Expression of Fragile Sites Triggers Intrachromosomal Mammalian Gene Amplification and Sets Boundaries to Early Amplicons

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Summary

Drug-selected intrachromosomal gene amplification by breakage-fusion-bridge (BFB) cycles is well documented in mammalian cells, but factors governing this mechanism are not clear. Here, we show that only some clastogenic drugs induce drug resistance through intrachromosomal amplification. We strictly correlate triggering of BFB cycles to induction of fragile site expression. We demonstrate a dual role for fragile sites in intrachromosomal amplification: a site telomeric to the selected gene is involved in initiation, while a centromeric site defines the size and organization of early amplified units. The positions of fragile sites relative to boundaries of amplicons found in human cancers support the hypothesis that fragile sites play a key role in the amplification of at least some oncogenes during tumor progression.

Introduction

Gene amplification is a frequent manifestation of genomic instability in cancer cells, where the amplification of proto-oncogenes plays a critical role in tumor progression (Bishop, 1991; Brison, 1993). Understanding the factors triggering this phenomenon is of major clinical importance, but reconstitution of the mechanism of amplification is obscured by a variety of secondary rearrangements in cells recovered from advanced tumors (Nowell, 1976). The development of fluorescence in situ hybridization (FISH) techniques has recently allowed a comprehensive analysis of early amplification events at three loci in model systems of cultured hamster cells selected for resistance to a cytotoxic drug. These studies have used an experimental design whereby secondary rearrangements, such as the well-documented interconversion between intrachromosomal and extrachromosomal units (Balaban-Mallenbaum et al., 1981; George and Powers, 1982), are limited. A major conclusion drawn is that at least two different mechanisms operate at the early stages. In some cases, amplification proceeds through an entirely intrachromosomal pathway, accumulating megabase-long inverted repeats on a chromosome arm bearing the original copy of the selected gene. This was first shown by studying early mutants of Chinese hamster fibroblasts that overcame the toxic action of coformycin through amplification of

the adenylate-deaminase 2 (*ampd2*) gene (Toledo et al., 1992b). These features are perfectly explained by the chromatid breakage-fusion-bridge (BFB) cycle model (McClintock, 1951; Figure 1). The same mechanism was shown to operate in methotrexate (MTX) and in N-(phosphonacetyl)-L-aspartate (PALA)- resistant mutants of hamster cell lines during amplification of the dihydrofolate-reductase (*dhfr*) and carbamyl-P-synthetase aspartate transcarbamylase dihydro-orotase (*cad*) genes, respectively (Smith et al., 1992; Ma et al., 1993; Bertoni et al., 1994). Analyses of early amplification events have shown that extra copies can also be acquired through unequal segregation at mitosis of acentromeric extrachromosomal elements bearing one or more copies of the selected gene (Windle et al., 1991; Toledo et al., 1992b, 1993), called "double minutes" (DMs) (Spriggs et al., 1962; Cowell, 1982) or "episomes" (Carroll et al, 1987; Maurer et al., 1987), depending on their size. In the *ampd2* system, early amplification by both the intrachromosomal and extrachromosomal pathways can occur at the same locus in cells of the same lineage (Toledo et al., 1992b, 1993).

The nature of the initial event(s) triggering either of the pathways remains uncertain. It was proposed that BFB cycles may be initiated by the fusion of sister chromatids with shortened telomeres (Smith et al., 1990, 1992) or by a chromatid break, telomeric to the amplified locus (Toledo et al., 1992b, 1993; Ma et al., 1993; Kuo et al., 1994). In support of the first hypothesis, fusion of sister chromatids with shortened telomeres has indeed been observed (Hastie and Allshire, 1989; Counter et al., 1992; Saltman et al., 1993). On the other hand, a role for breaks in the initiation of BFB cycles is suggested by the observation of cytological features of this mechanism following cell irradiation by X-ray or high linear energy transfer particles (reviewed by Evans, 1974; Moore et al, 1990; Martins et al., 1993). Chromatid breaks have also been thought to trigger extrachromosomal amplification (Windle et al., 1991). Breakage occurring within replication bubbles was proposed to explain the organization of episomes as inverted repeats. In contrast, Toledo et al. (1993) obtained evidence that intrachromosomal looping out of DNA circles could be responsible for the generation of rare DMs bearing the *ampd2* gene in cells with no cytogenetically detectable alteration other than the frequent deletion of one chromosomal copy of this gene.

Some clues as to the nature of the initial event(s) responsible for amplification can be expected from the identification and properties of agents that can induce that particular mutation. Early studies on the induction of *dhfr* gene amplification identified a large number of inducers, most of which are today known to inhibit DNA synthesis (summarized by Schimke, 1988). More recent experiments have documented the induction of intrachromosomal amplification by MTX or PALA, two agents that starve cells for DNA precursors (Rath et al., 1984; Tlsty et al., 1984; Kuo et al., 1994; Poupon et al., 1996). Because PALA and MTX are clastogenic drugs (Li and Kaminskas, 1984; Nelson and Kastan, 1994), their action
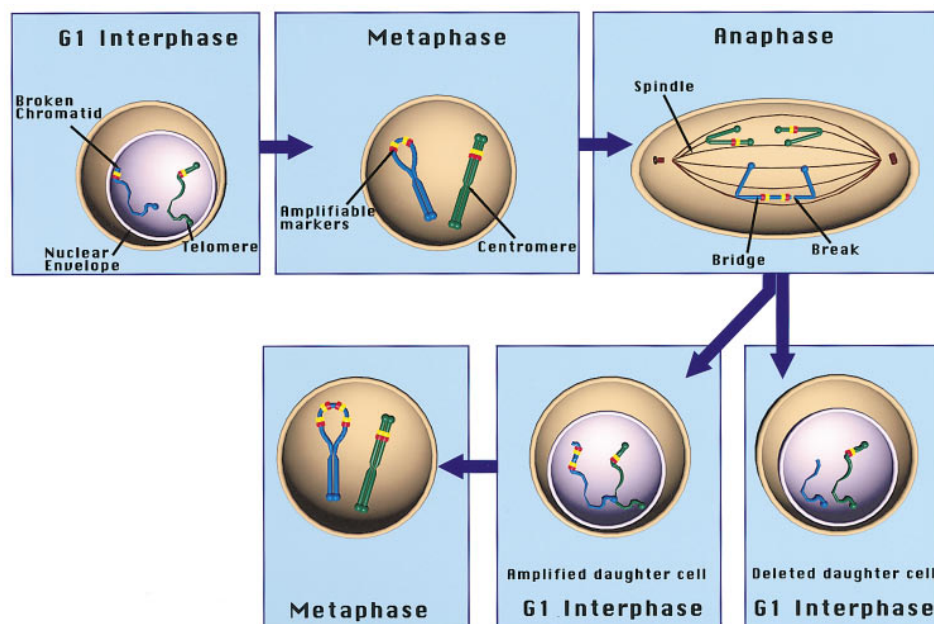
Figure 1. Breakage-Fusion-Bridge Cycles of the Chromatid Type

This model depicts the fate of a cell in which two sister chromatids bearing the selected gene have fused after replication of a broken chromatid. At anaphase, the dicentric chromatid appears as a bridge between centromeres moving to opposite poles of the mitotic spindle. Breakage of this giant inverted repeat leaves each daughter cell with a chromatid lacking one telomere, which again fuses after replication, perpetuating the BFB cycles. Amplification occurs in one daughter cell when the breakage is asymmetric, leading to unequal distribution of the selected gene in the daughter cells. The additional copies are located on the chromosome arm bearing the original copy of this gene and are organized as megabase-long inverted repeats with one or several orders of symmetry.

is consistent with the hypothesis that breaks can trigger amplification. The observation that out of two agents selective for asparagine synthetase gene amplification, the only one that is clastogenic allows the recovery of mutants amplified for this gene also supports this interpretation (Barrett and Andrulis, 1992). However, whether the ability of agents that break DNA to stimulate amplification is general, whether the clastogenic drugs identified as amplification inducers operate through a common mechanism, and whether the same agents can induce both BFB cycles and extrachromosomal amplification remain to be determined.

We have reevaluated here the ability of different agents to induce amplification through intrachromosomal or extrachromosomal pathways. We first analyzed the early stages of multi-drug resistance 1 (*mdr1*) gene amplification since several different drugs can be used to select mutants amplified for the same gene (for a review, see Veinot and Ling, 1992). Three selective agents—adriamycin (ADR), actinomycin D (AMD), and vinblastin (VB)—were chosen to screen for mutants of the GMA32 Chinese hamster cell line. ADR and AMD are DNA-damaging agents, known to induce p53 accumulation (Fritsche et al., 1993; Nelson and Kastan, 1994), while VB, a spindle poison, is not expected to have clastogenic properties and does not induce p53 (Nelson and Kastan, 1994). We found that only AMD induces *mdr1* gene amplification and that only the BFB cycles mechanism is significantly enhanced by this drug. We discovered that, at the drug concentrations used to select the amplified mutants, ADR is a powerful but random

clastogen, while AMD induces breaks clustered at the level of a few sites, one of which is telomeric to and close to the *mdr1* gene, an appropriate localization for triggering of *mdr1* amplification. These loci are specifically revealed on metaphase chromosome preparations from AMD-treated cells by a high frequency of gaps and breaks, a property that characterizes loci defined as drug-sensitive "fragile sites" (Sutherland, 1979; Yunis and Soreng, 1984; Yunis et al., 1987; Sutherland and Richards, 1995). Remarkably, induction of intrachromosomal *ampd2* and *dhfr* gene amplification was found associated with the presence of nearby drug-activated telomeric fragile sites. Moreover, the localization of distal and proximal fragile sites bracketing the *mdr1*, *ampd2*, and *dhfr* genes accounts in a simple manner for the structural peculiarities of amplified units generated at each of these loci during the initial stages of amplification. The results presented establish that site-specific breaks triggered by fragile site inducers account for the early features of intrachromosomal amplification.

Results

Recovery of Early Mutant Clones Resistant to ADR, AMD, or VB

To analyze independent resistant clones at a stage allowing the unequivocal identification of the molecular amplification mechanism(s) governing multidrug resistance, a protocol for the recovery of early mutants was adapted from the method described by Smith et al. (1990): eight cell subpopulations, small enough (50 cells)

Table 1. Mechanism of Resistance in Independent Mutant Clones

| Selective Agent | Number of Analyzed Clones | Not Amplified for *mdr1* | | Amplified for *mdr1* | | | | | | | |
| | | | | Total | | DMs | | BFBs | | Chromosome I Polysomy | |
		Number of Clones	%	Number of Clones	%	Number of Clones	%	Number of Clones	%	Number of Clones	%
VB	37	35	94.6	2	5.4	1	2.7	1	2.7	0	0
ADR	30	25	83.3	5	16.7	1	3.3	2	6.7	2	6.7
AMD	19	6	31.6	13	68.4	0	0	10	52.6	3	15.8

to be statistically devoid of preexisting resistant mutants, were isolated and independently expanded to 5.10^6 cells in nonselective medium. Aliquots of cells from each subpopulation were then independently plated in selective media containing ADR, AMD, or VB, each drug known to select for *mdr1* gene amplification, but only the first two being clastogenic. Three concentrations of each agent were used in parallel to adjust the selection stringencies. Seven of the initial subpopulations yielded a comparable small number of clones in the three drugs. However, the last subpopulation (designated 8) gave confluent plates in all three drugs after 5 days, suggesting that it contained a resistant mutant that had appeared prior to selection. Resistant cells of subpopulation 8 were pooled and analyzed by FISH. All were diploid with unrearranged chromosomes 1 and contained DMs bearing the *mdr1* gene, independently of the drug used for selection. This result suggests a clonal origin of the resistant cells, supporting the hypothesis that subpopulation 8 contained a preexisting mutant.

Resistant clones from the seven other subpopulations were recovered and independently expanded in their original selective medium for a few more generations before analysis. We examined 37, 30, and 19 clones resistant to VB, ADR, and AMD, respectively (Table 1). Dot blot and cytological analyses revealed that the *mdr1* gene was unamplified in some resistant mutants (not shown). Such clones were recovered from cells selected with AMD, ADR, or VB, and some were cross-resistant to the three drugs. They were not further studied and the mechanism of their resistance is unknown, but cross-resistance most likely results from overexpression or mutation of the *mdr1* gene or of a related gene involved in multidrug resistance (Veinot and Ling, 1992; Gottesman et al., 1995, 1996). The ratio of the unamplified mutants to the total number of mutants depended strikingly on the selective agent: they represented the great majority of resistant clones that grew in the presence of VB (94.6%) and ADR (83.3%), but the minority of those that grew in the presence of AMD (31.6%) (Table 1). These results identify AMD as the only potent inducer of amplification among the three drugs tested.

Molecular Mechanisms of *mdr1* Amplification in the Resistant Clones

Among the amplified clones, dot blot analysis revealed considerable heterogeneity in *mdr1* copy number from clone to clone (not shown). We screened amplified clones using double color FISH with probes for the *mdr1* gene (which maps at 1q26) (Sen et al., 1987; Biedler et

al., 1988) and P3C4, a telomeric marker for chromosome 1q (Toledo et al, 1992b; Figure 4B). Some clones, selected for resistance to ADR or AMD, identified by dot blot analysis as weakly amplified, were polysomic for chromosome 1 (Table 1). Since these mutants result from neither BFB cycles nor DM formation, they will not be discussed further. Other clones exhibited a higher copy number of the *mdr1* gene. In some of them, the great majority of cells contained DMs, frequently bearing more than one copy of the *mdr1* gene and two normal chromosomes 1, with no cytogenetically detectable alteration other than the deletion of one chromosomal copy of the *mdr1* gene (Figure 2). These observations
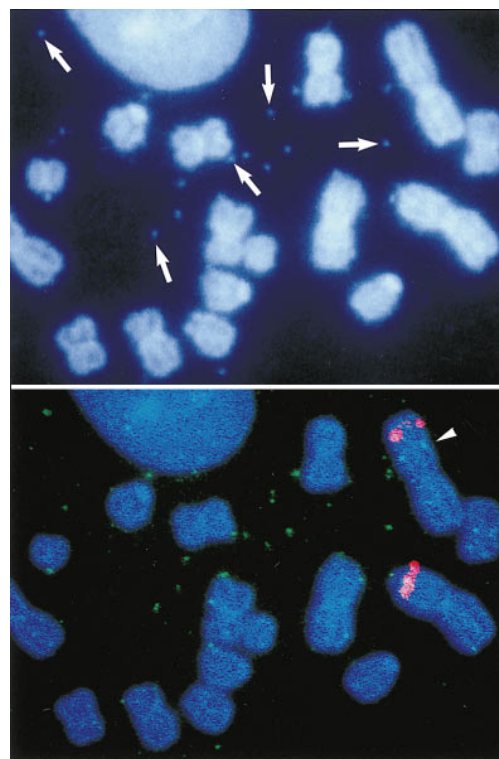

Figure 2. An Example of Extrachromosomal Amplification

Top: DAPI staining; the arrows point to some DMs. Bottom: FISH with an *mdr1* probe (yellow) and a P3C4 probe, a specific marker of the telomeric part of chromosome 1q (red). FISH shows that the DMs bear *mdr1* copies. Consistent with the looping-out mechanism, both chromosome 1 homologs are labeled by P3C4 spots at their wild-type locations. Note that only one chromosome 1 bears an *mdr1* gene at its wild-type location (arrowhead), while the homolog is deleted for this gene.
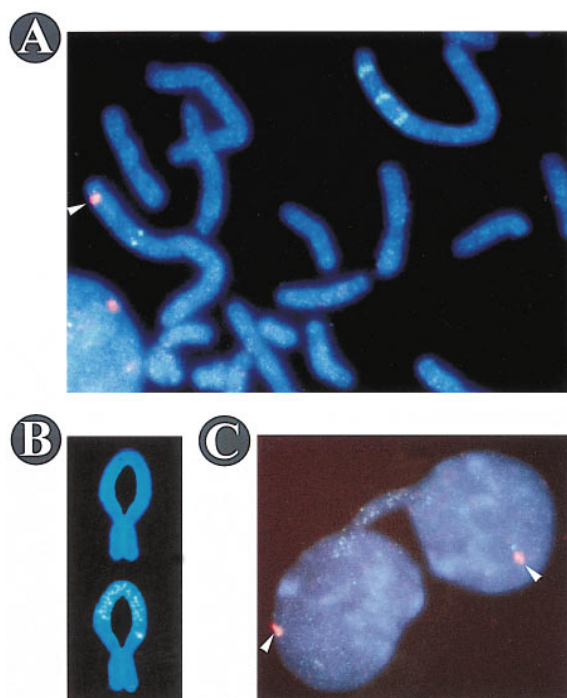
Figure 3. Examples of Structures Related to BFB Cycles

FISH with *mdr1* (yellow) and P3C4 (red) probes.

(A) An *mdr1* ladder on a chromosome 1. The homolog is normal, with a single copy of *mdr1* and P3C4 (arrowhead) at their wild-type locations. Note the low intensity of the *mdr1* single copy compared to a rung of the ladder, suggesting that each rung bears more than one *mdr1* copy.

(B) Top: DAPI staining of a sister chromatid fusion. Bottom: FISH showing *mdr1* extracopies on fused chromatids.

(C) A bridge, bearing numerous *mdr1* extracopies, between interphase nuclei. Note the single P3C4 (arrowhead) signal per nucleus, indicating that one of the two copies of this locus has been lost; this confirmed that the BFB mechanism is operating in these cells.

favor a looping-out mechanism for DM formation, as previously proposed from analysis of *ampd2* gene amplification (Toledo et al., 1993). Such clones were recorded as products of extrachromosomal amplification, although reintegration of extra copies was observed in some metaphases (not shown). Only a few clones were amplified by this extrachromosomal process: 1/37 in selection with VB, 1/30 with ADR, and 0/19 with AMD (Table 1).

The remaining clones exhibited intrachromosomal amplification. They contained a single copy of P3C4 on the unamplified chromosome 1 and ladders of extra copies of *mdr1* on the other chromosome 1, the telomeric part of which was deleted (Figure 3A). Occasionally, metaphases contained fused sister chromatids bearing the amplified *mdr1* genes (Figure 3B) or an amplified chromatid forming a bridge between two anaphase–telophase genomes (not shown). Bridges bearing the amplified copies of *mdr1* were also observed between interphase nuclei containing a high level of *mdr1* amplification (Figure 3C). These observations show that BFB cycles operate in these clones.

Screening of the few highly amplified clones recovered after VB or ADR selections showed that they were distributed with comparable frequencies as products of intrachromosomal and extrachromosomal amplification mechanisms (Table 1). In striking contrast, all of the highly amplified clones (10/10) recovered after selection with AMD displayed products of BFB cycles. We considered the possibility that cells containing DMs could be counterselected in AMD or, on the contrary, that cells containing intrachromosomal copies of the *mdr1* gene could be counterselected in VB and ADR. Cells of subpopulation 8 (which show DMs when selected with all three drugs) exhibited a normal growth rate in AMD when compared to VB or ADR. Moreover, the two clones with extrachromosomal *mdr1* amplification recovered from the other subpopulations after selection with VB or ADR also exhibited a normal growth rate when checked for cross-resistance to AMD. No selection against cells containing intrachromosomal copies of the *mdr1* gene selected in AMD was noticed during subsequent growth in VB or ADR. Taken together, our results indicate that AMD specifically induces the generation of *mdr1* amplification by initiating BFB cycles.

DNA Breaks Induced by the Clastogenic Drugs ADR and AMD

To understand the striking difference in the properties of ADR and AMD, we investigated the damages imposed on DNA by the two drugs at the concentrations used to select mutants. As a control, we verified that no significant increase in DNA breaks was observed in cells treated with VB, compared to untreated cells. We found that only AMD induces site-specific breaks. We focused on breaks on chromosome 1 and localized six major sensitive sites (Figure 4). The main site of breakage, expressed in 14% of mitoses and designated (a) in Figure 4, was very close to and telomeric to the *mdr1* gene. It probably corresponds to the Chinese hamster chromosome 1 fragile site located at 1q26–31 (Rassool et al., 1991). In contrast, in ADR-treated cells, multiple breaks distributed at random from cell to cell were observed with no preferential location. In experiments performed with lower concentrations of ADR, giving rise to only few breaks per cell, no evidence for preferential breakage sites was obtained either (not shown). The specific ability of AMD to activate the expression of fragile sites, particularly site (a), provides an explanation for its ability to stimulate the initiation of *mdr1* intrachromosomal amplification. Moreover, the fluorescence intensity of each rung of the *mdr1* ladders is systematically higher than the fluorescence intensity of the normal locus (Figure 3A), suggesting that each rung represents more than one *mdr1* copy. This observation is consistent with initiation of the process by a break close to the selected gene, since each rung would then be a doublet of *mdr1* copies.

Coformycin Induces Breaks at Fragile Sites Close to the *ampd2* Gene

Using the same cell line, we have previously selected mutants resistant to coformycin, an adenosine analog that acts as an inhibitor of adenylate-deaminase activity. No mechanism of resistance other than *ampd2* gene amplification has been detected in the large number of
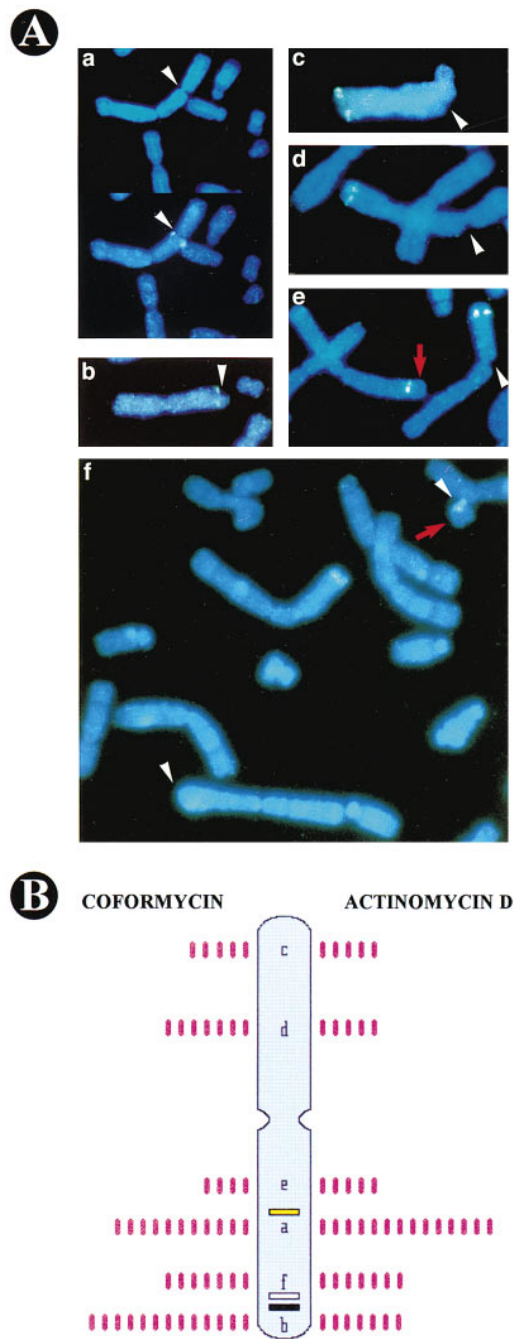
Figure 4. Breaks Induced by Coformycin or AMD at Six Major Fragile Sites on Chromosome 1

(A) Examples of breaks at fragile sites; arrowheads point to the breaks. (a): DAPI staining (top) and FISH with an *mdr1* probe (bottom) of the same metaphase, showing a break at fragile site (a) close to and telomeric to the *mdr1* gene. (b): FISH with an *ampd2* probe (yellow), locating fragile site (b) close to and telomeric to the *ampd2* signal. (c–f): localization of sites (c)–(f). FISH with a P3C4 probe identifies chromosome 1q. (e): the red arrow points to the telomeric part of chromosome 1a containing P3C4. As previously reported (Toledo et al., 1992), the distance between P3C4 and the telomere is longer on the 1a than on the 1b homolog. (f): as in (e), one P3C4 is on a normal chromosome 1b and the other is on a broken fragment of chromosome 1a.

(B) Localization and frequency of breaks induced by coformycin or

mutants tested and, with few exceptions, amplification proceeded by the BFB mechanism (Toledo et al., 1992a, 1992b). The situation disclosed by the analysis of the *mdr1* system encouraged us to check for the existence of a coformycin-activated fragile site telomeric to the *ampd2* gene. As represented in Figure 4B, coformycin induces breaks at sites colocalized with those activated by AMD, within the limits of precision of the mapping technique. Among them, the (b) site, telomeric to the *ampd2* gene (Figure 4Ab), is expressed most frequently following the coformycin challenge (13% of informative mitoses) (Figure 4B).

Thus, the break induced at site (b) again accounts for the triggering of amplification by BFB cycles of a gene centromeric to this fragile site. The frequent coamplification of *ampd2* and P3C4 during BFB cycles (Toledo et al., 1992b) could result from the existence of site (f), another coformycin-inducible fragile site centromeric to P3C4 (Figure 4Af). Indeed, induction of fragile site (f) cannot initiate intrachromosomal *ampd2* amplification since the gene, like any marker telomeric to the break, would be deleted. Induction of this site could instead efficiently resolve a bridge between sister chromatids fused at site (b) (Figure 5A). The two fragile sites flanking the *ampd2* locus would then define the size and organization of the first amplified unit. The rungs of *ampd2* gene copies appear as doublets because they are separated by twice the very short distance between *ampd2* and site (b). The next cell cycle leads to formation of a dicentric chromatid bearing two *ampd2* doublets, separated from each other by twice the distance between *ampd2* and fragile site (f). Repeated breaks at sites (f) accumulate repeats of these large units, giving rise to regular ladders of *ampd2* doublets on the amplified chromatid. Remarkably, the type of rearrangements predicted by this model explains entirely the generation of the regular repeats observed early in the amplification process, as well as the constant size of the repeats in independent clones during the very first stages of *ampd2* amplification (Toledo et al., 1992a) (Figure 5B). The induction of fragile site (f) is expected to stop as soon as accumulation of a sufficiently large number of *ampd2* gene copies relieves the metabolic pressure leading to its activation. During subsequent cell divisions, BFB cycles may be perpetuated by breakage of the bridge at random positions, leading to an increase in the heterogeneity of the amplified units, as observed in cells with a relatively high copy number of the *ampd2* gene (Toledo et al., 1992a).

In the model just described, we considered the case where a fragile site is broken only once. However, we also considered the possibility that a fragile site remains inducible after the first breakage. A secondary break at this site will only lead to a less efficient accumulation of the extra copies. As an example, if an activatable site (b), remaining between the two copies of an *ampd2*

AMD on chromosome 1. Each red oval represents 1% of mitoses with a break at the corresponding site. At least 250 informative mitoses were analyzed for each drug. Yellow rectangle: *mdr1* gene; white rectangle: P3C4; black rectangle: *ampd2* gene. (a)–(f) denote fragile sites illustrated in (A).
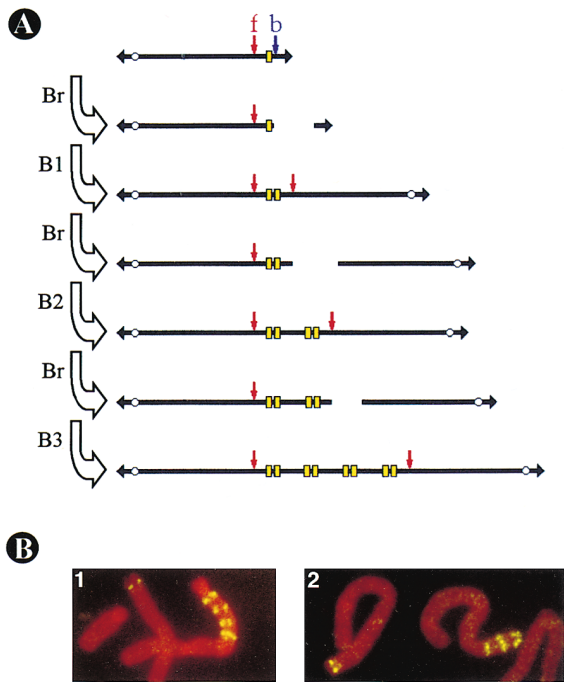
Figure 5. Role of Fragile Sites (b) and (f) in Early Stages of Intrachromosomal *ampd2* Gene Amplification

(A) A model of the role of sites (b) and (f) in *ampd2* amplification. Yellow rectangle: *ampd2* gene; red arrow: fragile site (f); blue arrow: fragile site (b); black arrowheads: telomeres; circles: centromeres; (Br): breakage; (B1), (B2), (B3): bridges of the first, second, and third cycles. Chromosome 1p is not drawn to scale.

(B) Examples of structures formed early. FISH with a probe for the *ampd2* gene (yellow). (1) and (2) left: normal chromosomes 1 with *ampd2* at its wild-type location; right: amplified chromosomes 1 from the same metaphases, bearing a ladder of *ampd2* genes with four (1) or three (2) regularly spaced rungs. Note that the spots at the level of each rung of a ladder are either split or more intense than the spots for the single copy on the normal chromosome 1.

doublet, is broken at stage B2, the break will lead to a ladder with an odd number of doublets, and may contribute to the formation of a ladder with three rungs (Figure 5B2).

MTX Induction of Fragile Site Expression and Gene Amplification

It has been demonstrated that pretreatment with MTX increases the frequency of mutants resistant to MTX, ADR, and PALA by amplification of the *dhfr*, *mdr1*, and *cad* genes, respectively (Rath et al., 1984; Tlsty et al., 1984; Kuo et al., 1994; Poupon et al., 1996). To evaluate how general the involvement of fragile sites in the induction of intrachromosomal amplification may be, we examined the pattern of breaks induced by MTX.

We focused on chromosome 1, which bears the *mdr1* and *ampd2* genes, and on chromosomes 2 and 7, which bear the *dhfr* and *cad* genes, respectively. Strikingly, MTX-induced sites on chromosome 1 appear to be colocalized with those described in Figure 4B. Among them, sites (a) and (b), involved respectively in AMD- and coformycin-induced amplification, were found to be also activated by MTX. Moreover, we identified a fragile site on

chromosome 7, telomeric to the *cad* gene, which could initiate its amplification (not shown). On chromosome 2, two fragile sites telomeric to the *dhfr* gene could trigger its amplification through BFB cycles, and a centromeric site could resolve the fused chromatids (Figure 6A). In this system, the two fragile sites that can initiate the process are close together but far from the selected gene, telomeric to the dark DAPI band specific for Chinese hamster chromosome 2 (Figure 6A). This accounts remarkably well for the large distance separating the *dhfr* gene at its wild-type location from the first extra copy (Figures 6B and 6C). FISH and banding experiments showed that this large duplication is an inverted repeat (Figure 6C). FISH with probes for the *dhfr* gene and a marker close to the 2p telomere (II6) showed that this marker is deleted on the chromosome arm bearing *dhfr* ladders (not shown). These observations are completely explained by BFB cycles starting at a fragile site telomeric to the dark DAPI band. Moreover, we observed two slightly different types of large inverted repeats, corresponding to the two possible initiator sites. These were characterized by a different length of the DAPI$^+$ band flanked by the two short symmetrical dark bands (not shown). In most early amplified mutants, localization of the third *dhfr* gene copy of the ladder (Figure 6C3) is explained perfectly by a break at fragile site (ce).

Discussion

Inducers of Fragile Sites Enhance Gene Amplification

To analyze the differential ability of clastogenic agents to induce gene amplification, we took advantage of the possibility to select mutants at the *mdr1* locus with different drugs in cells of the same line. Both *cis* and *trans* effects of the genetic background are thus avoided. In contrast to VB, ADR and AMD are clastogenic intercalating agents. Only AMD is a potent inducer of amplification, while VB and, surprisingly, ADR select for but do not induce *mdr1* amplification. In the rare amplified mutants resistant to ADR or VB, the extrachromosomal and intrachromosomal mechanisms appear at comparable frequencies. Strikingly, none of the amplified clones recovered from AMD selection exhibited extrachromosomal-amplified *mdr1* copies. Three clones were polysomic for chromosome 1, and ten presented the characteristic features of BFB cycles (Figures 3A–3C). Thus, most, if not all, AMD-induced amplified mutants resulted from the BFB mechanism. This property was correlated with the ability, unique to AMD, to induce expression of a fragile site telomeric to the *mdr1* gene. The expression of fragile sites telomeric to the selected gene was also examined at other loci, the amplification of which is usually selected with clastogenic drugs (*ampd2*/coformycin, *dhfr*/MTX). The conclusion in all cases examined is that the drugs induce fragile sites at locations that can trigger the amplification of the selected genes. The redundant character of such a correlation strongly supports the simple hypothesis that these drugs induce intrachromosomal amplification because they activate the expression of fragile sites telomeric to the selected gene.
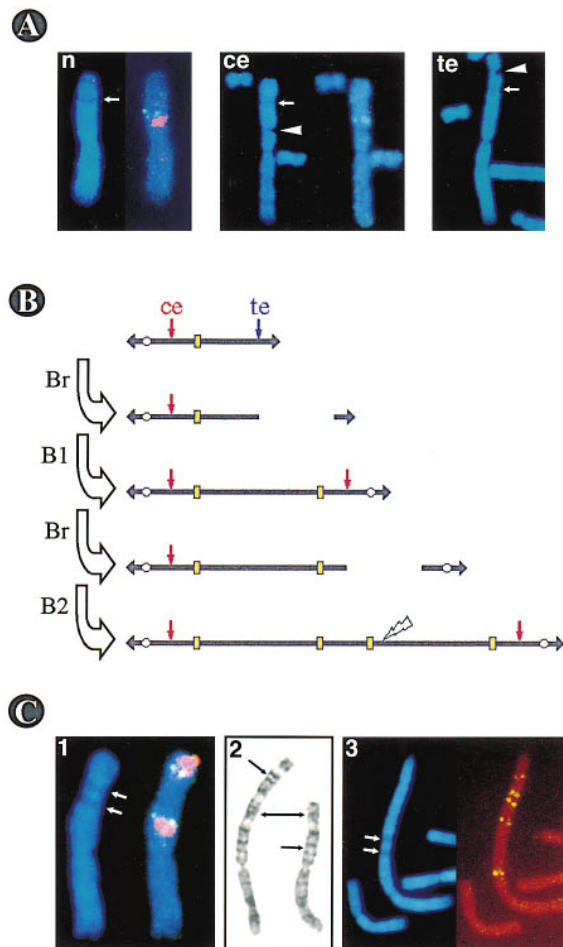
Figure 6. Role of Fragile Sites in Early Stages of Intrachromosomal *dhfr* Gene Amplification

(A) Mapping of fragile sites involved in *dhfr* gene amplification. (n): normal chromosome 2. Left: DAPI staining. The arrow shows the large DAPI dark band specific for Chinese hamster chromosome 2p. Note that this band is telomeric to the *dhfr* gene. Right: FISH with a *dhfr* probe (red) and an II4 probe, a marker close to and telomeric to the *dhfr* gene (yellow). (ce): break at fragile site centromeric to the *dhfr* gene. Left: DAPI staining. The arrowhead shows the break; arrow as in (n). Right: FISH with a *dhfr* probe (yellow). (te): break at fragile site telomeric to the *dhfr* gene. DAPI staining; symbols as in (ce).

(B) A model of the role of sites (te) and (ce) in *dhfr* gene amplification. Yellow rectangle: *dhfr* gene; red and blue arrows: (te) and (ce) fragile sites. The flash symbol points to the putative location of a break occuring during the third cycle, leading to the ladder shown in (C3). The other features are as described in Figure 5A. Chromosome 2q is not drawn to scale.

(C) Examples of amplified chromosomes. (1): DAPI (left) and FISH (right) with probes for the *dhfr* gene (red) and the II4 marker (yellow), showing an inverted duplication. The structure with the two DAPI dark bands (arrows) is consistent with an initial break at (te). (2): trypsin-banding of an amplified (left) and a normal (right) chromosome 2. The double-headed arrow shows the location of the initial break (te). The banding pattern discloses the presence of an inverted repeat on the amplified chromosome, probably from (te) to (ce) (black arrows). (3): DAPI (left) and FISH (right) with a probe for the *dhfr* gene (yellow) showing the large initial *dhfr* duplication (arrows denote the two DAPI dark bands) and a third copy spaced from the second by a distance consistent with a break at (ce).

Breaks induced by AMD and coformycin on chromosome 1 are colocalized, and most of them comap with those revealed by the use of aphidicolin or MTX in other studies (Rassool et al., 1991; Kuo et al., 1994), suggesting that the same fragile sites are induced by these different drugs. This offers an explanation for previous results showing that short pretreatment of cells with MTX increased the frequency of mutants amplified for *dhfr* (Rath et al., 1984; Tlsty et al., 1984), *mdr1* (Kuo et al., 1994), and *cad* genes (Poupon et al., 1996). Indeed, some of the MTX-inducible fragile sites that we mapped on chromosomes 1, 2, and 7 are appropriately located to trigger the amplification of the *mdr1*, *dhfr*, and *cad* genes, respectively. In the CHO cell line, a possible contribution of one of these sites to *mdr1* amplification has been proposed (Kuo et al., 1994). Pretreatment with PALA has also been proven to increase the frequency of amplified mutants resistant to MTX or PALA (Poupon et al., 1996). This suggests that PALA is also able to activate the same fragile sites as the drugs we have studied.

Breakpoints at Telomeric and Centromeric Fragile Sites Frame the Initial Intrachromosomal Amplicons

We obtained striking evidence that breaks at fragile sites control the initial steps of intrachromosomal amplification at different loci. Because the BFB mechanism leads to a progressive accumulation of extra copies of a gene during clonal cell expansion, the fragile sites will remain activated until enough copies to overcome the toxic effects of the selective drug are acquired. Thus, the sequential induction of two fragile sites flanking the selected gene can define the size of the amplified units and the distribution of marker genes along the amplified array. The distance between the fragile site telomeric to each *ampd2* and *mdr1* gene is very short, and doublets of these genes are observed along the initial ladders. In the case of the *dhfr* gene, two alternative fragile sites initiating BFB cycles are relatively close to the telomere and far from the selected gene, which readily explains the frequent occurence of very large initial inverted duplications observed here and in previous studies on CHO cells (Ma et al., 1993). In all three systems examined, the secondary activation of a site centromeric to the gene leads to the accumulation of regular repeats (Figures 5A and 6B). Chromosome translocations or deletions with breakpoints at fragile sites are well documented in cultured cells (Warren et al., 1987; Glover and Stein, 1988). Thus, gene amplification is another outcome of such site-specific breakages.

Secondary Events in Cells Undergoing BFB Cycles

When a selected gene is amplified enough, the selective pressure disappears and fragile site activation is likely to stop, making the resolution of chromatid bridges dependent on random breaks that alter the initial spacing along the ladders. In good agreement with this hypothesis is the observation of a "mixed ladder" organization in cells with a relatively high copy number of the *ampd2* (Toledo et al., 1992a) or *mdr1* and *dhfr* genes. Moreover,

we observed bridges between anaphase–telophase nuclei, but never between interphase nuclei, of daughter cells carrying only a few copies of the selected gene. On the contrary, bridges like those shown in Figure 3 were observed frequently in cells with a high copy number of the amplified gene, suggesting that resolution of the dicentromeric chromatid is delayed in these cases. These cytological features support the idea that at least two breakage mechanisms operate in sequence during the amplification process, and that their relative utilization is controlled by the copy number of the selected gene.

An expected consequence of the coexpression of several fragile sites in early mutants is the induction of chromosome rearrangements. During amplification, ring and dicentric chromosomes are observed at a strikingly high frequency from very early stages (Smith et al., 1992). We discussed previously the likely role of additional breakage events to generate these structures from chromosomes undergoing BFB cycles, but the origin of such additional breaks was not explained (Toledo et al., 1993). The coactivation of multiple fragile sites supplies a likely explanation for the generation of these early rearrangements.

Mutants Not Induced by the Selective Agent

Our study raises the question of whether additional copies of a gene can be acquired by the same mechanisms without induction by the selective drug. This question has been addressed previously in the *cad* system, where it was strongly suggested that BFB cycles operate in both cases (Poupon et al., 1996). We show here that the intrachromosomally amplified mutants obtained with ADR and VB exhibit cytological features typical of the operation of BFB cycles and that the amplified structures observed in these mutants were remarkably similar to those revealed in induced mutants. Indeed, doublets of the *mdr1* gene were present in each rung of the ladders, indicating that the initiating break occurred very close to the selected gene. This is consistent with initiation at the same fragile site as in induced mutants, which could have been activated in these cases by some uncontrolled stress imposed on the cells.

We also showed that the frequency of extrachromosomal events is not enhanced upon selection in ADR or AMD as compared to VB. Since we verified that cells containing DMs are not strongly counterselected in the two clastogenic drugs, our results show that neither AMD-induced site-specific breaks at fragile sites nor ADR-induced breaks at random stimulate formation of DMs in this system. These observations support the hypothesis that intrachromosomal and extrachromosomal amplifications follow independent pathways (Toledo et al., 1993), the products of which were observed at roughly similar frequencies in mutants selected with ADR or VB (Table 1). The latter result suggests that in model systems of drug-selected amplifications, the contribution of the BFB mechanism has probably been overestimated because the agents used to select mutants induce this mechanism specifically.

Fragile Sites and Gene Amplification in Tumor Cells

We have demonstrated here that activation of fragile sites plays a key role in initiating BFB cycles, as well as in determining the size and genetic content of amplified units at an early stage of drug-selected amplification. Because the amplification of oncogenes is frequently observed in tumor cells, it is of special interest to determine whether fragile sites are at the origin of breaks in the organism. Recently, Jacobsen Syndrome has been related to fragile site FRA11B, the activation of which leads to the deletion of the CBL2 proto-oncogene (Jones et al., 1995). The hypothesis that activation of fragile sites could be responsible for the recurrent chromosomal rearrangements identified in some cancers was proposed more than ten years ago (Yunis, 1983; Lebeau and Rowley, 1984; Yunis and Soreng, 1984), and evidence that fragile site FRA3B is involved in the deletion of the FHIT tumor suppressor gene has recently been obtained (Ohta et al., 1996; Sozzi et al., 1996). Taken together, these results suggest that fragile sites can be activated in vivo and can trigger targeted chromatid breaks. To evaluate the role of fragile sites in the initiation of oncogene amplification in tumors, we consulted the Genome Data Bank in an attempt to compare the locations of fragile sites with those of oncogenes amplified frequently in different cancers. Such an analysis is necessarily limited by the fact that not all fragile sites are localized precisely or even recorded, nor are all the gene maps reliable. Moreover, analysis of the amplification process in tumor cells is obscured by secondary rearrangements, as in advanced mutants isolated in vitro. We focused on the 11q13 and 12q13–14 loci, because these regions contain gene clusters allowing an analysis of coamplification patterns, and amplification by BFB cycles is suggested in some cases by localization of the extra copies on the chromosome arm bearing the locus in normal cells (Roelofs et al., 1993; Lese et al, 1995) or by their symmetrical organization (Pedeutour et al., 1994). At the 11q13 locus are linked the genes encoding the B cell CLL/lymphoma (BCL1), cyclin D1 (CCND1/PRAD1), fibroblast growth factor types 3 and 4 (FGF3/INT2 and FGF4/HSTF1), the cortactin protein (EMS1), and the glycoprotein A repetition predominant (GARP). These genes are frequently coamplified in breast, head, and neck carcinomas, in oral squamous cell carcinomas, and in other cancers (Schuuring, 1995). This gene cluster is flanked by two fragile sites: FRA11F (11q14.2) and FRA11A (11q13.3). Another fragile site not yet precisely mapped, FRA11H (11q13) could also be involved in the amplification process. The 12q13–q14 region is frequently amplified in glioblastomas (Collins, 1995) and in other cancers such as liposarcomas. Several genes at this locus were coamplified frequently, including the glioma-associated oncogene homolog (GLI), DNA damage–inducible transcript 3 (DDIT3/GADD153), cyclin-dependent kinase 4 (CDK4), sarcoma-amplified sequence (SAS), and human homolog of mouse double minute 2 (MDM2) genes. In this case, amplification could be initiated at FRA12B (12q21.3), and the size of the amplicon determined by FRA12A (12q13.1).

These data are consistent with the possibility that amplification of some oncogenes and their coamplification patterns may depend on induction of fragile sites in vivo as well as in vitro, an hypothesis having practical implications. For example, it has been proven that some

fragile sites are induced by environmental factors, such as consumption of coffee or cigarette smoking (Kao-San et al., 1987; Chen et al., 1989; Ban et al., 1995). Cigarette smoking could be involved in tumor progression both by inducing mutations in the P53 gene (Denissenko et al., 1996) and by triggering oncogene amplification through activation of fragile sites. An elevated expression of a fragile site at 11q13.3 (Kao-San et al., 1987) was indeed found in cells from smokers when compared to nonsmokers. It will be of special interest to investigate the possible link between this report and the repeated observation that additional copies of an 11q13 domain accumulate within HSR localized on chromosome 11 in patients with oral squamous cell carcinomas (Lese et al., 1995), recorded as a typical cigarette smoke–induced tumor.

An additional remark is that most drugs used here to select amplified mutants in vitro are frequently used in human cancer therapy. In some cases of treatment failure, amplification of oncogenes appeared in tumor cells of relapsing patients. As an example, 11q13 amplification has been identified in local recurrence of breast cancer (Champeme et al., 1995). Our results suggest that improved knowledge of the ability of chemotherapeutic agents to induce fragile sites in human cells, and precise mapping of these sites relative to oncogenes frequently amplified in tumors, may contribute to defining therapeutic protocols that can circumvent tumor progression in some cases.

Experimental Procedures

Selection of Amplified Mutants
The wild-type GMA32 cell line and regular culture medium have been previously described (Debatisse et al., 1982, 1984). The method used to observe cells at early stages of gene amplification is derived from Smith et al. (1990). For the *mdr1* selection, subpopulations of 50 cells were plated in separate dishes and grown in regular medium for about 12 generations. Each subpopulation was then plated in selective medium containing VB (15 or 19 ng/ml), ADR (188, 206, or 223 ng/ml), or AMD (88, 100, or 113 ng/ml). The same procedure was followed for the recovery of clones resistant to coformycin (0.5 or 0.7 μg/ml) or MTX (9 or 18 ng/ml). Drug-resistant colonies were recovered and expanded to 10^5 cells before cytogenetic analysis. Exponentially growing cells were treated with colcemid (100 ng/ml) or nocodazole (10 μM) for 2–4 hr, then spread on slides as previously described (Toledo et al., 1992a) and used for FISH studies.

Fragile Sites Induction
Exponentially growing GMA 32 cells (5.10^5 cells per 10 cm diameter petri dish) were treated with the drugs to be studied as previously described (Robinson and Elder, 1987; Yunis et al., 1987). Briefly, cells were incubated for 18 hr in medium supplemented with VB, ADR, AMD, coformycin, or MTX at the drug concentrations used to select mutants, then allowed to recover in normal medium for 8 hr, including the final 2 hr of incubation with nocodazole. Decreasing concentrations of ADR (200, 150, 100, 50 ng/ml) were also tested.

In Situ Hybridization and Giemsa-Trypsin Banding
FISH was performed essentially as described (Pinkel et al., 1988; Tkachuk et al., 1990) with minor modifications (Toledo et al., 1993). Lambda Charon 4A clones F 4-2 and R 97 (kind gifts of R. Zastawny and V. Ling) were used to probe the *mdr1* locus; cosmids KP 454 and KZ 381 (kind gifts of J. Hamlin), to probe the *dhfr* locus; cosmids C64 and C81 (kind gifts of G. Stark and E. Giulotto), to probe the *cad* locus; and cosmids 61W14, 56D3S1A1, and 56Y1B1, to probe the *ampd2* locus (Debatisse et al, 1988). Cosmids P3C3 and P3C4,

and II4 and II6 (isolated respectively from a Chinese hamster chromosome 1- and 2-specific library [M. D., B. Labidi, and P. Metezeau, unpublished data]) were respectively used as markers of chromosome 1 and 2. The probes were biotinylated by nick translation (BioNick Labeling System kit, GIBCO-BRL) or labeled with digoxigenin by random priming (DIG DNA labeling kit, Boehringer).

Giemsa-trypsin Banding was performed essentially as described (Seabright, 1971). Briefly, slides were incubated 12 sec in trypsin 0.8 mg/ml in PBS at 37°C, quickly rinsed in PBS, stained 7 min in 3% Giemsa (pH 6.8), and then washed in water.

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