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COMMENTARY

Chromosomal Fragile Sites and DNA Amplification in Drug-resistant Cells

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ABSTRACT. It has been well established that DNA amplification is one of the important mechanisms by which cultured cells acquire resistance to many cytotoxic compounds. Amplification of important genes including those encoding oncoproteins, growth factors, their receptors, and cell-cycle regulators has been reported in human neoplasms. Yet, despite intensive research since the first description of DNA amplification in cultured cells about 20 years ago, the mechanisms of DNA amplification remain largely unknown. Many models have been proposed to account for the diverse manifestations of amplified DNA in many different cell sources. It is not the intention of this commentary to review these many different models. Rather, we will focus on the recent advances in this area of research, made mainly via the fluorescence *in situ* hybridization technique, that have revealed a fairly common chromosomal manifestation of amplified DNA in the drug-resistant hamster cell lines and have demonstrated the association of chromosomal fragile site breakage with early events in DNA amplification. These new developments underscore the importance of future research toward understanding the molecular bases of chromosomal fragile sites, including mechanisms involved in DNA strand breakage and repair, chromosomal translocations, and deletions, which may, in turn, provide important new insights into genomic plasticity and neoplastic transformation. BIOCHEM PHARMACOL **56**;1:7–13, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. DNA amplification; fragile sites; chromosome breaks; drug resistance; cancers

FREQUENT ASSOCIATION OF DNA AMPLIFICATION INVOLVING THE B-F-B¶ MECHANISM WITH DRUG-RESISTANT HAMSTER CELLS

Amplified DNA can be either extrachromosomally located in DM or chromosomally borne at segments manifested as ABR. For reasons yet to be determined, amplified DNA in drug-resistant murine and human cell lines is often manifested as DM configuration, whereas in hamster cells it appears as ABR. The mechanisms of DNA amplification in drug-resistant cells and in cancer cells have been discussed in many previous reports [1–5]. The present commentary will focus on DNA amplification in the ABR. Most of the data discussed here are from recent studies in drug-resistant cell hamster lines using the FISH technique leading to the elucidation that B-F-B is a predominant mechanism of DNA amplification in such cells.

Early cytogenetic studies revealed ABR in a number of independently established MTX- or methasquine-resistant Chinese hamster lung cell lines [6] that overproduce the target enzyme DHFR. The ABR in these cell lines are found predominantly on 2q. *In situ* hybridization using a radioactively labeled *dhfr* probe also demonstrated that these ABR contained amplified sequences. The single-copy *dhfr* gene is also on chromosome 2q. These results suggested that the amplified DNA was located predominantly on the same chromosome where the single copy resides.

In another study, Sen *et al.* [7] analyzed the chromosomal amplification of the *P-gp* gene in three MDR CHO cell lines independently selected with vincristine, vinblastine, and Adriamycin®. All these cell lines contain ABR proximal to the telomere of chromosome 1q, where the amplified *P-gp* is located as determined by the conventional *in situ* hybridization. The single-copy *P-gp* gene, however, is located at 1q26–28 (about the middle portion of chromosome 1q [7, 8]). Together, these observations demonstrated a consistent intrachromosomal translocation of amplified DNA in the independently established hamster cells. Using FISH, which provides a better resolution of the hybridization signal than conventional *in situ* hybridization, these observations have been confirmed [9] (Fig. 1).

Since the first demonstration of the intrachromosomal translocation of amplified genes [7], several reports have demonstrated similar findings in other drug-resistant variants. Trask and Hamlin [10] analyzed nine independently

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¶ *Abbreviations:* ABR, abnormal banding regions; *ampd 2*, adenylate deaminase gene; B-F-B, breakage-fusion-bridge; *cad*, carbamyl-*P* synthetase/aspartate transcarbamylase/dihydro-orotase gene; DHFR, dihydrofolate reductase; DM, double minutes; FISH, fluorescence *in situ* hybridization; MDR, multidrug resistance; MTX, methotrexate; *P-gp*, P-glycoprotein *gene*; and PALA, *N*-phosphonacetyl-L-aspartate.

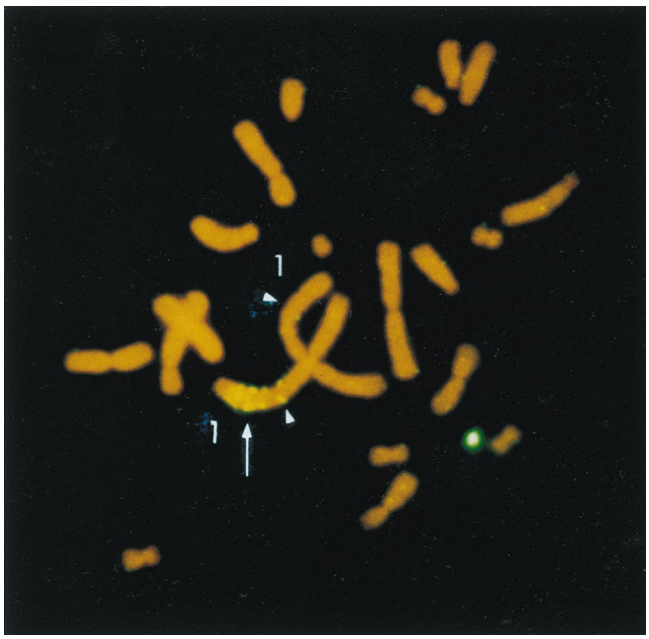

FIG. 1. FISH showing the locations of amplified (arrow) and single-copy *P-gp* genes (arrowheads) on chromosome 1q of a multidrug-resistant CHO cell.

established CHO lines selected to grow in low concentrations of MTX, and they demonstrated that in most of these cell lines amplified *dhfr* was found on 2q but was often located distal to the single-copy *dhfr* gene from the centromere. These authors also revealed unamplified *dhfr* sequences in the original locus, about one-third of the way out from the centromere of 2q. Smith *et al.* [11] studied the *cad* gene amplified in PALA-selected Syrian hamster cells and mapped to one B9 chromosome in which the single-copy *cad* gene is located. Toledo *et al.* [12, 13] showed the amplified *ampd 2* gene to be away from the single-copy gene toward the telomere of chromosome 1q in coformycin-resistant CHO cells. These observations collectively suggest a common chromosomal abnormality of DNA amplification in drug-resistant hamster cells, i.e. the amplified DNA usually coexists with the single-copy sequence on the same chromosome and the amplified DNA is located between the single-copy locus and the telomere.

The similar cytogenetic expression of amplified DNA in these drug-resistant hamster cells suggests that the same mechanism may be involved in DNA amplification in these cell lines. Based on these findings, the B-F-B mechanism of DNA amplification was proposed [12–15]. (It may be noteworthy that the B-F-B model was described previously by Kaufman *et al.* [16] to account for the amplification of transfected *dhfr* gene in CHO cells). In this model, the initial event of DNA amplification is chromosomal breakage. The sites of this breakage may depend upon the structure of the chromosome (see below). If the break occurs between the centromere and the target gene locus (Fig. 2, T), an acentric chromosome fragment containing the target gene will be produced (Fig. 2b). This fragment, because of its lack of a centromere, will not be transmitted properly to the following cell generation. However, a break between the target locus and the telomere should yield a chromosome containing a frayed end (Fig. 2c), and sister chromatid fusion may follow because of the close proximity of the broken chromatin ends (Fig. 2e). Replication of this chromosome in the subsequent cell cycle would generate a dicentric chromosome with duplicated copies of target sequence bridged by the fusion point (Fig. 2g). As this cell transverses to anaphase in the cell cycle, the movement of two centromeres toward opposite poles could cause additional breaks. Alternatively, unusual compartmentalization of the amplified DNA in the interphase nucleus may cause chromatin breakage [13]. A break at either of the A sites

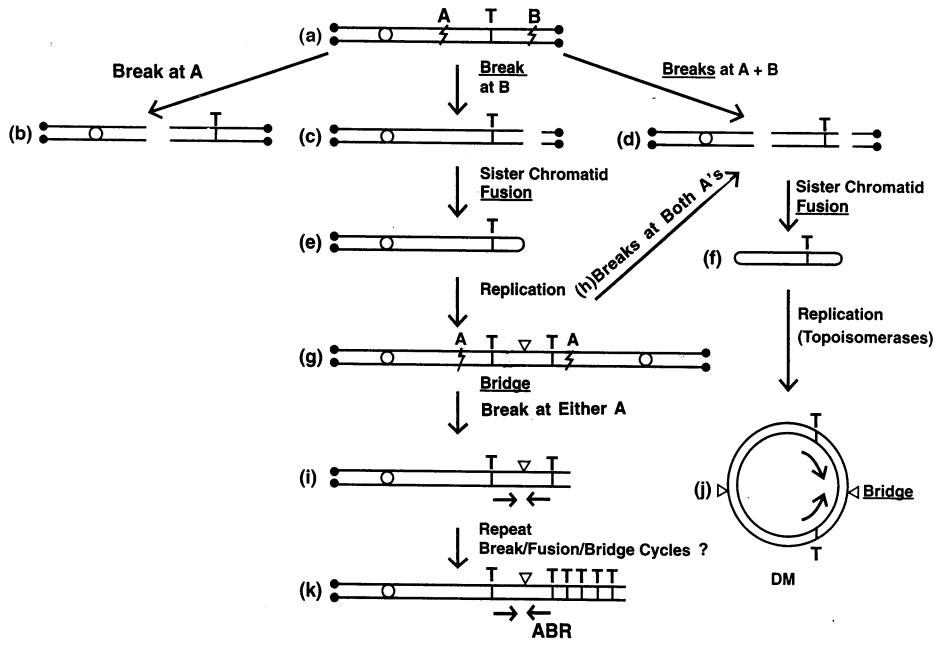

FIG. 2. Schematic diagram showing the formation of amplified DNA in the ABR and DM by the B-F-B cycle. Parallel lines represent two chromatids; zigzags, breaks; small horizontal arrows, centromere-telomere directionality; open circles, centromeres; T, target gene; closed circles, telomeres. Arrowheads point to sites of sister chromatid fusion. The B-F-B cycle starts with chromosome breaks (a), following multiple intermediate steps (b–i) as described in the text, resulting in the formation of chromosomally localized amplified DNA proximal to the telomere (k) or extrachromosomally localized circular DNA, as seen in DM (j).

(Fig. 2g), for example, will produce a chromosome with two copies of the target sequence that may be separated by megabases (Fig. 2i). There is still a possibility that the dicentric chromosome may be stabilized by inactivation of one of the two centromeres, but the frequency may be considered rare [17]. Completion of the B-F-B cycle should provide a cell with a growth advantage under selection pressure because of the gain of an additional target gene.

The diagram presented in Fig. 2 assumes chromatid breaks occurring in the S or G_2 phase, where each chromosome has two sister chromatids. Thus, the breaks should be regarded as isochromatid breaks. An alternative scheme may attain the same result when the cell is in the G_1 phase with a single chromatid per chromosome. A single break at the same locus will yield an isochromatid break after DNA replication and chromosome duplication.

Many cytogenetic observations support the B-F-B mechanism of DNA amplification, including the coexistence of the single-copy and intrachromosomally located amplified genes, as mentioned above [7–15], the high frequencies of sister chromatid fusion at the chromosome end [15, 18] and of dicentric chromosomes [11–13], the predicted giant inverted duplication within the duplicated target gene [13, 15] (Fig. 2i, arrows), and the initial breakage at specific sites [9, 14] (see below).

In many drug-resistant hamster cells, segments of amplified DNA are presented as ladders, indicating that multiple rounds of amplification have occurred. However, the mechanisms underlying the subsequent event are not well characterized, and whether the same B-F-B cycle remains involved requires further demonstration. This subsequent amplification often gives rise to excess gene copy numbers; therefore, the later events contribute more significantly to the development of the drug resistance phenotype.

The B-F-B model may also explain the formation of amplified DNA in extrachromosomal configurations loosely defined by the cytogenetic term DM in some cases. As shown in Fig. 2d, simultaneous breaks flanking the target gene would liberate a chromosome fragment with two broken ends. The broken ends could then be repaired by sister chromatid fusion. After replication, a circular DNA molecule with two copies of target sequences head-to-head should form (Fig. 2j). There are some supporting reports for this: DM DNA are often in circular form [19–23], but also see Ref. 24 for linear DNA in DM]. Also, the predicted inverted order of the DNA sequence within the circle was found in the extrachromosomal H-DNA amplified in the MTX-resistant *Leishmania* cells [25], in addition to the head-to-tail configuration.

CHROMOSOMAL FRAGILE SITES IN DNA AMPLIFICATION

The DNA amplification mechanism described above underscores the importance of chromosomal breakage as an initial event that generates the B-F-B cycle. Recent studies have suggested that the sites of chromosomal breakage are not random and, more strikingly, are consistent with chromosomal fragile sites. Kuo *et al.* [9] reported that the breakage sites can be readily identified due to chromosomal translocation in two independently established MDR CHO cell lines using vincristine and vinblastine as selecting agents. The breakage sites in these two cell lines were located at 1q31. The chromosomal break around 1q31 is also associated with the amplification of *P-gp* in the third cell line selected with Adriamycin. The observation that the same breakage site was associated with *P-gp* amplification under three different selecting agents strongly suggested that the breakage is independent of the selecting agent and, therefore, the breakage site can be considered a chromosomal fragile site. This was indeed demonstrated using the fragile site inducers MTX and aphidicolin [9].

The association of the chromosomal fragile site in the amplification of the hamster *P-gp* gene has been confirmed recently by Coquelle *et al.* [14]. These authors also revealed the association of fragile sites for the amplification of *ampd2*, *dhfr* and *cad* genes in hamster cells exposed to coformycin, MTX, and PALA, respectively. These studies thus highlight the importance of fragile sites in DNA amplification in hamster cell lines.

The observations of B-F-B as a predominant DNA amplification mechanism found in hamster drug-resistant cells has raised many important questions. For example, can the same mechanism account for the subsequent amplification of DNA that often exists in clusters (indicated by multiple Ts in Fig. 2k). In other words, are the amplified DNA ladders organized in the same inverted configurations? Several reports have provided direct molecular evidence of head-to-tail tandem amplicon repeats in human tumor cells containing amplified sequences [26–29], although whether these configurations are associated with the initial events of DNA amplification is unknown. Are the differences in amplified DNA configurations between humans and hamsters due to species variations? What events ultimately terminate the B-F-B cycle and stabilize the amplicons? These important questions require further experimental investigations. In so doing, one should keep in mind that multiple mechanisms are likely to be involved in the amplification of different targets in different cell settings, and the late events are likely to be different from the early events [for example see Refs. 30 and 31].

STRESS-INDUCED BREAKAGE AT CHROMOSOMAL FRAGILE SITES AND DNA AMPLIFICATION

The B-F-B model described above underscores the important role of chromosomal fragile site breakage in DNA amplification and raises important questions regarding the molecular bases of fragile site expression under assaults by cytotoxic agents.

Traditionally, fragile sites may be considered as loci that are intrinsically sensitive to extracellular stress [32]. Many cytotoxic agents used in DNA amplification studies have

no known mechanism of action directly on chromosomal DNA. Furthermore, some drugs known to induce DNA amplification (e.g. PALA) are not known to induce fragile sites. Therefore, the breakage associated with the DNA amplification described above must be considered as secondary to the primary targets of these agents, consistent with the hypothesis of stress-induced cellular response. Many of these agents are inhibitors of nucleotide biosynthesis and/or cell cycle arrestants. Cultured cells exposed to these agents develop intracellular nucleotide pool imbalance. Since the regulation of intracellular nucleotide pools plays an important role in controlling genomic stability [33–35], exposing cultured cells to these agents may therefore induce chromosome fragility. Furthermore, imbalanced nucleotide pools may arrest cells at particular stages of the cell cycle, resulting in chromosome disjunction and premature condensation [36] that may facilitate fragile site breakage and DNA amplification.

Consistent with this notion, Schimke and coworkers [37–39] demonstrated that pretreatments with agents that exert cellular stress result in enhanced DNA amplification frequency upon subsequent drug selections.

MOLECULAR CHARACTERIZATIONS OF CHROMOSOMAL FRAGILE SITES

The associations between chromosomal fragile sites and DNA amplification presented in the literature are mainly at the cytogenetic level with a resolution of megabase pairs (mb). To further evaluate the role of the fragile site in DNA amplification, it is necessary to resolve this association to nucleotide levels. Molecular characterizations of fragile sites have been mainly on the human genome, and very little is known about the Chinese hamster and Syrian hamster fragile sites [40, 41]. In humans, some fragile sites are common, whereas others are rare [40]. These fragile sites are inherited in a Mendelian codominant fashion. A well-known common fragile site is located at Xq27.3 with an occurrence frequency of 1 in 2,000–25,000 males. The affected individuals suffer a mental retardation illness known as the fragile X syndrome [42]. Rare fragile sites are distributed throughout the human genome with the exception of the Y chromosome [40]. These rare fragile sites are often associated with sites of chromosome translocation, deletion, or other chromosome abnormalities found in human neoplasms, consistent with the notion that fragile sites contain unstable DNA. For example, the rare fragile sites at 8q22 and 16q22 correspond to the breakpoints of chromosomal translocations seen in acute nonlymphocytic leukemia [43]. The fragile site at 3q14 is at the site of rearrangements seen in small cell carcinoma of the lung, hereditary renal cell carcinoma t(3;8), and rhabdomyosarcoma [43]. The fragile site at 11q13 is consistent with the translocation found in t(11,14)-positive B-cell tumors [44, 45]. However, the causal relationship between breakages at the respective fragile sites and tumor development remains to be critically evaluated. There is a possibility that breaks in a fragile site are not likely to associate with cancers [46].

Nevertheless, DNA has been isolated from many disease-associated fragile sites. Many of these sites contain amplified trinucleotide repeats $p(CCG)_n$ [for review, see Ref. 47]. Expansion of a 33-bp AT-rich sequence was found to associate with the distamycin A-sensitive fragile site FRA16B [48]. Fragile site FRA3B located at 3p14.2, which can be induced by aphidicolin, has also been characterized fairly extensively in recent years. Taking advantage of the fact that inducing fragile site breaks facilitates the preferential integration of exogenous DNA containing a selectable marker (pSV2neo) at the breakpoint, Rassool *et al.* [49] developed an elegant strategy that led to subsequent cloning of DNA sequences at this fragile site. At the present, >1 mb of DNA encompassing the fragile site are available. An expressed cDNA encoding the human *FHIT* gene spanning the entire fragile site region has been identified [50]. This gene contains 10 exons. Several important landmarks [51–53], including aphidicolin-induced breakage sites, pSV2neo integration sites, HPV16 integration sites, and a t(3;8) breakpoint, have been located (Fig. 3). Furthermore, frequent deletions in this region were often found in many tumor-derived cell lines [51]. In some of these cell lines, the deleted sequences were found entirely in introns that do not affect splicing, suggesting that *FHIT* is not the target of tumor development [51]. No expanded trinucleotide repeats have been found in this region. Together, these results suggest that the FRA3B fragile site contains many "hot spots" that are prone to breakage, insertion, and deletion.

Despite the characterization of DNA sequences associated with many of these fragile sites, much remains to be learned regarding the molecular bases of how these chromosomal fragile sites are expressed under stress conditions. It is possible that these unstable DNA sequences may have a greater propensity to form non-B configurations under stress conditions [54] and/or interact with unique DNA-binding protein [55]. Alternatively, these sequences may be error-prone during DNA replication and transcription [56]. All these possibilities remain hypothetical and require substantial experimental validations.

Moreover, the molecular mechanisms underlying many subsequent events following initial fragile site breakages are largely unknown. For example, what mechanisms are involved in the fusion of isochromatid breaks? Are the repair mechanisms involved in double-stranded DNA breaks [57] also involved in the repair of broken chromosome ends? How can large inverted chromosomal segments evolve and be regulated? In this regard, it may be relevant to note that induction of large inverted repeat DNA during amplification of rDNA in the developmental program of ciliated protozoa has been characterized recently [58, 59]. These studies revealed that chromosome breakage followed by intrachromosomal recombination controlled by a pair of 42 bp inverted repeats is involved in the formation of such a large palindrome. Moreover, RAD51, an *Escherichia coli*

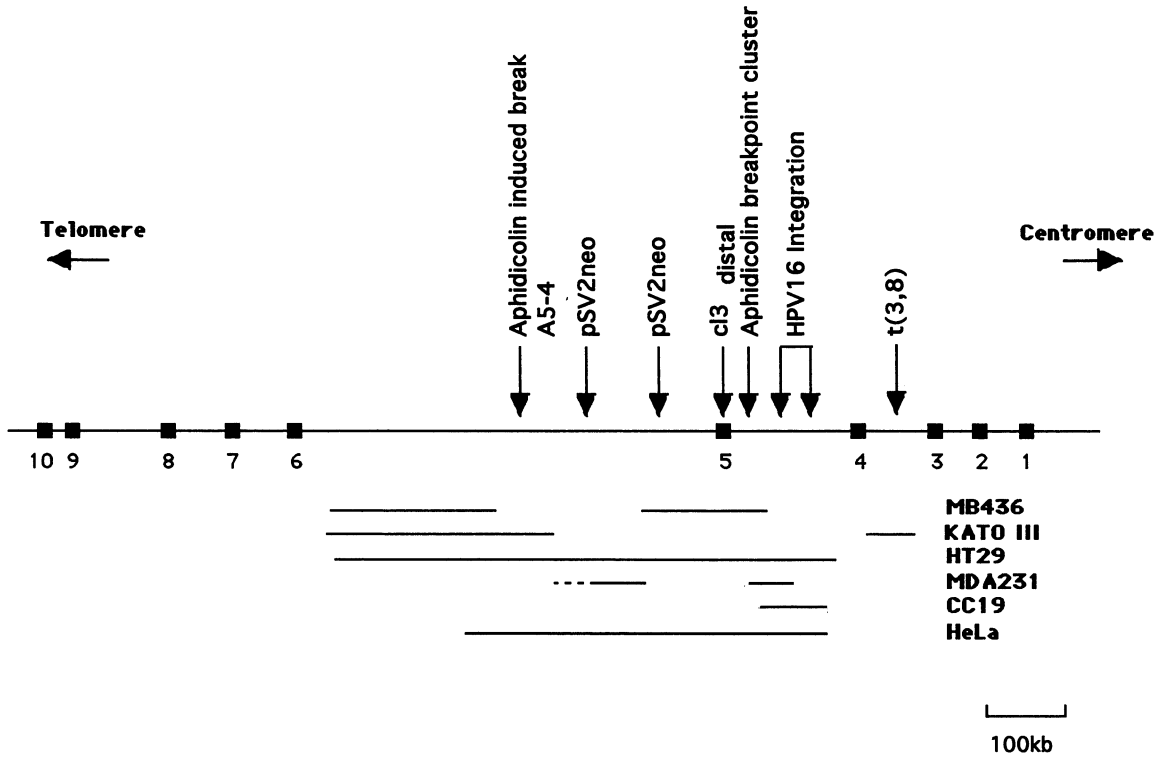

FIG. 3. Organization of the fragile site *FRA3B* region relative to the *FHIT* gene showing various landmarks including breakpoints, plasmid and viral integration sites, and homologous deletions found in cancer cells. The numbers 1–10 denote the locations of exons of the *FHIT* gene. The vertical arrows point to breakpoints induced by aphidicolin, by t(3,8) translocation, and by pSV2Neo plasmid and HPV16 viral sequence integration sites. Horizontal lines represent deletions found in several cancer cell lines; the dashed line indicates that the boundary has not been defined. Redrawn from Refs. 50–52.

recA protein homologue involved in homologous recombination and repair of double-stranded DNA breaks, is required for the formation of the giant palindrome [58]. The involvement of RAD51 in this process is particularly intriguing because RAD51 was demonstrated recently to physically interact with p53 [60, 61], the tumor suppressor gene product that plays an important role in the regulation of genomic stability, as manifested in enhanced frequency of gene amplification in cultured cells [62, 63].

CONCLUDING REMARKS

The frequently observed chromosomal breaks at major fragile sites found in drug-resistant Chinese hamster cells suggest an important role for the cytogenetically defined chromosomal fragile sites and DNA amplification. Indeed, many lines of cytogenetic evidence point to the possibility of fragile site breakage as a trigger for the B-F-B mechanism. Many of the events remain to be molecularly supported. Recent studies have provided impressive insights into the molecular organization of many human disease-associated chromosomal fragile sites but much remains to be learned, particularly about the mechanistic aspects of fragile site expression under stress conditions and how the chromosomal breaks translate into chromosomal translocations, deletions, and other abnormalities that may be associated with malignant transformation. Insights into all that has been learned thus far, plus more new insights into molecular details of fragile sites and DNA amplification, remain to be discovered. Future research may require chromosomal engineering technologies, such as site-specific integration of fragile sites into a surrogate system in which targeted chromosomal breaks can be manipulated, to determine the many cytogenetic observations relevant to chromosomal breaks and DNA amplification.

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