A role for common fragile site induction in amplification of human oncogenes

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Summary

Oncogene amplification is an important process in human tumorigenesis, but its underlying mechanism is currently unknown. Cytogenetic analysis indicates that amplification of drug-selected genes in rodent cells is driven by recurrent breaks within chromosomal common fragile sites (CFSs), via the breakage-fusion-bridge (BFB) mechanism. Here we show that BFB cycles drive the intrachromosomal amplification of the *MET* oncogene in a human gastric carcinoma. Our molecular evidence includes a "ladder-like" structure and inverted repeat organization of the *MET* amplicons. Furthermore, we show that the breakpoints, setting the centromeric amplicon boundaries, are within the CFS FRA7G region. Upon replication stress, this region showed perturbed chromatin organization, predisposing it to breakage. Thus, in vivo induction of CFSs can play an important role in human oncogenesis.

Introduction

A complex pattern of chromosomal aberrations is a common phenomenon in many cancers (Mitelman et al., 1997), but the mechanisms that initiate, direct, and enable this instability are still poorly understood. One form of cancer instability is intrachromosomal amplification of large genomic regions containing oncogenes. Amplification and subsequent overexpression of human oncogenes has been demonstrated for a variety of different neoplasias, and is thought to play an important role in the progression of tumor cells toward an increased malignancy (Brison, 1993).

Early events of gene amplification in cancer are usually unavailable for studying. Thus, model systems in cultured rodent cells were developed, in which amplification of genes conferring drug resistance is induced and selected for. Analysis of the structure and organization of such induced amplicons suggested that the mechanism underlying the early amplification events is breakage-fusion-bridge (BFB) cycles (Toledo et al., 1992). According to this model (McClintock, 1951) (Figure 1), an initial break (or telomere dysfunction) of a chromatid bearing the selected gene might lead to fusion of the uncapped sister chromatids after replication. The resulted dicentric chromosome forms an anaphase bridge between the centromeres, which will break while moving to opposite poles of the mitotic spindle. If this break occurs centromeric to the selected gene, a duplication of the region between the breaks is gained. Several recurrent cycles of chromosomal fusion and breakage under the appropriate selection lead to intrachromosomal amplification. Importantly, the recurrent breaks defining the boundaries of the induced amplicons were found to coincide with the cytogenetic location of specific hamster chromosomal loci (Kuo et al., 1994; Coquelle et al., 1997), defining as common fragile sites (CFSs) (Glover et al., 1984).

CFSs are specific regions in mammalian chromosomes that are prone to breakage and rearrangements. They appear as constrictions, gaps, or breaks in metaphase chromosomes of cells exposed to inhibitors of DNA replication, among which are known mutagens and carcinogens (Yunis et al., 1987), as well as inducers and enhancers of amplification events (Stark et al.,

SIGNIFICANCE

Here we provide molecular evidence that the chromosomal breakpoints that drive oncogene amplification occur nonrandomly. Our results show that the breaks setting the boundaries of amplified genomic regions might occur at specific chromosomal loci defined as fragile sites. There are ~100 fragile sites in the human genome estimated to encompass >100 Mb of DNA. The fragility of these sites is induced under conditions which interfere with DNA replication. We suggest that during in vivo tumorigenesis, cells can undergo genetic changes and/or be exposed to environmental factors that interfere with DNA replication and induce fragile site expression. Since many of the drugs used in cancer therapy are potential inducers of both fragile sites and gene amplification, they can lead to chromosomal rearrangements and further contribute to cancer development. Better understanding the effect of these drugs on the mechanisms that initiate, direct, and enable chromosomal instability is of major clinical importance and might lead to the development of better therapeutic approaches.

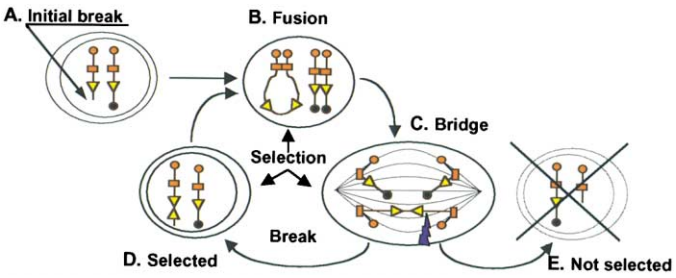

Figure 1. A scheme illustrating gene amplification via breakage-fusion-bridge cycles

Amplicons, yellow triangles; telomeres, orange (p-arm) or black (q-arm) circles; centromeres, orange rectangles. **A:** Interphase—an initial break gives rise to an uncapped chromatid carrying the selected gene. **B:** Metaphase—fusion of the 2 uncapped sister chromatids results in a dicentric chromosome. **C:** Anaphase—the dicentric chromosome forms a bridge between the opposite poles. A break of this chromosome leaves one daughter cell (**D**) with 3 copies of the selected gene, and only one copy in the other cell (**E**). Under a selection, recurrent cycles of BFB will occur, resulting in further accumulation of amplicon copies.

1989; Windle et al., 1991). They are classified as either rare or common, depending on their frequency within the population and their mode of induction. CFSs are considered to be part of the normal chromosome structure and thought to be present in all individuals (Sutherland and Richards, 1995). In tissue cultures, induced CFSs are preferential targets for chromosomal rearrangements, sister chromatid exchange, and integration of foreign DNA (Smith et al., 1998). So far, molecular analysis of CFSs has only been performed in human and mouse. This includes the partial identification of 4 sites (FRA3B, FRA7H, FRA7G, and FRA16D) out of ~90 that have been cytogenetically defined in the human genome (Boldog et al., 1997; Mishmar et al., 1998; Huang et al., 1998a; Paige et al., 2000; Shiraishi et al., 2001). These studies reveal that the fragile regions (the regions which exhibit fragility under the induction conditions) might encompass hundreds of kilobases (kb) of DNA. The molecular basis underlying their fragility is largely unknown; however, several studies show that intrinsic features of the fragile sequences might lead to perturbed fork progression. This can result in delayed replication along the fragile regions (Le Beau et al., 1998; Wang et al., 1999; Hellman et al., 2000), which is thought to interfere with the normal chromatin organization of the fragile region in metaphase, leading to fragility (Laird et al., 1987).

The ex vivo induction of CFSs was suggested to trigger and drive the amplification of drug-selected genes in rodent cells (Coquelle et al., 1997). However, the role of CFSs in the in vivo amplification of human oncogenes remains unclear. Here we investigate the possibility that recurrent chromosomal breaks in common fragile regions can drive BFB cycles, leading to amplification of human oncogenes. Under this model (Figure 1), the following would be predicted: (1) equal-spaced organization of intrachromosomal amplicons, visualized by fluorescence in situ hybridization (FISH) as a "ladder-like structure;" (2) clustering of the recurrent breaks within a CFS region; (3) inverted repeat organization of all the amplified copies along the same chromosomal arm; and (4) absence of sequences telomeric to the endogenous amplified region in the chromosome carrying the amplified copies. In this study, we reconstructed the molecular events that led to the amplification of the *MET* oncogene in a human gastric carcinoma and found that they fit with the BFB-CFS model. Our results suggest that recurrent breakage at CFS can lead to in vivo amplification of human oncogenes and other chromosomal rearrangements, thus shedding a new light on the role of CFSs in cancer.

Results

Equal-spaced organization of an amplified oncogene in a human cancer

If BFB cycles and recurrent breaks underlie oncogene amplifications, an equal-spaced organization of the amplified copies would be expected to be preserved in some amplification events, in which the initial organization was stabilized and preserved. We thus searched the literature for amplification of human oncogenes that resemble a ladder-like structure. Such a possible structure was noticed in the GTL-16 cell line, originating from a human gastric carcinoma (Motoyama et al., 1986). In this hypotetraploid cell line, 2 copies of a marker chromosome containing ~10 copies of the *MET* oncogene appear in all cells (Ponzetto et al., 1991), accompanied by overexpression and high activity of the MET tyrosine-kinase (Giordano et al., 1989). To study the amplicon organization in these cells, we applied FISH using clones from the vicinity of *MET*, and evaluated the results using confocal microscopy and computational image analysis (Hellman et al., 2000). As shown in Figure 2A, a clear ladder-like structure with 4 distinct intervals was visualized on the chromosomes carrying the amplification. This equal-spaced organization suggests that all the amplicons were bordered by breakpoints at the same chromosomal region. Thus, GTL-16 cells provide a suitable system to investigate the involvement of a BFB mechanism and the role of fragile site expression in cancer amplification.

Induction of perturbed chromatin organization along the CFS FRA7G region

Previous analysis in GTL-16 cells defined the centromeric boundary of the amplicons within an interval of several Mb at 7q31.1-7q31.2 (Ponzetto et al., 1991). The only CFS in this interval is FRA7G, cytogenetically mapped to 7q31.2 (Yunis et al., 1987). Although Huang et al. characterized ~300 kb spanning the fragile region (Huang et al., 1998a; 1998b), the entire FRA7G region and its location relative to *MET* have not yet been defined (Tatarelli et al., 2000).

To further define and characterize the entire FRA7G region, we constructed a physical map covering ~10 Mb of 7q31 by isolating BAC clones that bridged contiguous sequenced regions from the public and the Celera databases (Figure 3E). We further determined the location of clones relative to FRA7G gaps and constrictions by using FISH on metaphase chromosomes induced to exhibit CFSs. A clone was considered as spanning the fragile region if on different chromosomes from the same preparation its hybridization signals appeared centromeric or telomeric to the FRA7G gaps, or crossed the gaps ("both sides") (Mishmar et al., 1998). Since there are several CFSs along 7q, we used computational image analysis to identify FRA7G (Experimental Procedures). We were able to define 2 distinct zones within the region of FRA7G. One zone encompassed ~700 kb (dark blue in Figures 3B and 3F) and comprised clones (c169h6, AC002066, and V203C) that appear to span the FRA7G gaps (Figure 2B, Table 1). The more telomeric 19d10 clone (*RAY1/*

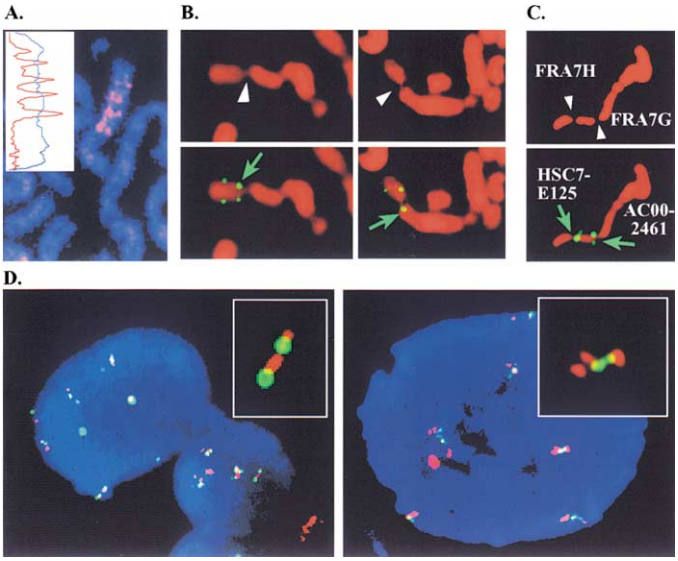

Figure 2. Organization of the *MET* amplicons relative to FRA7G region

A: The organization of the *MET* amplicons along a GTL-16 metaphase chromosome, analyzed by FISH using clone V193A as a probe. Insert: Computational representation of the FISH signals (red) and the DAPI staining (blue) along the amplified marker chromosome. **B:** FISH analysis of the FRA7G region. Upper panels: metaphase chromosomes expressing FRA7G (white triangles), stained with propidium iodide (PI). FRA7G are seen as unstained gaps. Bottom: the same chromosomes probed with a *MET* clone (cosmid c169h6, green arrow) and a reference clone from 7q32 to mark chromosome 7. The *MET* signals appear telomeric (left) or centromeric (right) to FRA7G gaps. **C:** Upper panel: A metaphase chromosome stained with PI, expressing both FRA7H and FRA7G. Bottom: The same chromosome probed with YAC HSC7E125 (FRA7H) and BAC AC002461 (FRA7G). Note the opposite orientation of both clones relative to the physical map. **D:** The order of clones along the FRA7G region, analyzed by FISH on interphase nuclei from the GM00847 cells (each has 4–6 chromosomes 7). Left: AC003080 (green); AC025297 (red); AC002461 (green); AC002465 (red). Right: HSC7E160 (7q31.1) (red); AC073137 (green); AC002461 (green); AC002465 (red). Inserts: a magnification of one chromosome from each nucleus. Note that the order found in these FISH experiments is the expected order, based on the sequenced contigs (Figure 3).

ST7 locus) showed hybridization signals only telomeric to the FRA7G gaps, indicating that the telomeric border of this zone must be between the *MET* and the *RAY1/ST7* loci (Figure 3 and Table 1).

The other zone encompassed 3–4 Mb (light blue in Figures 3B and 3F) and was comprised of clones showing an unusual hybridization pattern, since they hybridize mostly to the telomeric side of the FRA7G gaps, even though they are located more centromeric on the physical map (clones AC034112 to AC002463, Figure 2C and 3F and Table 1). The centromeric border of this zone was found between AC002463 and AC003080. To exclude the possibility that the opposite orientation of these clones resulted from an inversion or translocation of the region between AC002463 and AC034112 in the GM00847 cells, we performed a dual-color FISH analysis on interphase nuclei from cells grown under normal growth conditions. The signal order of 2 sets of clones was analyzed in at least 50 interphase chromosomes lying in a linear position (Figure 2D). This analysis showed that the order of clones along the entire region was as expected from the physical map, excluding the possibility of a chromosomal rearrangement in the GM00847 cells. To confirm the mapping of the opposite orientation region and to further exclude the possibility of a chromosomal rearrangement, we performed FISH on metaphase chromosomes expressing FRA7G in another human cell line, PANC-1. A similar telomeric orientation of clone AC002089 was identified in these cells (data not shown). These results suggest that the opposite-orientation pattern reflects an unusual chromatin organization of the fragile region in metaphase chromosomes exhibiting fragility. Thus, the analysis of the entire FRA7G region showed that fragile site induction can lead to an unusual chromatin organization (gaps, breaks, and spanning or opposite-orientation organization) along ~5 Mb of DNA.

Perturbed DNA replication along FRA7G

Perturbed DNA replication was previously found along CFS sequences (Le Beau et al., 1998; Wang et al., 1999; Hellman et al., 2000). To further define the region encompassed by FRA7G, we analyzed the replication pattern along that region, using FISH on S-phase nuclei. In this method, a high percentage of unreplicated alleles (single hybridization dot, S signal) indicates that the region is replicated relatively late in the S-phase, while a high percentage of replicated alleles (double dot, D signals), indicates a relatively early replication time. For most sequences, the 2 alleles replicate in a synchronous manner and have a low percentage (10%–20%) of SD signals (Selig et al., 1992).

First we analyzed the replication pattern of the nonfragile region telomeric to FRA7G (Figure 3). This analysis has revealed 2 distinct replication time zones: the region adjacent to FRA7G (cosmid clones c172d6 and c19d10, orange in Figures 3C and 3G) showed a relatively early replication time and an allelic synchronous replication pattern (18% and 19% SD, Table 2) while the more telomeric part (CW44 and CNH24, dark green in Figures 3C and 3G) showed a late and synchronous pattern (12% and 10% SD). Thus, the region defined by the cytogenetic analysis as the telomeric nonfragile region indeed presented a normal replication pattern with early and late replication time zones, which likely correspond to the R-band 7q31.2 and the G-band 7q31.3, respectively (Figure 3A).

We then analyzed the replication pattern of clones that span the FRA7G gaps. All of these clones (149e12, 182b3, 63e3, 19d5, and V193A, pink in Figures 3C and 3G) showed early replication time, but with high levels of allelic asynchrony (29%–32% SD, Table 2). Such high levels of asynchrony have previously been found along the CFS FRA7H and might reflect perturbed DNA replication (Hellman et al., 2000). However, it might also reflect allele-specific replication time of regions harboring parentally imprinted genes (Kitsberg et al., 1993a). We excluded this possibility by analyzing the replication pattern of this region in an isodisomic cell line (CF33-3) carrying 2 maternal chromosomes 7 (Hellman et al., 2000). The FRA7G clone V193A showed asynchronous replication in both normal (CF33-2) and the isodisomic cells (30% and 27% SD, respectively), indicating that its asynchrony is not a result of allele-specific replication time. It is important to note that the identified asynchrony might also result from an abnormal separation of the sister chromatids as part of the unusual chromatin organization of the region.

We further investigated the replication pattern along the FRA7G region by analyzing the replication time differences between adjacent sequences. The cosmid clone c63e3 replicated before c182b3 (35 kb apart) in 62% of the nuclei, and the clone c169h6 replicated before c149e12 (150 kb apart) in 76% of the

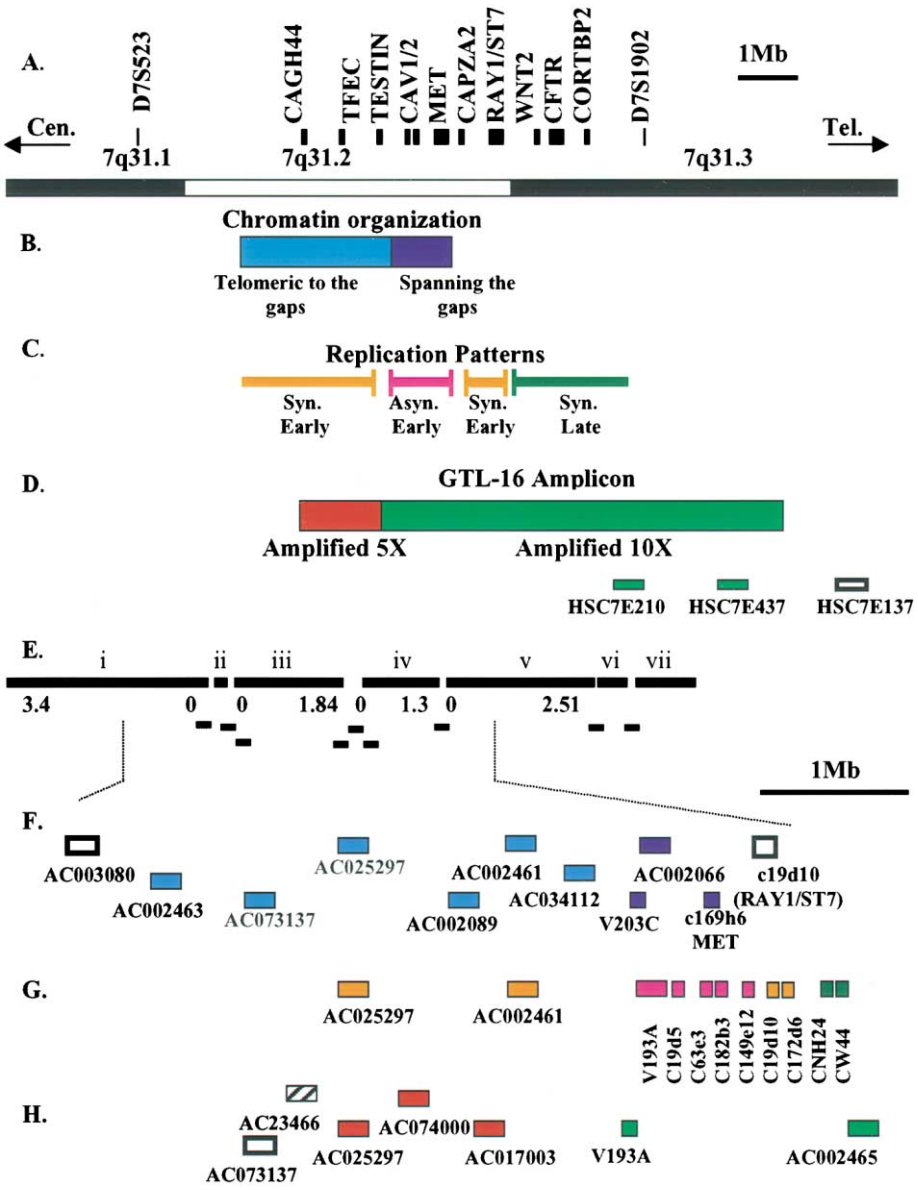

Figure 3. Maps of the FRA7G region

A: Reference genes and polymorphic markers at 7q31.1–7q31.3. **B:** A region spanning the cytogenetic gaps and constrictions (dark blue), and a region showing telomeric signals of clones that are located more centromeric on the physical map ("opposite oriented") (light blue). **C:** Regions showing normal (synchronous) and early replication pattern in orange, normal and late in dark green, and unusual (asynchronous) and early in pink. **D:** The boundaries of the GTL-16 amplicons, 5× region in red, 10× in green. Below are indicated the YAC clones used in the analysis of the telomeric boundary. **E:** The set of Celera DNA sequence scaffolds (upper line) connected by BAC clones (bottom lines). The numbers indicate the size of each sequence in Mb. The sequence names from left to right are: i-GA_x8Y8FG7; ii-GA_x8Y8FKQ; iii-GA_X2HTBKNJPOH; iv-GA_x2HTBL4GTR2; v-GA-x8WU11E; vi-GA_x2HTBKNGALA; vii-GA_x2HTBI2VJAN. The BAC clones or accession numbers from left to right are: AC073346; CIT-2172F3; AC032017; AC073901; CIT-2012G19; AC015621; NH032P06; NH516A21; AC005016. **F:** Clones used for the cytogenetic analysis of FRA7G gaps and constrictions. **G:** Clones used for the replication analysis. **H:** Clones used for the analysis of the amplicon boundaries and the copy number within the amplified region. The colors of the clones in **F–H** are the same as in **B–D**.

nuclei. The ability to clearly detect the replication time order of such adjacent clones suggested an unusual replication rate along FRA7G, as previously found along FRA7H (Hellman et al., 2000). Hence, the replication analysis supported the cytogenetic identification of the FRA7G region, and showed that this region has an intrinsic replication perturbation, in addition to its induced chromatin perturbation.

Breakpoints within FRA7G set the centromeric boundaries of the *MET* amplicons

If the amplification of *MET* in GTL-16 cells originated by a BFB-CFS mechanism, then the centromeric boundaries of the amplicons are expected to lie within the FRA7G region. In a previous study, the boundaries of the *MET* amplified region were estimated to lie at least 1 Mb centromeric and 2 Mb telomeric to *MET* (Ponzetto et al., 1991). To further define the boundaries, we applied dual-color FISH on GTL-16 nuclei using clones from 7q31. In this analysis, amplified sequences were detected as 2 groups of multiple hybridization signals (representing the 2 amplified chromosomes), while nonamplified sequences were detected as 4 isolated signals (representing the 4 copies of chromosome 7 in these cells; examples in Figure 4A). Using this approach, we defined the telomeric boundary of the amplified region between YACs HSC7E437 and HSC7E137 (Figure 3D) and the centromeric boundary between BACs AC025297 and AC073137 (Figure 3H). Very weak signals were seen with AC023466, indicating that the breakpoint is probably within this clone. These results show that the centromeric boundary of the amplified region is within the chromatin perturbation region of FRA7G, as expected from the model.

Since FRA7G encompasses a large genomic region, and assuming that the 10 amplified copies were generated by BFB cycles, more than one breakpoint was expected within the fragile region. In order to identify additional breakpoints, we ana-

Table 1. FISH analysis of hybridization signals on metaphase chromosomes exhibiting FRA7G

Clones	Number of signals		
	Centromeric	Both sides	Telomeric
c19d10 (*RAY1/ST7*)	0	0	21
c169h6 (*MET*)	2	1	17
AC002066/V203C	12	1	21
AC034112/AC002461	0	0	40
AC002089	1	2	43
AC025297	2	1	33
AC073137	1	0	54
AC002463	1	1	40
AC003080	18	0	3

The clones are ordered in accordance with the physical map, telomeric (top of the table) to centromeric (bottom). The results of clones covering the same genomic region were combined.

Table 2. Replication pattern (% SD, SS, and DD) of S-phase nuclei

Clones	SD	SS	DD
Synchronous and late			
CW44	12	73	15
CNH24	10	77	13
Synchronous and early			
C172d6	18	37	45
C19d10	19	45	36
Asynchronous and early			
C149e12	31	34	35
C182b3	29	31	40
C63e3	32	27	41
C19d5	30	33	37
V193A	29	28	43
Synchronous and early			
AC002461	18	25	57
AC025297	16	28	56

The clones are ordered according to the physical map, telomeric (top of the table) to centromeric (bottom).

lyzed the level of amplification along the amplified region by counting FISH signals in interphase nuclei (only nuclei in which the analyzed region was replicated were considered, Experimental Procedures). The analysis has revealed 2 distinct levels of amplification. Most (8/10 Mb) of the amplified region, covered by clones HSC7E437, AC002465, and V193A, showed 20 $\pm$ 5 signals per group (green in Figures 3D and 3H, and Figure 4B). This indicated that each marker chromosome carried 10 copies of the amplified region, in agreement with previous studies (Ponzetto et al., 1991). However, the centromeric 2 Mb of the amplified region, encompassed by BAC clones AC017003, AC074000, and AC025297 (red in Figures 3D and 3H), showed only 10 $\pm$ 3 signals/group, indicating only 5 copies/chromosome (Figure 4B). Hence, 2 breakpoints were identified in the centromeric boundary of the *MET* amplification: one between the high- and the low-amplified regions (between V193A and AC017003), and the other between the low- and the nonamplified regions (between AC025297 and AC073137). These 2 breakpoints lie within the FRA7G region, as predicted by the BFB-CFS mechanism. The final amplification break (accounting for $>$8 *MET* copies, Figure 5A, stage iv) is expected to appear in only one copy (Figure 5A, stage v). This final breakpoint could not be identified since a change in only one FISH signal is below the resolution of the analysis.

The GTL-16 amplicons are organized as inverted repeats

Another important prediction of the BFB model is an inverted repeat organization of the amplicons (Figure 1). The first indication for such an organization in GTL-16 cells was the 5-step ladder as demonstrated by hybridization with clone V193A, which is 2 Mb from the edge of the amplicon (Figure 2A). This type of pattern would be expected using a FISH probe from the edge of the amplicon, assuming an inverted repeat organization. An alternating pattern between close signals (below the resolution of FISH at metaphase) and distant signals should be observed, and this could result in a 5-step ladder, with each rung corresponding to 2 copies of the *MET* amplicon (Figure 5A). As can be seen in Figure 2A, this was indeed the identified pattern.

Since FISH resolution is much higher in interphase ($>$100 kb), we further examined the amplicon organization in interphase chromosomes. In the case of an inverted repeat organization, signals of a clone from the edge of the amplicon are expected to appear adjacent to each other, while signals of a clone from the center are expected to flank them (Figure 5A). In contrast, in the case of a direct repeat (head to tail) organization, the signals from the edge and those from the center are expected to alternate, and in a case of a random organization, no pattern is expected. The analysis of paired probes, one from the center and the other from the centromeric edge of the GTL-16 amplicon, showed a pattern (Figure 5B) consistent with an inverted repeat organization, as predicted by the BFB model.

The structure of the amplified chromosome

In many amplification events in cancer cells, the initial amplicon organization is hampered by secondary rearrangements which occur during the cancer development. However, in GTL-16 cells, the amplicon organization indicates that such secondary rearrangements did not occur in these cells. By using spectral karyotyping (SKY) and FISH analyses we found that the chromosome arm harboring the *MET* amplicons is fused to the centromere and the short arm of chromosome 12 (Figures 4C and 4D). Such a fusion could have prevented secondary BFB cycles, due to the recapping of the broken chromosome end (Toledo et al., 1992). Importantly, FISH analysis of clones from the telomeric part of 7q (clones HSC7E137 from 7q31.3 and c53g3 from the *MEST* locus in 7q32) showed that sequences telomeric to the endogenous amplified region are absent in the marker chromosome, as predicted by the BFB model. In addition, the karyotype analysis of GTL-16 showed that most chromosomes have 4 copies, while chromosome 7 has 2 normal and 2 deleted copies, and chromosome 12 and the 7-12 fused marker chromosome each appear in only 2 copies (Rege-Cambrin et al., 1992). This organization further supports our hypothesis that the amplified chromosome was stabilized by fusion with the short arm of chromosome 12 following the amplification. However, the fusion between the amplicons and chromosome 12 sequences could have also resulted from a recombination event prior to the BFB cycles.

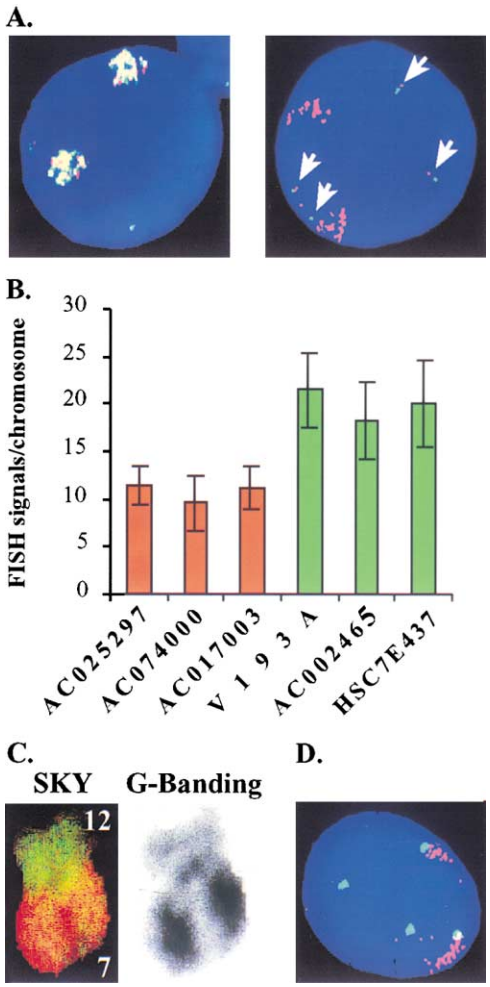

Figure 4. The organization of the amplified chromosome in the GTL-16 cells

A: FISH analysis of the amplicon boundaries. Left: an interphase nucleus hybridized with the *MET* clone c169h6 (green) and with clone V193A (red). The signals of both probes colocalize and appear yellow. Right: an interphase nucleus hybridized with V193A (red) and HSC7E137 (green). HSC7E137 signals appear only on the 4 nonamplified copies of chromosome 7 (arrowheads), indicating that this clone is not included in the amplicon. **B:** The level of amplification of clones encompasses the amplified region, determined by counting FISH signals in interphase nuclei. Only nuclei in which the analyzed region was replicated were considered. **C:** Left: SKY analysis of the amplified chromosome. Chromosome 12 sequences in green, chromosome 7 in orange. Right: G-banding of the same chromosome. **D:** An interphase nucleus from GTL-16 cells hybridized with a chromosome 12 centromeric probe (green) and V193A (red).

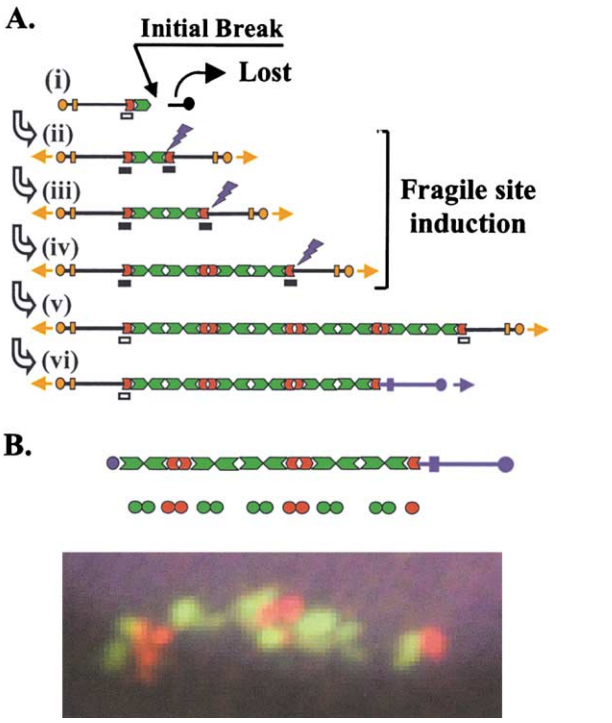

Figure 5. Reconstruction of the events which led to the *MET* amplification in GTL-16 cells

A: The sequence of events, leading to amplification of *MET* in the GTL-16 cells. The 5× region is shown in red, the 10× region in green, induced FRA7G as black rectangles, uninduced as empty rectangles. Only intact (nonrearranged) fragile regions are marked. Other elements have the same color code as in Figure 1. **B:** Upper: The expected hybridization pattern along one chromatid of the amplified chromosome. Bottom: A replicated amplified chromosome of a GTL-16 interphase, hybridized with clone AC025297, from the 5× amplified region (red) and AC002465 from the 10× amplified region (green).

Discussion

Here we demonstrate the role of a BFB-CFS mechanism in the amplification of a human oncogene in vivo. Our study provides evidence for all the predictions of this model: (1) An equal-spaced organization of the amplified units, visualized as a ladder-like structure. (2) Clustering of the recurrent breaks within CFS regions (rather then distribution of the breaks along the distance between the oncogene locus and the centromere). Our study provides molecular evidence that breakage leading to oncogene amplification preferentially occurs within CFS regions. Since human CFSs encompass large genomic regions, different breakpoints within the fragile region are expected to occur during subsequent BFB cycles. The regions between the breakpoints are expected to be included only in a subset of the amplicons (Figure 5). Here we identified 2 breakpoints (2 Mb apart) that set the centromeric boundaries of the *MET* amplicons. Both breakpoints lie in a region adjacent to the FRA7G spanning region, which showed unusual chromatin organization upon fragile site induction. Such an unusual organization (the "opposite orientated" phenomenon) was found around the spanning region of another CFS, FRA7H (Mishmar et al., 1998) (Figure 1C). Thus, we suggest that CFSs might consist of "core sequences" and "affected sequences." The core sequences (the spanning regions) have an unusual replication pattern (Le Beau et al., 1998; Wang et al., 1999; Hellman et al., 2000) (Table 2), which predispose them to additional replication delay under conditions that induce fragility. The replication delay might perturb the condensation of the chromatin, resulting in large regions showing perturbed chromatin organization at metaphase (Figure 1C). The unusual organization of the chromatin loops around the gaps might account for the "opposite-orientation" phenomenon. (3) Symmetric and alternate organization of the amplified copies, which fits with an inverted repeat organization of the amplified region. As can be seen in Figure 5B, such an organization was found for the 10× and 5× amplified regions in GTL-16 cells. (4) Absence of sequences telomeric to the endogenous

amplified region, in chromosomes carrying the amplified copies. In the GTL16 cells, no sequences telomeric to HSC7E137 were identified in the marker chromosome. Hence, for the amplification of *MET* in the GTL-16 cells, all the predictions of the BFB-CFS model have been verified at the molecular level. It therefore appears that intrachromosomal amplification of human oncogenes may arise by the same mechanism as suggested for the amplification of drug resistant genes in rodent cells.

We suggest the following sequence of events (diagrammed in Figure 5) for in vivo oncogene amplification in tumor cells: (1) An initial break sets the telomeric boundary of the amplified unit and leads to deletion of the region telomeric to the break (Figure 5A). The break might occur spontaneously, or as part of general chromosomal instability that characterizes early stages of many cancer types (Lengauer et al., 1998), such as nonreciprocal translocations caused by telomere dysfunction (Artandi et al., 2000). However, the initial break might also occur at CFSs due to exposure to induction conditions. In GTL-16 cells, this break occurred at 7q31.3, between YACs HSC7E437 and HSC7E137. (2) The initial break generates uncapped chromosomal ends, which can lead to end-fusion of the sister chromatids, resulting in a dicentric chromosome (Figure 5A). (3) At the same stage, the cells can undergo additional genetic changes and/or be exposed to environmental factors (e.g., hypoxia, deregulation of the nucleotides pools, and treatment with cytotoxic drugs) that interfere with DNA replication and induce fragile site expression (Yunis et al., 1987). All these conditions are also enhancers of gene amplification (Stark et al., 1989; Coquelle et al., 1998; Poupon et al., 1996). Importantly, FRA7G was shown to be induced by several agents, such as methotrexate or actinomycin D (Yunis et al., 1987), which are inducers of gene amplification. The unusual chromatin organization of induced fragile regions predisposes them to chromosomal breaks during anaphase, when the dicentric chromosome is segregating to opposite poles (Figure 5). In GTL-16 cells, this evidently led to a break within FRA7G, between the 10× and the 5× amplified regions. (4) Additional fusion-bridge-breakage cycles occur, giving rise to the amplification of the region between the breaks. In GTL-16 cells, 3 such BFB cycles presumably occurred, resulting in 16 extra copies of the *MET* amplicon. (5) Eventually, the selection pressure disappears once the oncogene has attained sufficient amplification. Since *MET* protein is known as a scatter factor, which triggers cell proliferation, cell survival, cell motility, invasion of extracellular matrix, and induced angiogenesis (Prat et al., 1998), we postulate that in the GTL-16 cells, the disappearance of the selection pressure (which also induces fragility) might have occurred as a result of the development of motility and/or angiogenesis ability in cells carrying extra copies of MET, leading to reoxygenation of the tumor cells. (6) Secondary BFB cycles can occur, hampering the initial organization of the amplicons, unless the uncapped chromosomal ends stabilize within a short time following amplification. In the GTL-16 cells, the amplified chromosome fused with chromosome 12, and the breakage of this dicentric 7-12 chromosome was stabilized, giving rise to the marker chromosome (Figures 4C and 4D and Figure 5A, stage vi), probably shortly following the amplification.

Recent studies suggest that BFB cycles represent a general mechanism leading to cancer instability. Gisselson et al. investigated cancer cells with high intratumor heterogeneity, and found evidence for frequent BFB events, including anaphase bridges, telomeric associations, and dicentric ring chromosomes, in both tissues and cell lines from a variety of solid tissues cancers (2000a). Artandi et al. showed that BFB cycles promote nonreciprocal translocations and epithelial cancers in mice (2000). Shuster et al. reported a pattern of rearrangements in oral squamous cell carcinoma cell lines that fit to the BFB mechanism of amplification in human chromosome 11q13, a region that harbors several oncogenes (2000). Our search of the literature identified additional examples of symmetric amplicon organization, which might indicate ladder-like organizations in amplifications of several human oncogenes, including *KRAS2* (Figure 7 in Gisselsson et al., 2000b), *MLL* (Figure 3C in Kakazu et al., 1999) and *C-MYC* (Figure 4 in Falzetti et al., 2000). Hence, the lack of visual ladder-like structures in most human tumors containing intrachromosomal amplifications probably reflects their late stage in the cancer progression, during which secondary rearrangements can hamper the recognition of the initial amplicon organization.

These findings show that the expected results of BFB-CFS amplification could be found in a variety of human tumors, suggesting that fragile sites may play an important role in cancer. Yunis and Soreng (1984) put forward the hypothesis that CFSs play a role in cancer instability, based on the correlation between chromosomal bands in which CFSs are mapped and bands harboring cancer breakpoints and/or oncogenes. Subsequently, deletions of regions containing tumor suppressor gene(s) were found at the human CFSs FRA3B, FRA7G, and FRA16D regions, indicating instability in cancer cells (reviewed in Smith et al., 1998; Mangelsdorf et al., 2000). However, these studies left open the question of whether this instability is the outcome of an intrinsic instability conferred by the fragile sites or due to the selection of altered cancer genes located in these regions (Smith et al., 1998). Our study suggests that these cancer breakpoints are indeed the outcome of an intrinsic instability conferred by the fragile site sequences, since in the case of intrachromosomal amplification of large genomic regions, the breaks are distant from the targeted genes and thus are not affected by selection. Thus, it is important to investigate the molecular organization of additional intrachromosomal oncogene amplifications (such as *C-MYC*, *cyclin D1*, *MLL*) and the role of the fragile sites in their vicinity (FRA8C or D, FRA11F or A, and FRA11G, respectively).

In many cancers, chromosomal instability precedes and promotes the dysfunction of specific cancer genes (Lengauer et al., 1998), leading to the generation and/or to the progression of malignancy (Rennstam et al., 2001). Our results suggest that under conditions which induce the expression of CFSs, these regions can direct chromosomal rearrangements that play a significant role in cancer development. Since many of the drugs used in cancer therapy are potential inducers of both fragile sites and amplification, they can lead to chromosomal rearrangements and further contribute to cancer development. Thus, a better understanding of the causal relationship between cancer therapeutic agents and their specific targets (specific fragile sites and oncogenes) will provide the required information for developing better therapeutic approaches.

Experimental procedures

Cells and growth conditions

The cell lines used in this study: GTL-16, a gastric carcinoma cell line with amplification of the *MET* oncogene (Motoyama et al., 1986); Manca, a lym-

phoma cell line; CF33-2, a normal lymphocyte cell line; and CF33-3, a chromosome 7 isodisomic lymphocyte cell line (Hellman et al., 2000). GM00847 (National Institute of General Medical Sciences, Camden, NJ), a simian virus 40 (SV40)-transformed human fibroblast cell line. PANC-1 (American Type Culture Collection, Rockville, MD), a tumor-cell line established from a carcinoma of the pancreas. All cell lines except GM00847 were grown in RPMI medium containing 10% fetal calf serum. GM00847 was grown in MEM-EAGLE medium supplemented with 10% fetal calf serum.

Contigs, sequences, and DNA probes

A DNA sequence-based map of chromosome 7q31.1 to 7q31.3 was constructed by comparing and combining information acquired from the Celera scaffold assemblies and from high-throughput (HTGS) and finished sequence from the public GenBank database. Physical gaps were bridged by BAC clones that have either been sequenced or whose end-sequence (TIGR BAC End Sequence Database) was known or generated. BLAST2 analysis was used to align all sequences. The assembled map with no physical (clone) gaps represents a consistent presentation of order of DNA markers, in comparison to our other studies which used the additional technologies of radiation and somatic cell hybrid mapping, and FISH (see http://www.genet.sickkids.on.ca/chromosome7/ for any additional information on clones required).

Fluorescent in situ hybridization (FISH) on interphase nuclei

FISH experiments on interphase nuclei (for mapping of FRA7G region, replication time analysis, and mapping of the amplicon boundaries and copy number) were performed as previously described (Hellman et al., 2000). To avoid misinterpretation of the amplification signals, only nuclei in which the 4 nonamplified copies of chromosome 7 were replicated (showed D signals) were considered in the analysis. Signals of 50 replicated amplified chromosomes were counted for each probe.

Cytogenetic analysis of the FRA7G region

GM00847 cells were grown on coverslips, and fragile sites were induced by growing the cells in M-199 medium in the presence of 0.4 μM aphidicolin and 0.5% ethanol for 24 hr prior to chromosome fixation. FISH mapping of the region encompassed by FRA7G was performed as previously described (Mishmar et al., 1998). Since there are several aphidicolin induced fragile sites on 7q, their positions had to be carefully determined. Using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD), the distance of the fragile sites from the tip of the long arm of chromosome 7 was measured, relative to the total length of the chromosome. According to the Genome DataBase mapping of the fragile sites, this value should be $\sim$15% for FRA7H, $\sim$30% for FRA7G, and $\sim$37% for FRA7F. Analysis based on 150 FRA7G measurements indicated that it is located at 29% $\pm$ 2.5%. Observed cytogenetic gaps within 2 SD were considered to be FRA7G.

Spectral karyotyping analysis

Chromosome labeling was performed with the SKY fluorescent labeling kit (Applied Spectral Imaging, Migdal HaEmek, Israel) according to the manufacturer's protocol. Chromosomes were counterstained with DAPI. Image acquisition was performed by use of a SD200 Spectracube (Applied Spectral Imaging, Inc.) mounted on an Olympus BH-2 microscope using a custom designed optical filter (SKY-1, Chroma Technology, Brattleboro, VT, USA). Automatic identification of chromosomes was based on the measurement of the spectrum for each chromosome.

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