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Initiation of the breakage–fusion-bridge mechanism through common fragile site activation in human breast cancer cells: the model of *PIP* gene duplication from a break at FRA7I

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Gene amplification plays a critical role in tumor progression. Hence, understanding the factors triggering this process in human cancers is an important concern. Unfortunately, the structures formed at early stages are usually unavailable for study, hampering the identification of the initiating events in tumors. Here, we show that the region containing the *PIP* gene, which is overexpressed in 80% of primary and metastatic breast cancers, is duplicated in the breast carcinoma cell line T47D. The two copies are organized as a large palindrome, lying '*in loco*' on one chromosome 7. Such features constitute the landmark of the breakage–fusion-bridge (BFB) cycle mechanism. In hamster cells selected *in vitro* to resist cytotoxic drugs, common fragile site (CFS) activation has been shown to trigger this mechanism. Here, we characterize FRA7I at the molecular level and demonstrate that it lies 2 Mb telomeric to the *PIP* gene and sets the distal end of the repeated sequence. Moreover, our results suggest that the BFB process was frozen within the first cycle by healing of the broken chromosome. T47D cells thus offer a unique opportunity to observe the earliest products of the BFB cycle mechanism. Our findings constitute the first evidence that this amplification mechanism can be initiated *in vivo* by fragile site activation.

INTRODUCTION

Gene amplification is a genetic alteration through which a cell gains additional copies of a small part of its genome. In mammalian cells, such mutations significantly contribute to tumor progression and possibly to tumorigenesis (1,2). The mechanisms responsible for amplification have been extensively studied in model systems of drug-resistant mutants selected *in vitro* from rodent cell lines (3). More recently, fluorescence *in situ* hybridization (FISH) has proven to be a very powerful tool to analyse the structures formed in the early events of amplification. This allowed the discovery that at least two different mechanisms, relying on unequal segregation of chromosomal sequences at mitosis, can drive amplification. One of them, the BFB cycle mechanism (4), is responsible for the accumulation of intra-chromosomal extra-copies creating chromosomal expansions known as homogeneously staining regions (HSR) (5–9). This amplification mechanism can be triggered by a double-strand break distal to the selected gene (10), followed by fusion of the broken sister chromatids after replication, which in turn leads to the formation of a bridge and further break in mitosis. The cytogenetic manifestations of this mechanism, such as accumulation of extra-copies organized as large inverted repeats within a chromosome have also been

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observed in tumor cells (11–14). Thus, it is now generally agreed that the BFB cycle mechanism operates, at least in some cases, in tumors. The demonstration that breaks are able to initiate this amplification mechanism suggested that the drugs used *in vitro* to select resistant mutants, particularly when they are well known DNA damaging agents, could contribute to trigger the process (15). Indeed, we have established that the ability of a drug to induce amplification directly relies on its ability to activate a CFS distal to the selected gene (9).

Fragile sites are chromosomal regions prone to breakage under specific culture conditions (16,17). They are classified as common and rare depending on their frequency in the populations [reviewed in (18,19)]. CFS are present in cells of all individuals and are well conserved when examined within linkage groups of different mammalian species (20–22). These observations identify them as full part components of the chromosomes. So far, seven human CFS have been characterized at the molecular level (23–29). In each case, a fragile region extending over hundreds of kilobases was identified. Several works suggested that fragility relies on particular sequences and/or chromatin structures that delay replication of the fragile locus (30–32). CFS have been involved in a growing number of chromosome rearrangements that drive tumor progression, such as deletions of tumor suppressor genes (33,34), virus integration (28) and translocations (26,35). More recently, they have also been involved in breakage of anaphase bridges formed during the amplification process (14). However, whether breaks at CFS are also responsible for the initiation of the BFB cycles in cells of human cancers remains to be established.

The *PIP* gene encodes a secreted factor known as prolactin-inducible protein (PIP) (36), which binds to CD4 (37–39) and carries a fibronectin-specific aspartyl protease (40). According to the human genome sequence public database (http://www.nhgri.nih.gov/genome_hub.html), the *PIP* gene maps to 7q34. It is present as a single copy and spans ~8 kb of genomic DNA (41). We have recently found that the *PIP* gene exhibits various rearrangements in several solid tumors (42,43). It is also overexpressed in 60 to 80% of primary and metastatic breast cancers (36,44), as well as in some breast carcinoma cell lines. However, the mechanisms responsible for the overexpression of this gene are still poorly understood. Cytogenetic analyses and comparative genomic hybridization have shown that breast cancers frequently exhibit numerical and structural alterations of chromosome 7 (45–48). Strikingly, six CFS sensitive to aphidicolin, the archetype of CFS inducing-drugs (49), have been described along the long arm of chromosome 7 (17). Only two of them, FRA7G and FRA7H, which map at 7q31.2 and 7q32.3, respectively, have been characterized at the molecular level (24,25). Poor information is available about FRA7I, which was assigned to the 7q36 region by G/Q banding (17).

Using the breast carcinoma cell line T47D, which constitutively overexpresses *PIP*, we demonstrate that the *PIP* gene is duplicated as part of a large inverted repeat, localized at a normal position on 7q. Moreover, we localize FRA7I to 7q35 in a chromosomal region spanning 2 Mb and we show that FRA7I sets the telomeric boundary of the duplicated region. Altogether, our results strongly suggest that the duplication was generated by one cycle of BFB initiated from a break at FRA7I.

RESULTS

Inverted repeat of the region containing *PIP*

By using the FISH technique, we examined whether the *PIP* gene is amplified in the breast carcinoma cell line T47D. Upon hybridization of T47D metaphase spreads with two independently isolated BAC clones, H2D and AC027522, overlapping *PIP* (Fig. 1A), duplicated signals were observed on one chromosome (Fig. 1B). To verify the specificity of this double signal, FISH was performed with the same probes on cells of the HT1080 fibrosarcoma line. The HT1080 karyotype is essentially normal and rearrangements of chromosome 7 were not detected by chromosome painting or G banding. We observed a single hybridization signal on each chromatid of the two chromosomes 7 in a relatively distal position (not shown) as expected from the position of *PIP* at 7q34. In T47D cells, chromosome painting failed to detect chromosome 7 alterations (data not shown) and the duplicated copies of *PIP* were observed at an apparently normal localization on one homologue, indicating that the duplication was generated '*in loco*'. With both probes, duplicated spots were observed in only a fraction of the metaphase spreads, which could reflect either cell to cell heterogeneity in line T47D or technical limitations due to a tight linkage of the duplicated copies. The observation of interphase nuclei, in which chromosomes are far less condensed than in metaphase spreads, allowed us to exclude the first possibility. Indeed, an extra-spot was clearly visible in almost all nuclei (not shown).

In order to further characterize the duplication involving *PIP*, two-color FISH was performed with a probe located about 2 Mb centromeric to *PIP* (AC091742, red) and a probe overlapping the gene (AC027522, green). We observed two red signals flanking the green signals (Fig. 1C). This result confirms the existence of a duplication and indicates that the two copies are organized as a large inverted repeat. This pattern and the normal localization of the extra-copy strongly suggest that the duplication was generated by a BFB cycle. It is now agreed that this mechanism can operate in tumors, but whether CFS activation contributes to initiate the process in these cases is still unknown.

Mapping of FRA7I

To address this issue, we have examined the distribution of known CFS on the long arm of chromosome 7. Since FRA7I was assigned to 7q36 (17), the formation of the inverted duplication identified in T47D cells may rely on that site. In order to test this hypothesis, we mapped FRA7I by using HT1080 cells. In aphidicolin-treated cells, breaks in this region represented about 2% of the total gaps scored. FISH analysis was performed with a panel of probes spanning the 7q34–q36 region (Fig. 2A). We identified a first set of probes that gave only signals centromeric to all the breaks observed in this chromosomal domain (Fig. 2B, panels 1 and 2; Table 1). A second series of BAC clones revealed both centromeric and telomeric signals and occasionally signals crossing the breaks, indicating that they all span the fragile region (Fig. 2B, panels 3 and 4; Table 1). A third set of probes gave only signals telomeric to the breaks (Fig. 2B, panels 5 and 6; Table 1).

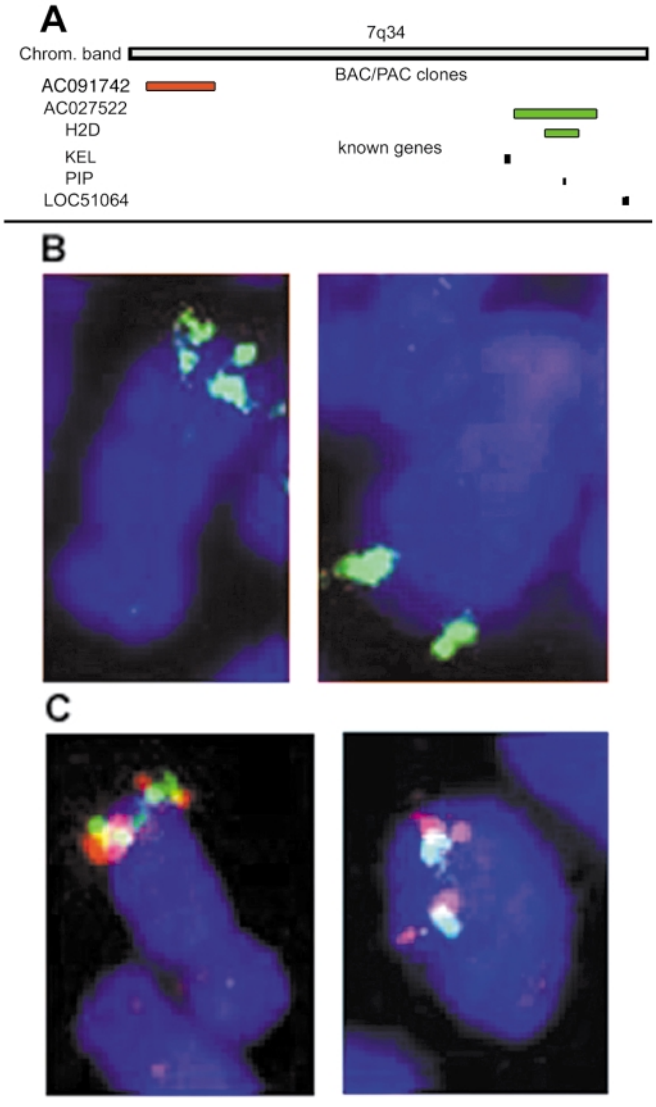

Figure 1. Inverted duplication of the chromosomal region containing *PIP*. (**A**) BAC/PAC clones used as probes: mapping of clones (colored bars) and genes (black bars) is derived from http://www.nhgri.nih.gov/genome_hub.html. The clone H2D was isolated from a human DNA library constructed by us (see Materials and Methods) and contains the whole *PIP* gene; clones AC027522, overlapping H2D and AC091742, located ~2 Mb centromeric to *PIP*, are from commercial sources. Clones are designated by their accession number. (**B**) FISH of T47D breast carcinoma cell metaphase spreads with H2D and AC027522 probes: two green signals are visible on each chromatid of one chromosome. (**C**) Two-color FISH of T47D metaphase spreads with probes AC091742 (red) and AC027522 (green): two red signals flank the green signals. The results shown are representative of four independent experiments.

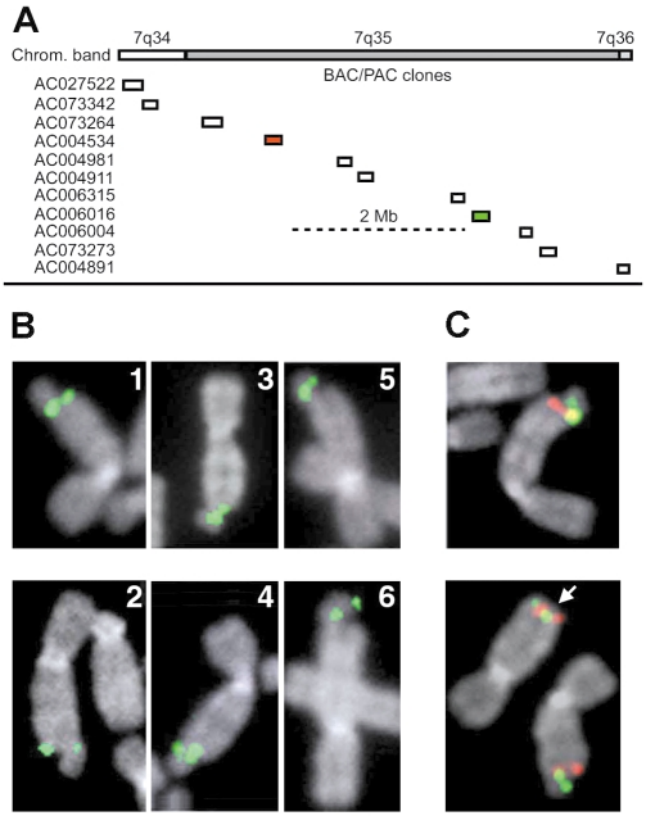

Figure 2. Localization of FRA7I by FISH in HT1080 cells. (**A**) Probes spanning the 7q34–q36 region used to hybridize metaphase spreads of aphidicolin-treated HT1080 fibrosarcoma cell line. (**B**) Examples of hybridization signals centromeric to (probe AC004534, panels 1 and 2), crossing (probe AC006315, panels 3 and 4) and telomeric to (probe AC006016, panels 5 and 6) the breaks. (**C**) Two color FISH with probes AC004534 (red) and AC006016 (green): the signals line the breaks. Arrow points to the normal homologue. Representative results of three independent experiments are shown.

Two-color FISH experiments with probes from the first (AC004534, red) and the third (AC006016, green) group repeatedly showed hybridization signals on both sides of the breaks (Fig. 2C). This definitely establishes that these probes flank a fragile region. Thus, our results identified a CFS extending over an ~2 Mb long sequence that lies at 7q35 (Fig. 2A, dotted line). Since no other loci activated by aphidicolin were detected in the 7q34–q36 region, we concluded that this site is FRA7I.

Table 1. BAC clone hybridization signals on chromosomes expressing FRA7I

BAC/PAC clones	No. of signals		
	Centromeric	On	Telomeric
AC073264	25	—	—
AC004534	27	—	—
AC004981	3	—	9
AC004911	4	1	9
AC006315	3	3	7
AC006016	—	—	21
AC006004	—	—	29

Analysis and counting of hybridization signals observed on chromosomes expressing FRA7I in aphidicolin-treated HT1080 cells. The clones are ordered in accordance to the physical map, centromeric (top of the table) to telomeric (bottom).

To determine whether the unstable region detected in HT1080 fibrosarcoma cells is also expressed in normal human cells, FISH experiments were performed on aphidicolin-treated human lymphocytes isolated from the blood of a healthy donor.

Previous works have established that this site is expressed at very low level in lymphocytes (19,34). In agreement with these results, we observed only 3 breaks in the 7q35 region out of 160 scored on chromosome 7, which prevents statistical analysis of the results. However, two-color FISH experiments with probes AC004534 (green) and AC006016 (red) clearly showed red and green hybridization signals on each side of all 3 breakpoints (Fig. 3). Hence, these results support our conclusions that we characterized FRA7I and indicate that this site is more active in HT1080 cells than in lymphocytes.

Flexibility analysis of the region containing FRA7I

Previous work proposed that hotspots of breakage within common fragile sites correlate with peaks of enhanced flexibility (25). Accordingly, the FlexStab computer program was used to determine flexibility variations of the 2 Mb long sequence spanning FRA7I. This analysis revealed several regions deviating significantly (>4.5 SD) from the average flexibility value of the analysed sequence (Fig. 4). For example, an about 300 kb long sequence located within FRA7I showed up to 17 peaks of high flexibility (Fig. 4B). Very few peaks were found in the region proximal to FRA7I (Fig. 4A). Surprisingly, analysis of sequences distal to FRA7I also disclosed regions highly enriched in peaks of flexibility (Fig. 4C). These results confirm that common fragile sites contain highly flexible regions but also indicate that such structural features can also be found outside the core fragile domain. Therefore, if DNA flexibility is a hallmark of CFS, additional yet unknown structural components also contribute to fragility.

Inverted duplication of the 7q34 region as a result of one BFB cycle

We then addressed the question as to whether the inverted duplication of the region containing *PIP* in T47D cells was generated by a cycle of BFB initiated at FRA7I. According to this model, sequences centromeric to the break could be amplified, whereas regions telomeric to the break should be excluded from the repeat (9,10). To check this point, we performed two-color FISH on T47D metaphase spreads using a probe overlapping *PIP* (green) and probes telomeric to FRA7I (red) (Fig. 5A). A single red signal was observed on each chromatid of all chromosomes that exhibited a duplication of the green signal, suggesting that the region telomeric to FRA7I is not duplicated (Fig. 5B). This was confirmed by using a second set of probes more distant from each other: AC091742 (red) and AC004891 (green) (Fig. 5C and D). The observation of interphase nuclei definitely established this point (Fig. 5D). Hence, we concluded that activation of FRA7I triggered the rearrangement.

Moreover, the position of the green signal on chromosome 7q (Fig. 5D) suggested that the telomeric part of the chromosome caps the duplicated region. This hypothesis was confirmed by using more distal probes, up to the sub-telomeric region of the long arm of chromosome 7 (Fig. 5C). In each case, we observed a single hybridization spot, distal to the duplication (data not shown). These results support the hypothesis that the inverted duplication was generated by one cycle of BFB

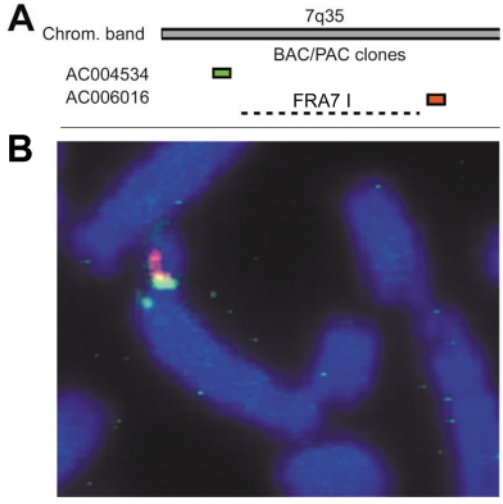

Figure 3. Localization of FRA7I in human lymphocytes. (**A**) Probes used to hybridize metaphase spreads of aphidicolin-treated lymphocytes from a healthy donor. (**B**) Two color FISH with probes AC004534 (green) and AC006016 (red): example of signals lining the break.

initiated from a break at FRA7I and that amplification was frozen within the first cycle by recapture of the chromosome fragment bearing the telomeric part of the long arm of chromosome 7. We therefore propose that the following steps lead to duplicate the *PIP* region (Fig. 6): in G1 interphase, a break at FRA7I on one homologue initiated the BFB cycle mechanism (Fig. 6A); the two broken sister chromatids fused after replication (Fig. 6B); at anaphase, a bridge was formed when the two centromeres of the dicentric chromatid moved to opposite poles of the mitotic spindle and was broken upon completion of anaphase or telophase (Fig. 6C). The broken chromatid bearing the duplication was healed by recapture of one copy of the chromosome fragment bearing the telomeric part of 7q (Fig. 6D), giving rise to one deleted and counter-selected (Fig. 6E), and one amplified selected (Fig. 6F) daughter cell.

DISCUSSION

Growing evidence indicates that gene amplification may arise from the BFB cycle mechanism in cancer cells and that recurrent breaks resolve the mitotic bridge formed at each cell cycle (11–14). However, it is presently unknown whether breaks at CFS, especially in cells of patients that were neither treated by chemotherapy or submitted to drastic environmental injury, contribute to initiate BFB cycles *in vivo*.

Here we show that a region containing the *PIP* gene at 7q34 is duplicated in the breast carcinoma cell line T47D, which overexpresses this gene. The duplicated *PIP* region appears as part of a large palindrome lying '*in loco*' on the distal part of the long arm of one chromosome 7 and exhibits the inverted repeat organization generated by the BFB cycle mechanism. Moreover, upon identification of FRA7I at 7q35, ~2 Mb telomeric to the *PIP* gene, we were able to show that FRA7I sets the distal boundary of the inverted repeat. These results

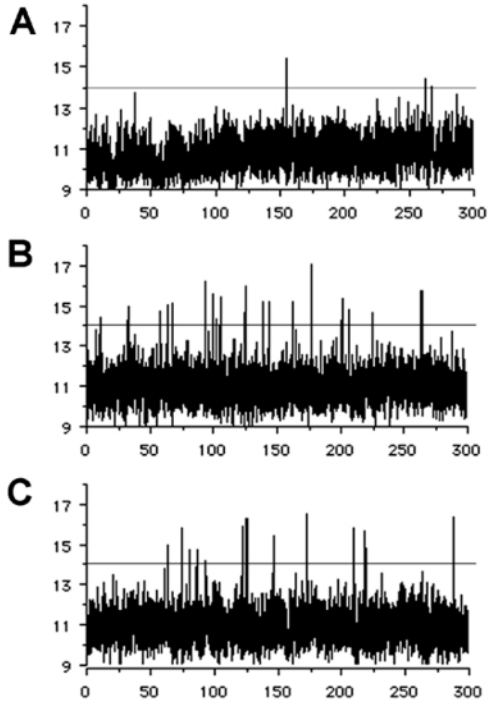

Figure 4. Flexibility analysis of the 7q35 region. Analysis of DNA flexibility of a 300 kb long region was performed using FlexStab computer program. x Axis, nucleotide position, y axis, degree of inclination in the twist angle. The value corresponding to 4.5 SD above the average value is indicated with a line. Peaks higher than this threshold value correspond to regions with significant high flexibility. Examples of sequences located (**A**) centromeric to FRA7I (AC004534), (**B**) within FRA7I (AC006315) and (**C**) telomeric to FRA7I (AC073273).

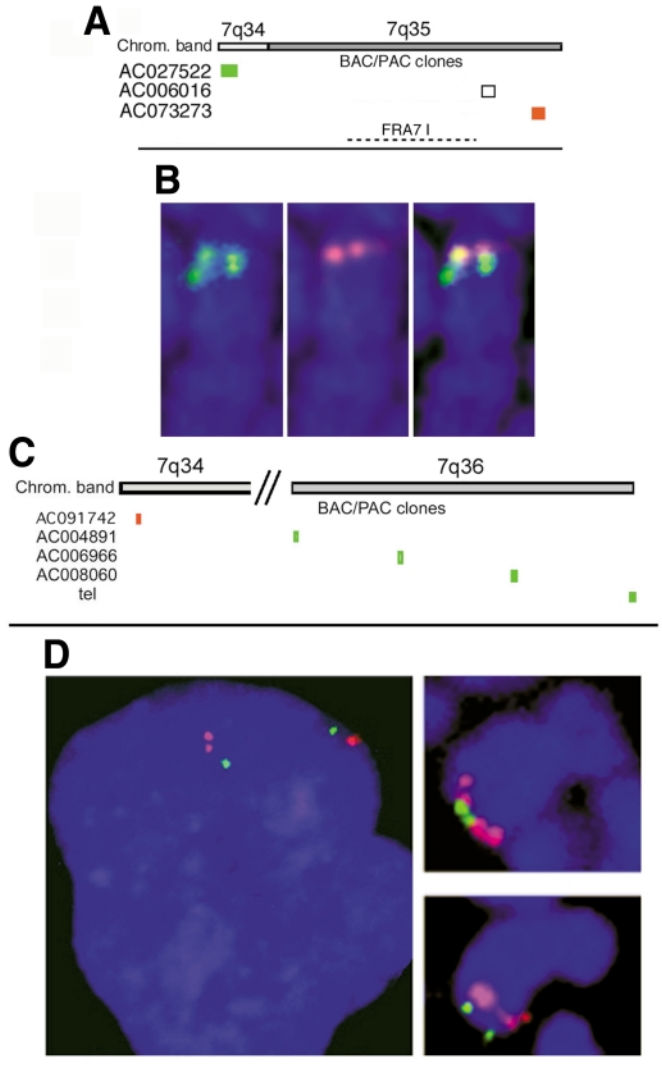

Figure 5. Lack of duplication of the region telomeric to FRA7I and recapture of the telomeric fragment. (**A** and **C**) Probes telomeric to FRA7I used to hybridize metaphase spreads of T47D cells; tel: a 7q telomere DNA probe. (**B**) Two-color FISH with AC027522 (green) containing *PIP* and AC073273 (red) telomeric to FRA7I: a single red signal and two green signals are visible on each chromatid of one chromosome. Identical results were observed with probe AC006016. FITC (left) and rhodamine (middle) labeling are shown separately and merged (right). (**D**) Two-color FISH with AC091742 (red) centromeric to *PIP* and AC004891 (green) telomeric to FRA7I. Example of an interphase nucleus with one chromosome 7 bearing duplicated red signals, specific for the inverted duplication of the *PIP* containing region and one green signal distal to FRA7I; note the presence of the normal homologue with a single red signal. A single green signal is also observed on each chromatid of all metaphase chromosomes bearing duplicated red signals. The results shown are representative of four independent experiments.

support the conclusion that the event initiating the BFB cycle took place within FRA7I.

In Hamster cells selected *in vitro* to resist cytotoxic drugs, BFB cycles have been shown to accumulate intra-chromosomal copies of the target gene within large inverted repeats lying on a chromosome arm where one gene copy maps in normal cells (6–9). Fused sister chromatids and anaphase bridges, two typical intermediates of this mechanism, were repeatedly observed in cell populations undergoing intra-chromosomal amplification (7,9). The cytogenetic manifestations of the BFB cycles have also been observed in some tumor cells, indicating that this mechanism also operates in human cancers (11–14). In all cases, recurrent cycles of BFB did occur, leading to progressive accumulation of extra copies during clonal expansion. In the breast carcinoma cell line T47D, we show that the BFB cycle mechanism generates an inverted repeat involving the *PIP* gene region rather than typical ladder-like structures, such as those previously described in rodent cells (6–9) and a human gastric carcinoma cell line (14). Moreover, the detection of sequences telomeric to the duplicated region and FRA7I in T47D cells strongly suggests that amplification was frozen within the first cycle of BFB by recapture of the chromosome fragment bearing the telomeric part of the broken chromosome (Fig. 6).

Repair of amplified chromosomes with fragments generated by the initial break was not observed previously (6–9,14). In mutant cells selected *in vitro* for drug resistance (6–9), this may be due to prolonged selection pressure. Indeed, as long as copies sufficient to overcome the toxic effects of the selective drug are not acquired, early repair of the amplified chromosome would block amplification and prevent the cells from growing in selective medium. Thus, the acentric fragment is probably lost or degraded long before healing of the broken chromosome (6–9). The same constraint can account for the lack of

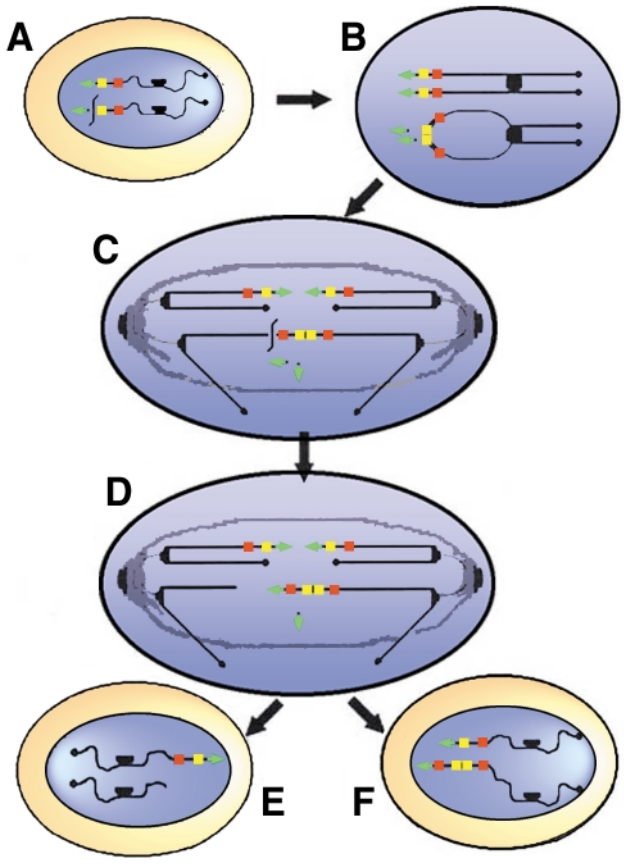

Figure 6. Model for the generation of the inverted duplication found in T47D cells. The two chromosomes 7 are represented with the *PIP* gene region (yellow), the region centromeric to *PIP* (red) and the telomeric part of the 7q arm distal to FRA7I (green). (**A**) Initial break at FRA7I (black broken line), (**B**) fusion of the broken sister chromatids after replication, (**C**) break (black broken line) of the bridge formed at anaphase, (**D**) healing of the broken chromatid by recapture of one copy of the chromosome fragment bearing the telomeric part of 7q. The behavior of the second copy of the fragment is unknown. (**E**) One deleted and (**F**) one amplified daughter cell are formed. The normal homologue is shown above the rearranged chromosome at each step (A–F).

sequences telomeric to the *MET* amplicons derived from multiple BFB cycles, as recently described (14). Here, the results suggest that a single extra copy of *PIP* and/or of a neighboring gene gives a growth advantage to T47D breast carcinoma cells. It is unlikely that the weak increase in *PIP* copy number by itself accounts for the ~5-fold higher level of *PIP* RNA detected in these cells as compared with the *PIP* expression level in normal mammary gland (M.A.D., unpublished data). We may thus hypothesize that, following a first event leading to gene overexpression in T47D cells, BFB cycle-driven duplication significantly increased the *PIP* expression level, thereby permitting an early repair of the broken chromatid.

In model systems, evidence has been obtained that breaks at fragile sites distal and proximal to the selected gene frame the repeated sequences at early stages of the process. Indeed, it has been demonstrated that a targeted break telomeric to the gene efficiently triggered the initial sister chromatid fusion (9,10,50), while a break centromeric to the gene recurrently resolved the bridge formed in anaphase at each cell cycle (9). Amplicons limited by two CFS have been identified in mutant cells recovered upon selection with drugs that are potent CFS activators, thus indicating that the selective agent both selects for amplification and induces the BFB cycle mechanism (9). In tumor cells exhibiting BFB cycle-driven *MET* amplification, breaks resolving anaphase bridges have been shown to repeatedly occur at FRA7G, a CFS proximal to *MET* (14). Here we identified FRA7I at the molecular level and showed that the fragile region encompasses a megabase long sequence and is enriched in peaks of enhanced flexibility. Both the size and the properties of the fragile sequence disclosed are typical of CFS. FRA7I lies at 7q35, about two Mb telomeric to the *PIP* gene and we demonstrate that it sets the distal boundary of the repeated sequence in T47D breast carcinoma cells. This finding establishes for the first time that the BFB cycle mechanism can be initiated by a break occurring within a CFS in human cancer cells.

It has been reported that telomere dysfunction may play a role in the initiation of the BFB cycle mechanism. Fusions of sister chromatids with shortened telomeres in rodent cell models (5,51) and examples of chromosome or chromatid fusions in cells with short or unprotected telomeres have been reported *in vitro* and *in vivo* in a variety of genetic backgrounds in mice and possibly in human cancers (52–55). In the T47D breast carcinoma cells it is most unlikely that the initial break and subsequent sister chromatid fusion originate from such a mechanism. The finding that FRA7I sets the telomeric boundary of the inverted repeat, and that the fragment of the long arm of chromosome 7 telomeric to FRA7I retained its original structure, points to the initial break occuring within FRA7I.

The mechanisms responsible for activation of CFS in cancer cells are not fully understood. It has been demonstrated that stresses resulting from variations in tumor microenvironment, such as oxygen starvation, activate some CFS (56). Alternatively, fragile site expression may be consecutive to spontaneous changes in chromatin organization and/or impairment of the rate or the timing of DNA replication (14,30–32). Whether chromosomal instability, tumor microenvironment variations or other unknown events led to FRA7I activation remain to be elucidated.

In conclusion, our results highlight for the first time that BFB cycles can be initiated *in vivo* by events taking place within CFS in tumor cells. Moreover, the observation of the earliest products of the BFB cycle mechanism in the breast carcinoma cell line T47D is unprecedented.

MATERIALS AND METHODS

Genomic library construction and BAC/PAC clones

A BAC library was contructed from human seminal vesicle DNA and cloned into the pBeloBAC11 vector, as reported (57). The H2D BAC clone was isolated by screening the library with a *PIP* cDNA probe (58). The other BAC/PAC clones were chosen according to their position in draft genome sequence assembly (http://www.nhgri.nih.gov/genome_hub.html) and were purchased from BACPAC Resources, web site (http://

www.chori.org/bacpac). A 7q telomere DNA probe (QBiogene, Illkirch, France) was also used.

Cell culture and fragile site induction

T47D breast carcinoma and HT1080 fibrosarcoma cell lines were obtained from American Type Culture Collection and grown in classical culture medium. The induction of fragile sites in HT1080 cells and lymphocytes and metaphase spreads were performed as described (59).

Fluorescent *in situ* hybridization (FISH)

BAC and PAC DNA was labeled using the BioPrime labeling system (Life Technology, Cergy-Pontoise, France) with digoxigenin (DIG)-11-dUTP (Boehringer, Mannheim, Germany) or biotin. Competitor Cot-1 DNA was added to the probes, samples were denaturated at 70°C for 5 min and incubated for 3 h at 37°C. FISH hybridization was performed as adapted (60). After an overnight hybridization in a moist chamber at 37°C, slides were washed in 4× SSC/0.3% Tween 20 for 1 min at 70°C, then in PBS for 5 min at room temperature. Probe detection was performed using FITC-avidin/rhodamine anti-DIG antibody (Oncor, Illkirch, France) and DAPI was used as counterstain. The slides were examined under a Zeiss (Jena, Germany) epifluorescence microscope with a combination of filters for DAPI, FITC and TRITC. Pictures were acquired using a tri-CCD camera and Vysis (Downer Grove, Il) computer software (Smart capture).

Computer analysis of helix flexibility

DNA flexibility was measured using the FlexStab program available at the Hebrew University of Jerusalem, web site (http://leonardo.ls.huji.ac.il/departements/genesite/faculty/bkerem.htm), as previously reported (25).

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