those that appear to span the fragile site (85% on one side or other of FRA16D); black boxes, those that are distal to the fragile site (on the basis that >85% of their FISH signals were distal to FRA16D). A clones that gave high background on FISH were not scored. These and other λ clones for which FISH data were not obtained are included as thin boxes. STS localization of the AGS homozygous breakpoints are indicated by the presence (shaded circles) and absence (open circles) of PCR products.

Fig. 3. FISH of λ subclones against FRA16D expressing chromosomes. Each panel contains two FRA16D expressing partial metaphases, with and without FISH signal merged. In each case, the width of the gap or break at the fragile site is greater than the width of the chromatid. a, A504 showing signal proximal (P) to FRA16D; b, A181 showing signal proximal and distal (D) to FRA16D; c, A191 (top) and A8 (bottom) showing signal distal to FRA16D. Images of metaphase preparations were captured by a cooled CCD camera using the ChromoScan image collection and enhancement system (Applied Imaging International Ltd.). FISH signals and the 4',6-diamidino-2-phenylindole banding pattern were merged for figure preparation.
RESULTS

Positional Cloning of FRA16D. A contig of YAC clones was established in the 16q23.2 region between markers c306D2 and c307A12, which were found by FISH to map proximal and distal to FRA16D, respectively (Fig. 1B). The individual YACs from this contig were also used as hybridization probes to further localize the fragile site. These experiments identified the YAC 801B6 as spanning FRA16D, and therefore, this YAC was used as a source of DNA for subcloning the region to provide shorter DNA fragments for further refinement of the fragile site position. In addition, BAC clones were identified from the region to provide redundancy of cloned human DNA in an effort to avoid potential problems of instability of human DNA in YACs, as has been noted previously for other fragile site regions, including FRAXA (36), FRA10B (37), and a Chinese hamster aphidicolin-inducible fragile site region (38).

A pulsed-field gel restriction map of YAC 801B6 was constructed by using HincII restriction fragment subclones of the YAC for use as hybridization probes (H13m, H22s, H23m, H29m, and H40m; Fig. 2A). The position of the BACs (379C2, 325M3, and 353B15) with respect to the YAC restriction map was determined by both the restriction mapping of the BACs and the positioning of common markers by PCR or hybridization (Fig. 2A). The STS (D16S518, Afma336yg9, WI2755, STSG-10102, and D16S3029) content of the YACs and BACs was also determined to assist in map construction.

Subclone libraries of DNA from YAC 801B6 and BAC 325M3 were used to isolate overlapping DNA fragments by hybridization. A pulsed-field gel restriction map of YAC 801B6 was constructed by using HincII restriction fragment subclones of the YAC for use as hybridization probes (H13m, H22s, H23m, H29m, and H40m; Fig. 2A). The position of the BACs (379C2, 325M3, and 353B15) with respect to the YAC restriction map was determined by both the restriction mapping of the BACs and the positioning of common markers by PCR or hybridization (Fig. 2A). The STS (D16S518, Afma336yg9, WI2755, STSG-10102, and D16S3029) content of the YACs and BACs was also determined to assist in map construction.

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were generated using the λ vectors AGEM12 and AGEM11 (Promega), respectively, and assembled into a contig by end-fragment hybridization and restriction mapping. The integrity of the YAC restriction map was verified by comparison with that of the BACs 325M3 and 353B15. For the region between the BACs, the integrity was verified by the use of long-range PCR using human chromosomal DNA as template (data not shown).

**Localization of FRA16D by FISH.** There have been difficulties in determining the precise localization of common chromosomal fragile sites using FISH [FRA3B (12, 39–41), FRA7G (17, 18), and FRA7H (42)]. The FISH data have been interpreted as due to the fragile sites being spread out over long DNA sequences (e.g., hundreds of kilobases) or that there are multiple fragile sites at a single locus. An alternative explanation is that the DNA in the immediate vicinity of the fragile site is not tightly “packaged” into chromatin. We therefore chose to score only those chromosomes in which the width of the gap or break at the FRA16D fragile site was greater than that of one chromatid (Fig. 3). This approach was intended to reduce the possibility that the “unpackaged fragile site DNA” might be looping back over the distant side of the fragile site and therefore give a false “spanning” signal, particularly for probes that are very close to or within the fragile site region. In addition, although the use of preassociation in the hybridization process dramatically improved the signal to noise ratio, it did cause repeat-rich regions to be poor hybridization probes. This was particularly evident in the FRA16D region, where there is an abundance of DNA repeat sequences of various kinds.

The results of the FISH experiments are plotted in Fig. 4. The closest clearly proximal probe to FRA16D is λ1–44, whereas the closest unequivocally distal probe is λ433. These probes map at a distance of ~200 kb apart. However, this 200-kb region includes consistent scatter of distal signal around λ1–38 and λ1–27 and the poor hybridization between A181 and A511 (due to repetitive DNA content). Therefore, this 200-kb region defined by FISH analysis is likely to be the maximum sequence required to define FRA16D, rather than provide any evidence that the fragile site is spread over such a distance.

**Detection of Homozygous Deletion in Tumor Cell Lines.** The FRA3B fragile site HFT gene intron 4 region is a frequent site of deletion in various types of cancer (7). Homozygous FRA3B deletions have been detected in various human adenocarcinoma cell lines, including (gastric) AGS and Kato III; (breast) MDA-MB436; and (colon) LoVo, HT29, SW480, and LS180 (7). Because these deletions are somatic events that presumably occur as a result of exposure of these cells to certain environmental factors (10), we chose to analyze tumor cell lines that exhibit FRA3B deletions for the presence of homozygous deletion at the FRA16D locus.

STSs that were either mapped to the FRA16D region (Fig. 1) or generated from partial sequence analysis through the region (data not shown) were used to screen for homozygous deletion in various tumor cell line DNAs. The STSs were duplexed with a PCR from the dystrophin locus as an internal control. The results for the analysis of one of the FRA16D region markers, STSG-10102, is shown in Fig. 5. Of the seven tumor cell lines tested, the stomach tumor cell line AGS was found to be homozygously deleted at STSG-10102 and a series of contiguous markers through the region (Table 1), thus suggesting the presence of minimal deletions spanning the FRA16D region in each chromosome 16 present in the AGS cell line.

The proximal boundary of the AGS homozygous deletion (Fig. 2B) falls within the same interval (Alu20–178poly) as that of the HCT116 colon adenocarcinoma cell line homzygous deletion proximal boundary described by Paige et al. (28). This is also the most likely location of FRA16D (Fig. 2). The distal boundary of the AGS homozygous deletion is within the same interval (IM7–IM9) of the PEO4 ovarian adenocarcinoma cell line distal boundary (28). This

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Table 1 PCR results for markers in the FRA16D region in various tumor cell lines

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<sup>a</sup> Peripheral blood leukocyte (blood bank) DNA.
<sup>b</sup> No added DNA.
<sup>c</sup> +, present; −, absent.

**---**

V. Watson, personal communication.
coincidence of breakpoint boundaries suggests the likelihood of a common mechanism of deletion.

**Detection of Heterozygous Deletion in AGS Tumor Cell Line DNA.** The maximal extent of heterozygous deletion in the AGS tumor cell line in the FRA16D region was determined by genotyping polymorphic markers. The markers D16S518 and D16S3029 both gave two alleles indicating proximal and distal outer limits to the deletion of either chromosome 16 in AGS cells (Fig. 2A). The markers Afma336yg9 and 504CA were uninformative and therefore did not aid in delineating the limits of heterozygous deletion.

**DISCUSSION**

The region in which the chromosomal fragile site FRA16D is located has recently been shown to be associated with two types of chromosomal instability in cancer. In multiple myeloma, translocation of immunoglobulin loci into the 16q23 region causes the dysregulation of the c-MAF proto-oncogene on the affected allele. Although these breakpoints are spread over at least 500 kb, they bracket both the c-MAF gene and the FRA16D fragile site (Ref. 43 and Fig. 1). The dysregulated expression results in elevated c-MAF expression. The FRA16D deletion, therefore, if the DNA instability at FRA16D and FRA7G really is a consequence of the sequences that cause this class of fragile site, then one would expect FRA16D to behave in a similar manner.

What, then, are the possible biological consequences of FRA16D deletion? Given that the observed deletions are homozygous, they are therefore likely to represent the loss of a negative function (e.g., tumor suppressor) rather than the gain of a tumor promoting function. If the analogy with the FRA3B locus holds, then a gene either spanning or at least partially within the FRA16D commonly deleted region may contribute to neoplasia as a consequence of quantitative and/or qualitative effects of the deletion. Alternatively, the proximity of the FRA16D deletions to the c-MAF gene suggests that they have the potential to affect c-MAF expression. The FRA3B fragile site is located has recently been shown to be associated with two types of fragile site loci and given the coupling of replication with transcription, the deletion of the FRA16D region may lead to an alteration in the timing, with respect to the cell cycle, of the expression of genes in the area, including c-MAF.

**ACKNOWLEDGMENTS**

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Chromosomal Fragile Site \textit{FRA16D} and DNA Instability in Cancer

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