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Chromosome Breakage at a Major Fragile Site Associated with P-Glycoprotein Gene Amplification in Multidrug-Resistant CHO Cells

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Recent studies of several drug-resistant Chinese hamster cell lines suggested that a breakage-fusion-bridge mechanism is frequently involved in the amplification of drug resistance genes. These observations underscore the importance of chromosome breakage in the initiation of DNA amplification in mammalian cells. However, the mechanism of this breakage is unknown. Here, we propose that the site of chromosome breakage consistent with the initial event of P-glycoprotein (*P-gp*) gene amplification via the breakage-fusion-bridge cycle in three independently established multidrug-resistant CHO cells was located at 1q31. This site is a major chromosome fragile site that can be induced by methotrexate and aphidicolin treatments. Pretreatments of CHO cells with methotrexate or aphidicolin enhanced the frequencies of resistance to vinca alkaloid and amplification of the *P-gp* gene. These observations suggest that chromosome fragile sites play a pivotal role in DNA amplification in mammalian cells. Our data are also consistent with the hypothesis that gene amplification can be initiated by stress-induced chromosome breakage that is independent of modes of action of cytotoxic agents. Drug-resistant variants may arise by their growth advantage due to overproduction of cellular target molecules via gene amplification.

Acquisition of resistance to cytotoxic agents via amplification of target genes has been well documented (for reviews, see references 12, 32, 40, and 50). In addition, genes that encode oncoproteins, growth factors and their receptors, and cell cycle regulators are often amplified in many tumor cells (1, 4, 33). Two types of cytogenetic abnormalities associated with amplified DNA have been described: extrachromosomal double minutes (DM) and homogeneously staining region (HSR) (3). DM are often associated with murine and human cell lines, whereas HSRs are found in hamster cell lines.

Many models describing the mechanisms of DM and HSR formation in various drug-resistant systems have been published (12, 22, 32, 40, 51). Recent studies using fluorescence in situ hybridization (FISH) suggested that a breakage-fusion-bridge (B-F-B) cycle is frequently involved in DNA amplification in Chinese hamster drug-resistant variants (17, 22, 38–40, 45, 46, 51). According to this model (Fig. 1), a chromatid break occurs between the target locus and the telomere, yielding a chromosome with a frayed end. After replication, the broken chromosome end is then repaired by sister chromatid fusion. Replication of this chromosome would generate a dicentric chromosome with duplicated copies of the target sequence bridged by the fusion point. Repetition of the B-F-B cycle, or other mechanisms, would generate multiple copies of the target DNA located distal to the original locus.

Many cytogenetic observations have supported the B-F-B mechanism. (i) The amplified DNA localized distally to the single-copy target gene in the same chromosomal arm has been described for many drug-resistant hamster cells selected with various cytotoxic agents (22, 35, 38–40, 45–47, 51). (ii) A deletion of the chromosome arm containing amplified DNA has been noted (35). (iii) Frequent sister chromatid fusion and formation of dicentric chromosomes have also been observed to occur in many drug-resistant hamster cell lines (17, 22, 39, 46, 51). (iv) The B-F-B mechanism predicts a very large inverted duplication of the chromosome arm between the single-copy locus and the amplified DNA cluster and between each amplification unit within the amplified cluster. By using multiple DNA probes with two-color FISH, these have indeed been found in the methotrexate (MTX)- and deoxycoformycin-resistant lines (22, 46).

As chromosome breakage is an important mechanism that generates the B-F-B process, understanding the mechanism of chromosome breakage is of importance in determining how DNA amplification starts. In this communication, we present cytogenetic evidence that chromosome breakage associated with DNA amplification in the multidrug-resistant (MDR) CHO cells occurred at a major fragile site induced by MTX and aphidicolin (APC), suggesting that fragile sites have an important role in mammalian DNA amplification.

MATERIALS AND METHODS

Cell lines. The cell lines VCR15, VBR1.5, and ADR10 were originally established by stepwise selection protocols involving progressively increasing concentrations of vincristine (VC), vinblastine (VB), and adriamycin to final concentrations of 15, 1.5, and 10 μg/ml, respectively (35, 42). VBR1.5-1 and ADR10-1 were subclones of VBR1.5 and ADR10, respectively. These cells and drug-sensitive CHO cells were maintained as monolayer cultures at 37°C in 5% CO_2 in air in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (GIBCO BRL).

FISH. Three cosmid DNAs were used as probes: (i) cosDR6 (a gift from Larry D. Teeter, M. D. Anderson Cancer Center),

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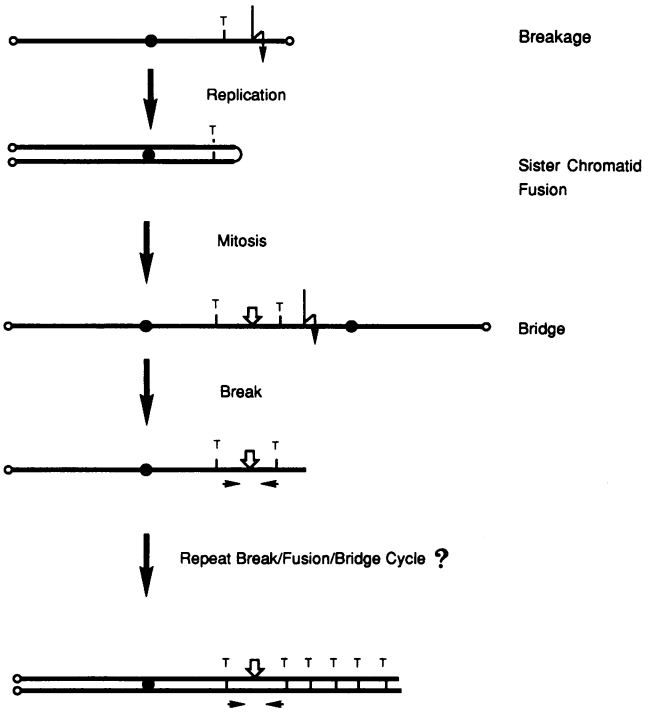

FIG. 1. Schematic diagram showing the formation of amplified DNA in the abnormal banding region by the B-F-B mechanism. Each line represents one chromatid, zigzag arrows represent chromatid breaks, open arrows point to sister chromatid fusion sites, small arrows indicate centromere-telomere directionality, open circles indicate telomeres, filled circles indicate centromeres, and T indicates the target gene. Details are given in the text.

which contains about 40 kb of the Chinese hamster *P-gp* gene (42); (ii) cosDR30.1, which was isolated by screening a cosmid VCR15 genomic library with the cDNA probe cp30 (designated gene class 5 [8, 48], a gift from Alexander M. Van der Bliek and Piet Borst, The Netherlands Cancer Center, Amsterdam, The Netherlands); and (iii) P3C4, a DNA marker located near the terminus of Chinese hamster chromosome 1q (46) (a gift of M. Debatissee, Pasteur Institute, Paris, France). The cosmid DNA was labeled with biotin-11-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.) by using a nick translation kit purchased from Promega (Madison, Wis.).

FISH was carried out as described by Pinkel et al. (25), with some modifications. The air-dried chromosome preparations were denatured for 5 min in a solution containing 70% formamide, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 6.8), and 10 mM sodium phosphate and sequentially dehydrated in 70, 90, and 100% ethanol.

The probe (1 μg) was denatured in a solution containing 50% formamide, 2× SSC, 10% dextran sulfate, and 20 μg of CHO DNA at 80°C for 5 min and immediately applied to the chromosome preparations. The slides were incubated at 37°C for 2 to 3 days and washed sequentially with 50% formamide–2× SSC, 2× SSC (both at 45°C), and 4× SSC with 0.05% Tween 20 and 5% nonfat dried milk. The biotin-labeled signal was detected by sandwiched layers of fluoresceinated avidin (Vector Laboratories) and biotin-conjugated goat antiavidin (Vector Laboratories). Each avidin or antiavidin treatment, both 5 μg/ml in 4× SSC containing 0.5% Tween 20, was followed by three washes in 4× SSC–0.05% Tween 20 at 22°C for 5 min. The digoxigenin-labeled probe was detected by an antidigoxigenin antibody (1:500 dilution; Sigma, St. Louis, Mo.) and then incubated first with rhodamine-conjugated rabbit anti-mouse antibody (1:1,000 dilution; Sigma) and then with rhodamine-conjugated goat anti-rabbit antibody (1:1,500 dilution; Sigma). The chromosomes were counterstained with 0.5 μg of propidium iodide (Sigma) per ml or with 0.5 μg of diamidinophenylindole (DAPI) per ml and mounted in 1,4-diazabicyclo(2.2.2)octane (DABCO) antifade solution (Sigma). Chromosome preparations were analyzed with a Nikon microscope equipped with fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, and UV epifluorescence optics by using appropriate filters for different fluorochromes.

Induction of the chromosome fragile site. Exponentially growing CHO cells at 2×10^5 cells per 10-cm-diameter petri plate were treated with three fragile-site-inducing agents according to the protocols described by Elder and Robinson (9, 30), with slight modifications. Briefly, cells were treated with (i) 1 μg of MTX (Lederle, Pearl River, N.Y.) per ml for 18 h followed by incubation in the regular medium for 6 h, including the final 2-h incubation with 0.2 μg of VC per ml; (ii) 0.1 μg of APC (Sigma) per ml for 24 h; and (iii) 0.4 μM 5-fluorodeoxyuridine (FUdR; Sigma) plus 2.2 mM caffeine (Sigma) for 24 h followed by a 6-h incubation in the regular medium with the addition of 0.2 μg of VC per ml for the final 2 h.

Measurements of frequencies of drug resistance and DNA amplification in one-step selection. CHO cells (5×10^5) in 10 ml of the regular medium were plated in a 10-mm-diameter petri dish and cultured for 24 h. Cells were treated with different concentrations of MTX (0.05 or 0.1 μg/ml) or APC (0.5 or 1.0 μg/ml) for 24 h and allowed to recover in the regular medium for 24 h. After trypsinization, cells were replated in quadruplicate at 10^5 cells per 10 ml of medium and treated with 0.1 μg of VB per ml for 2 weeks with one change of drug-containing medium. The frequency of drug resistance was measured by counting the surviving cell colonies after normalization against plating efficiency.

The cell colonies, after expanding to mass cultures in the presence of selecting drugs (3 to 4 weeks of total drug exposure), were used directly for FISH using biotin-labeled cosDR6 probe and detected by fluoresceinated avidin as described above.

Cytogenetic analysis. Cells were harvested by routine cytogenetic procedures, and chromosomes were Giemsa-trypsin banded by a modification of the procedure of Seabright (34).

RESULTS

FISH of amplified DNA in MDR CHO cells. Previous in situ hybridization studies using radioactively labeled cosDR6 (*P-gp*) probe localized the amplified *P-gp* gene near the terminus of chromosome 1q (or Z1q by the nomenclature of Deaven and Peterson [7]) in VCR15 (42), VBR1.5-1, and ADR10-1 cells (35). Because FISH provides high resolution of hybridization signal and multiple probes can be used simultaneously, we employed FISH to reinvestigate gene amplification in these MDR CHO cells.

(i) VCR15 cells. cosDR6 (*P-gp*) and cosDR30.1 (gene class 5) probes were used either for single- or for double-color FISH with this cell line. A total of 73 metaphases were analyzed, and examples of these hybridizations are shown in Fig. 2A and B. More than 94% of the metaphases showed amplification of both *P-gp* and gene class 5. The amplified signals were located at the q arm of one of the largest chromosomes (Fig. 2A and B, arrows), which was previously identified as chromosome 1 by Giemsa-trypsin banding (42). The probes also hybridized to the single-copy locus on chromosome 1q (the middle portion

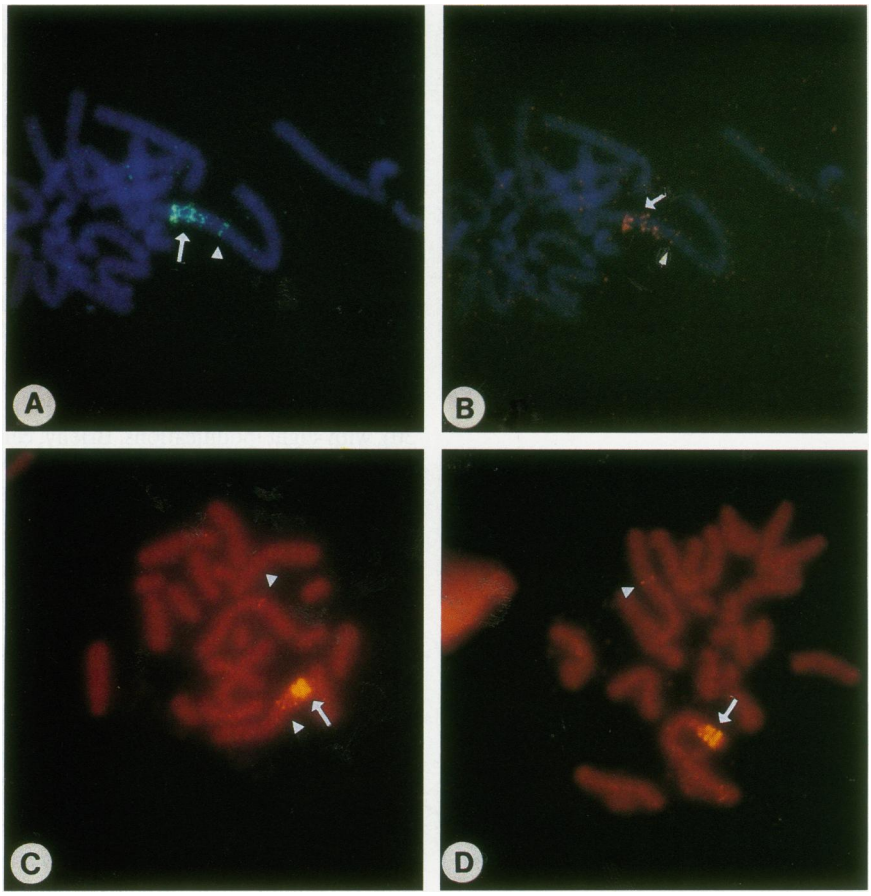

FIG. 2. FISH localization of DNA amplification in MDR CHO cells. (A and B) Same metaphase from a VCR15 cell showing hybridizations with cosDR6 (biotin-labelled *P-gp*) and cosDR30.1 (digoxigenin-labelled gene class 5) probes, respectively. (C and D) FISH localizations of the *P-gp* gene in VBR1.5-1 and ADR10-1 cells, respectively. The arrows point to the distinct amplification zones, and arrowheads point to the single-copy loci. Note that the amplified DNAs are near the telomeres of chromosomes 1q, whereas the single-copy genes remain at their native loci.

of the long arm) in most of these metaphases (Fig. 2A and B, arrowheads) (2, 35). Three distinct hybridization zones were found in the amplified chromosome segments for both genes by double-color FISH (Fig. 2A and B, arrows). These results indicate that these two genes were coamplified.

(ii) VBR1.5-1 cells. Thirty-six metaphases were analyzed by using cosDR6 and cosDR30.1 probes. In more than 83% of the cells, both probes revealed DNA amplification in similar places, i.e., in a region proximal to the telomere (Fig. 2C, arrows). The single-copy sequences were usually detected at their original sites (Fig. 2C, arrowheads). In many metaphases, characteristic hybridization zones were discernible in the amplified segments. Occasional tetraploid cells and endoreduplicated metaphases with duplicate hybridization patterns were also observed (data not shown).

(iii) ADR10-1 cells. Seventy-four informative metaphase spreads were analyzed; 78% showed DNA amplification at the terminal region of chromosome 1q with cosDR30.1 (Fig. 2D, arrow) and cosDR6 (not shown). Hybridization to the single-copy sequence at its native region was found in more than 50% of the metaphases.

These results, consistent with our initial observation (35), indicate that the amplified DNAs in the three independently established MDR CHO cell lines are distally localized on the chromosome where the single-copy sequence resides. These observations are also consistent with the fact that many drug-resistant Chinese hamster variants contain amplified DNA (22, 45–47).

Site-specific breakage associated with DNA amplification in the MDR cells. Conventional Giemsa bandings of VCR15 and VBR1.5-1 cells revealed two marker chromosomes that are distinct from those of the parental CHO cells, derivatives of chromosome 1 (M1) and chromosome Z7 (M). Figure 3 presents the banding pattern of these marker chromosomes in MDR CHO cells in comparison with their normal counterparts in CHO cells. Although chromosome 1q contains amplified DNA sequences, no obvious increase in its length was observed.

The two probes used in this study were physically located at least 1 megabase (Mb) apart by pulsed-field electrophoresis (48). Southern blot hybridizations have shown that both *P-gp* (35, 42) and gene class 5 sequences (unpublished data) are coamplified 30- to 40-fold in these MDR cells. Therefore, these MDR cells must contain at least 30 to 40 Mb of amplified DNA sequences. Assuming that the genome size for a CHO cell is 2×10^9 bp, of which chromosome 1q contributes approximately 5%, on the basis of a measurement of the relative lengths of chromosome arms, addition of 30 to 40 Mb

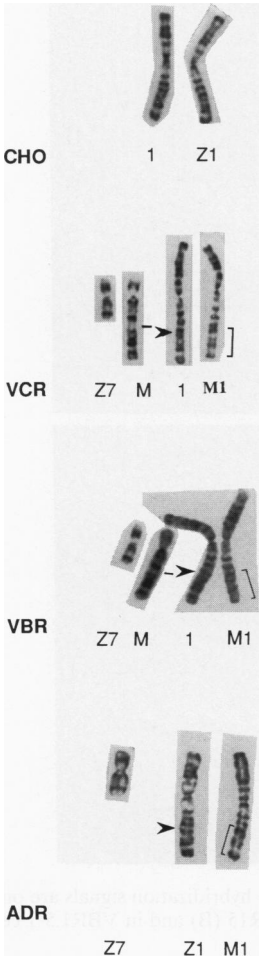

FIG. 3. Giemsa-trypsin-banded CHO and MDR CHO chromosomes. Only the chromosomes discussed in the text are shown here. In VCR15, VBR1.5-1, and ADR10-1 cells, only one of the two chromosomes (1 or Z1) (labeled M1) contains the amplified DNA sequences at the bracketed regions. Unidirectional translocation of the telomeric segment from chromosomes 1 to Z7 forming the marker chromosome M is proposed to occur in VCR and VBR cells. This translocation involves a break on chromosome 1 (arrowheads) and a fusion point (bar) at marker chromosome M. It is proposed that deletion of chromosome 1q preceded DNA amplification for ADR10-1 cells. Bracketed regions contain putative giant inverted repeats, as shown in Fig. 1.

of amplified DNA to chromosome 1q should have increased the length of chromosome 1q by at least 30%. The observation that chromosome 1q remains its normal length suggests that deletion or translocation may have accompanied DNA amplification in these MDR cells.

Indeed, we found that the Z7 marker chromosome (M) gained an additional segment with a size approximating that of the missing part in chromosome 1q. Furthermore, the Giemsa-banding pattern in the Z7 addition matched that of the distal portion of chromosome 1q. No loss or gain of other chromosome segments was evident in these two MDR cells (35, 42). This observation suggests that a unidirectional translocation from chromosomes 1 to Z7 was associated with DNA amplification in these two cell lines. In ADR10-1 cells, no obvious translocation was observed. A deletion in chromosome 1q must have occurred.

To test this hypothesis, we performed FISH using cosmid P3C4 DNA, which hybridizes to a sequence near lqter (46), as a probe. As expected, CHO cells contained two P3C4 alleles on each chromosome 1q (Fig. 4A). However, in VCR15 (Fig. 4B) and VBR1.5-1 (Fig. 4C) cells, only one allele remained on chromosome 1q, the other being found in a chromosome resembling the Z7 fusion chromosome. In ADR10-1 cells (Fig. 4D), only one P3C4 allele on 1q was found in the entire metaphase. This is consistent with loss of the distal portion of one chromosome 1 in ADR10-1, as mentioned earlier. Thus, we conclude that chromosome breakage on 1q is associated with DNA amplification, an important element in the B-F-B mechanism.

It is possible to deduce the sites of chromosome breakage involved in the translocation or deletion. As shown in Fig. 3, the breakage sites in VCR15 and VBR1.5-1 cells were similar, i.e., 1q31 (indicated by arrowheads and by the nomenclature proposed by Ray and Mohandas [26]) (see also Fig. 6C for the nomenclature). It is difficult to precisely determine the breakage site in ADR10-1 cells from the Giemsa-banded chromosome; however, according to the principle of B-F-B, the breakpoint should be located midway between the single copy and the amplified DNA cluster (Fig. 1, open arrows). This suggests that the breakage site should be located very close to 1q31, if not at 1q31 itself. These results suggest that a common breakage site is associated with DNA amplification in three different MDR CHO cell lines.

Chromosome breakage associated with *P-gp* amplification is located at a major fragile site. Since it has been reported that chromosome fragile sites are frequent targets of cytotoxic agents (54), it was of interest to determine whether 1q31 is a major fragile site in CHO cells. Toward this aim, we treated CHO cells with agents that are known to induce chromosome fragile sites, i.e., MTX, APC, and FUdR-caffeine (9, 24, 30, 41). Treatment of CHO cells with 1 μg of MTX per ml for 18 h induced two major types of abnormalities on chromosome 1: induction of constrictions and chromatid breaks. Constriction was defined as a narrowing of the chromosome width (aside from the centromere, which is the primary constriction site). Four constriction sites, located at 1p21, 1q17, 1q31, and 1q36, were seen in the MTX-treated CHO cells. The degrees of constriction varied from minor (Fig. 5A) to very distinct (Fig. 5B). The CHO cells grown in the regular medium exhibited no constriction except in centromere areas (data not shown).

The second type of chromosome abnormality in the MTX-treated CHO cells was chromatid breaks, consistent with the definition of fragile-site expression (9, 24, 41). The breaks were not random. We examined 68 informative Giemsa-banded metaphases, and a cumulative frequency of breakage at various sites is presented in Fig. 6C (left). Examples of these breaks are shown in Fig. 5C and D and Fig. 6A. Strikingly, 12 metaphases (17%) showed breaks at 1q31, and 7 of these 12 metaphases showed breaks on both chromosomes 1. Three minor breakage sites (1p21, 1q17, and 1q36) were also observed on chromosome 1. All of these sites were consistent with the constriction sites, suggesting that chromosome constriction may precede fragile-site breakage.

Treating CHO cells with APC induced chromosome breakage but not constriction. The frequency of chromosome breakage was much lower than that in the MTX-treated cells. Examining the sites of breakage on 182 informative Giemsa-banded chromosomes 1 revealed a total of 16 chromatid breaks on 16 metaphase cells. The distribution of these breaks was consistent with those in the MTX-treated cells (Fig. 6B and C, right).

Treating CHO cells with FUdR-caffeine did not induce

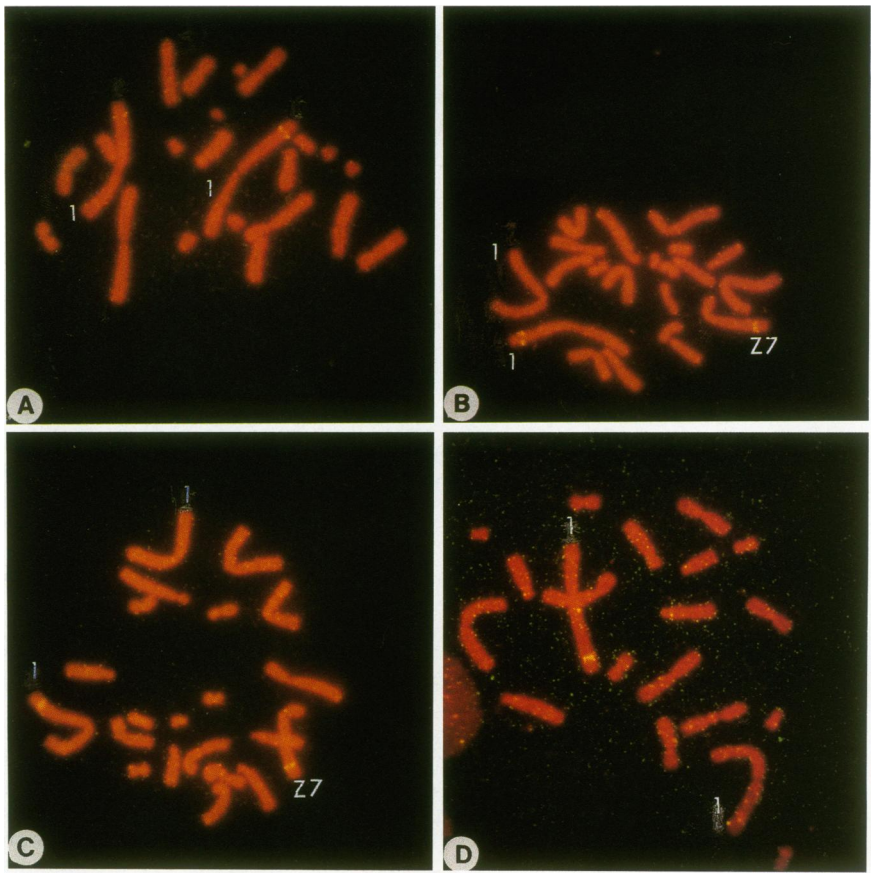

FIG. 4. Visualization of P3C4 sequences in CHO and MDR CHO chromosomes by FISH. The hybridization signals are on both chromosomes 1 in the CHO cell (A), on one of the two chromosomes 1 and the Z7 fusion chromosome in VCR15 (B) and in VBR1.5-1 cells (C), and on only one of the two chromosomes 1 in ADR10-1 (D).

chromosome breakage in 320 metaphase cells examined, indicating that these agents were not good fragile-site inducers in CHO cells. These results are consistent with previous reports that not all the fragile-site inducers are equally effective in the same cell line (24, 41).

Enhancement of frequencies of drug resistance and DNA amplification in CHO cells by pretreatments with MTX and APC. Since it appears that fragile sites are involved in the initial events of DNA amplification, it was of importance to investigate whether pretreatments of CHO cells with fragile-site inducers, MTX and APC, could enhance the frequency of DNA amplification-mediated drug resistance. CHO cells were treated with 0.05 or 0.1 μg of MTX per ml, and after recovery, the pretreated cells were selected with 0.1 μg of VB per ml. As shown in Table 1, the frequencies of resistant colonies were 7- to 17-fold higher than those in the cultures that were not pretreated. Pretreating CHO cells with 0.5 or 1.0 μg of APC per ml enhanced at least 10-fold the frequency of resistance to subsequent VB selection. Pretreating CHO cells with 0.3 μg of MTX or 5.0 μg of APC per ml by a similar selection protocol showed no surviving colonies in 4×10^5 cells plated (data not shown). These results show that prior exposure of CHO cells to fragile-site-inducing agents enhanced the evolution of drug resistance in cultured cells.

FISH using cosDR6 as a probe revealed that more than 80% of the cells in the resistant populations exhibited cytogenetic localizations of amplified *P-gp* DNA distal to the single-copy locus on chromosome 1 (Fig. 7B through F, arrows), whereas in most cases, the single-copy sequence remained in its residential locus (Fig. 7B, C, and E, arrowheads), as also demonstrated for the drug-sensitive CHO cell (Fig. 7A). These cytological localizations of the amplified DNA are in general agreement with those found in VCR15, VBR1.5-1, and ADR10-1 cells. These results suggest that the observed cytogenetic manifestations of DNA amplification occurred at early stages of drug selection.

The majority of metaphases showed only one amplified locus on one of the two chromosomes 1 (Fig. 7C through E). Occasionally, amplification of both alleles was observed (Fig. 7B and F). Furthermore, the hybridization signals for the amplified DNA were variable, ranging from a narrow zone (Fig. 7B) to approximately one-third of the length of chromosome 1q (Fig. 7D through F), suggesting that the copy numbers of amplified DNA and/or the sizes of the amplification units can be highly heterogeneous in one-step selection.

Virtually similar cytogenetic features of *P-gp* amplification were found in the VB-resistant variants selected with MTX or APC pretreatments (data not shown), suggesting that the enhancement of DNA amplification by these agents proceeded by a mechanism similar to the B-F-B process. Thus, we conclude that fragile-site-inducing agents can facilitate the development of DNA amplification-linked drug resistance.

Most of those metaphases that displayed no cytogenetic evidence of DNA amplification (less than 20% of the total

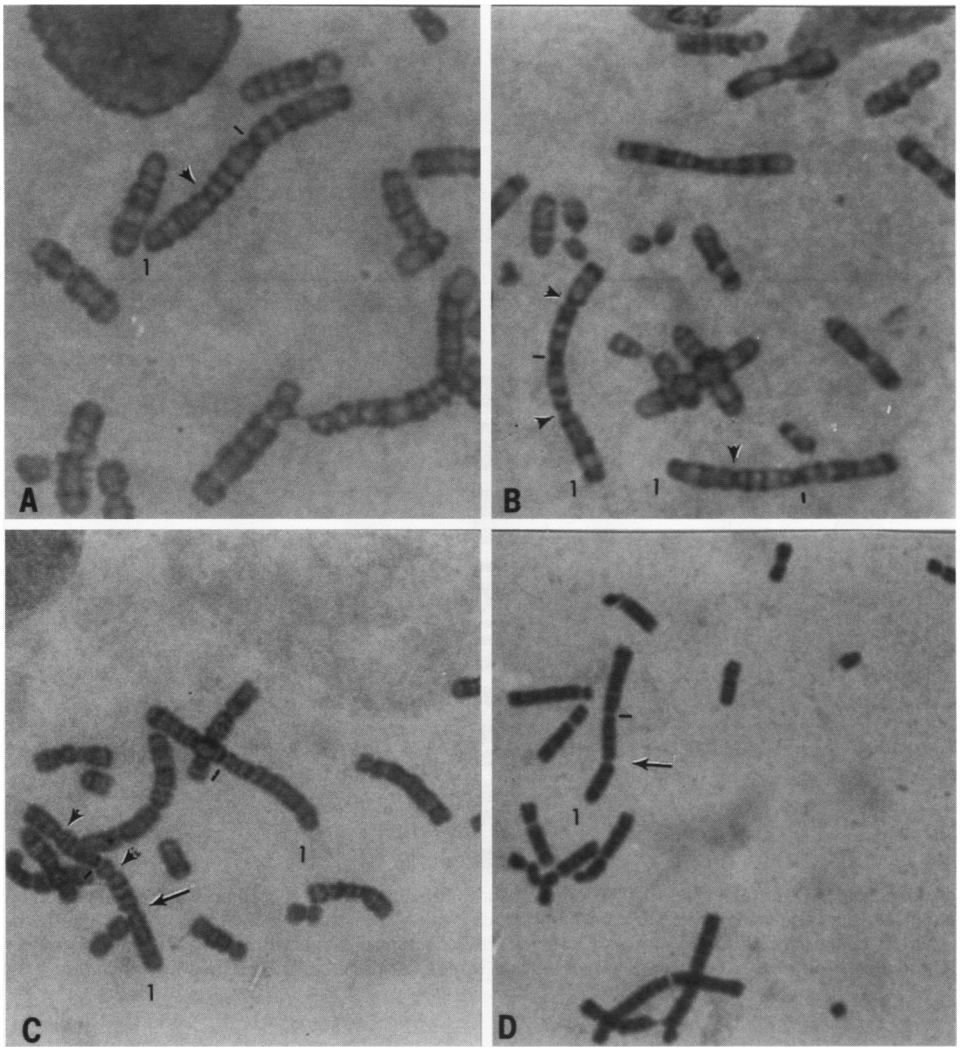

FIG. 5. Giemsa-trypsin banding of metaphase chromosomes in CHO cells treated with MTX showing chromosome constrictions (arrowheads) and breaks (arrows) on chromosome 1. The centromere is marked by a bar. Note that some constriction sites (A and B) are the sites of breakage (C and D).

metaphases examined [Table 1]) showed hybridization signals in the single-copy loci. The mechanisms of drug resistance in these populations are not known but may be due to structural mutations of tubulin genes (6) or other unknown cellular targets. Alternatively, these resistant populations may overproduce P-gp by transcriptional or translational activation without gene amplification (5, 27, 37).

DISCUSSION

The identification of B-F-B as an important mechanism of DNA amplification in the Chinese hamster genome has been mentioned only in recent years, although the mechanism was proposed more than 50 years ago by McClintock (23) to describe the movement of specific genetic loci. The cytogenetic features of amplified *P-gp* genes in the MDR CHO cells described in the present study are consistent with the B-F-B mechanism, suggesting that this mechanism is widely involved in the amplification of hamster DNA.

We demonstrated that the sites of chromosome breakage associated with *P-gp* amplification in the three MDR CHO cell lines occurred at 1q31 (or very close to it for ADR10-1 cells). This site is distal to the *P-gp* locus, which was previously mapped to 1q26 (2, 35), and approximately midway between the single-copy locus and the amplified cluster. According to the B-F-B model (Fig. 1), breakage at this site is consistent with the initial event of *P-gp* amplification.

The MDR cells described in this study were selected by three different cytotoxic agents (two vinca alkaloids and one anthracyclin), suggesting that the proposed initial breakage event is independent of the selecting agents. Consistent with this notion is the observation that the same breakage site can be induced by MTX and APC treatments. These results suggest that the chromosome breakage at 1q31 may be regarded as a stress-induced cytological event and independent of the modes by which these agents exert cytotoxic effects. Since MTX and APC are known fragile-site inducers, our results also suggest an important role of fragile site in the amplification of mammalian DNA.

Four fragile sites have been located on Chinese hamster chromosome 1 (Fig. 6C), but only 1q31 was associated with the *P-gp* amplification discussed in this communication, suggesting

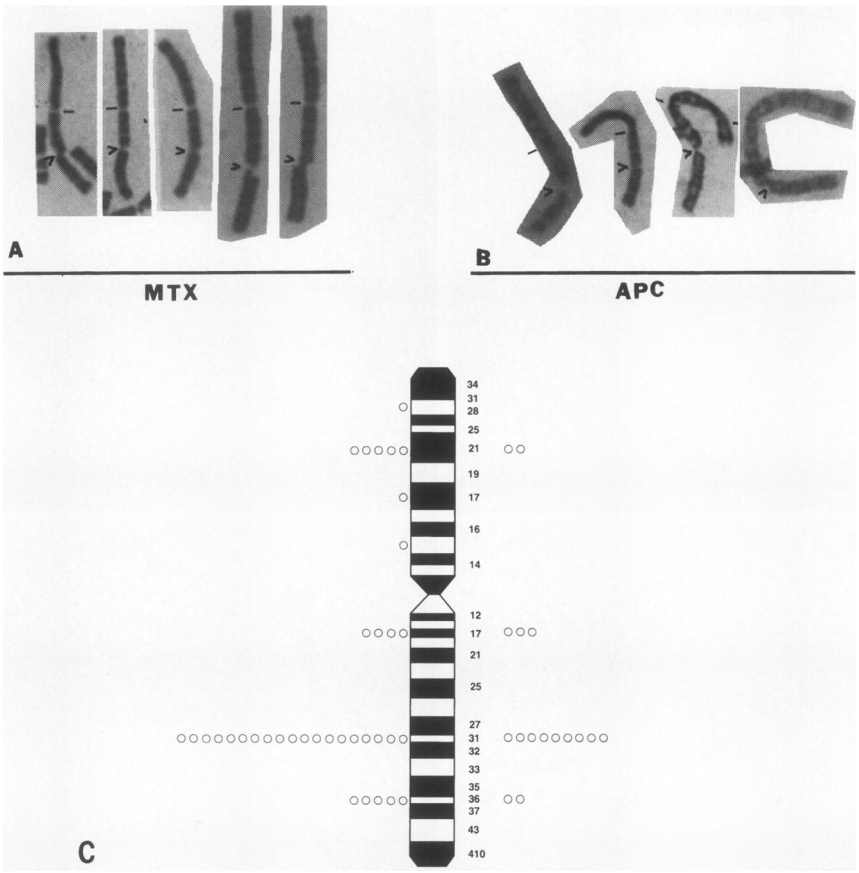

FIG. 6. Localizations of chromosome fragile sites on CHO chromosome 1. Examples of isochromatid breaks (indicated by >) induced by MTX and APC are shown in panels A and B, respectively. The centromeric area is shown by a bar. Panel C indicates the number of isochromatid breaks induced by MTX (to the left of the chromosome) and APC (right). Each circle represents a break at the site indicated. The banding pattern presented here is a simplified version of the one proposed by Ray and Mohandas (26), but the original nomenclature remains unchanged.

that not all the fragile sites contribute equally to the amplification of specific DNA. As stated, only the fragile sites located between the single-copy locus (designated T in Fig. 1) and the chromosome terminus can generate multiple copies of target gene that subsequently provide a growth advantage under continuous selection pressure. Major fragile site 1q31 and minor site 1q36 fall into this category. Fragile site 1q36 may be involved in *P-gp* amplification in some (about 5 to 10%) VBR1.5 and ADR10 cells, in which an elongated chromosome 1q with distally located amplified DNA was found (35).

TABLE 1. Frequencies of VB-resistant CHO cells selected following pretreatments with MTX and APC[a]

Pretreatment drug (concn [μg/ml])	Result with selecting drug (VB) (0.1 μg/ml)		
	Plating efficiency (%)	Frequency of:	
		Resistance[b] (fold increase)	*P-gp* amplification[c] (%)
None	96	1.8×10^{-5}	84/98 (85)
MTX (0.05)	75	14×10^{-5} (7)	145/176 (82)
MTX (0.1)	56	22×10^{-5} (12)	ND
APC (0.5)	84	25×10^{-5} (13)	79/89 (88)
APC (1.0)	78	32×10^{-5} (17)	132/159 (83)

[a] Protocols for drug treatment are described in Materials and Methods.
[b] Determined by dividing the number of cell colonies by the total number of cells plated after normalization against plating efficiency. Average for quadruplicate plates.
[c] Number of mitotic figures displaying distally localized amplified *P-gp* on chromosome 1/total number of mitotic figures showing hybridization signals. ND, not determined.

We also demonstrated that treatments of CHO cells with the fragile-site inducers prior to drug selection resulted in an increased frequency of DNA amplification. These observations reinforce the important role of fragile sites in DNA amplification. It has previously been demonstrated that pretreatments of CHO cells with a variety of stress-inducing agents, i.e., APC (14), hydroxyurea (13, 14), hypoxia (28), and UV (44) and γ-radiations (36), resulted in an increased frequency of resistance to MTX and amplification of *dhfr* sequence in the resistant populations. In another study, a 10- to 100-fold-higher frequency of acquisition of simultaneous resistance to MTX and AD than that predicted from the frequency with which each type of resistance occurred by independent selection was reported (29). Those investigators proposed that the same basic process may contribute to the emergence of resistance to different cytotoxic agents. Our present results are consistent with these findings. Furthermore, our data also suggest that fragile-site breakage may be one of the rate-limiting steps that regulate DNA amplification.

From a mechanistic point of view, it is reasonable to speculate that the rates of DNA amplification in the initial step would be higher than those in the later steps in a stepwise

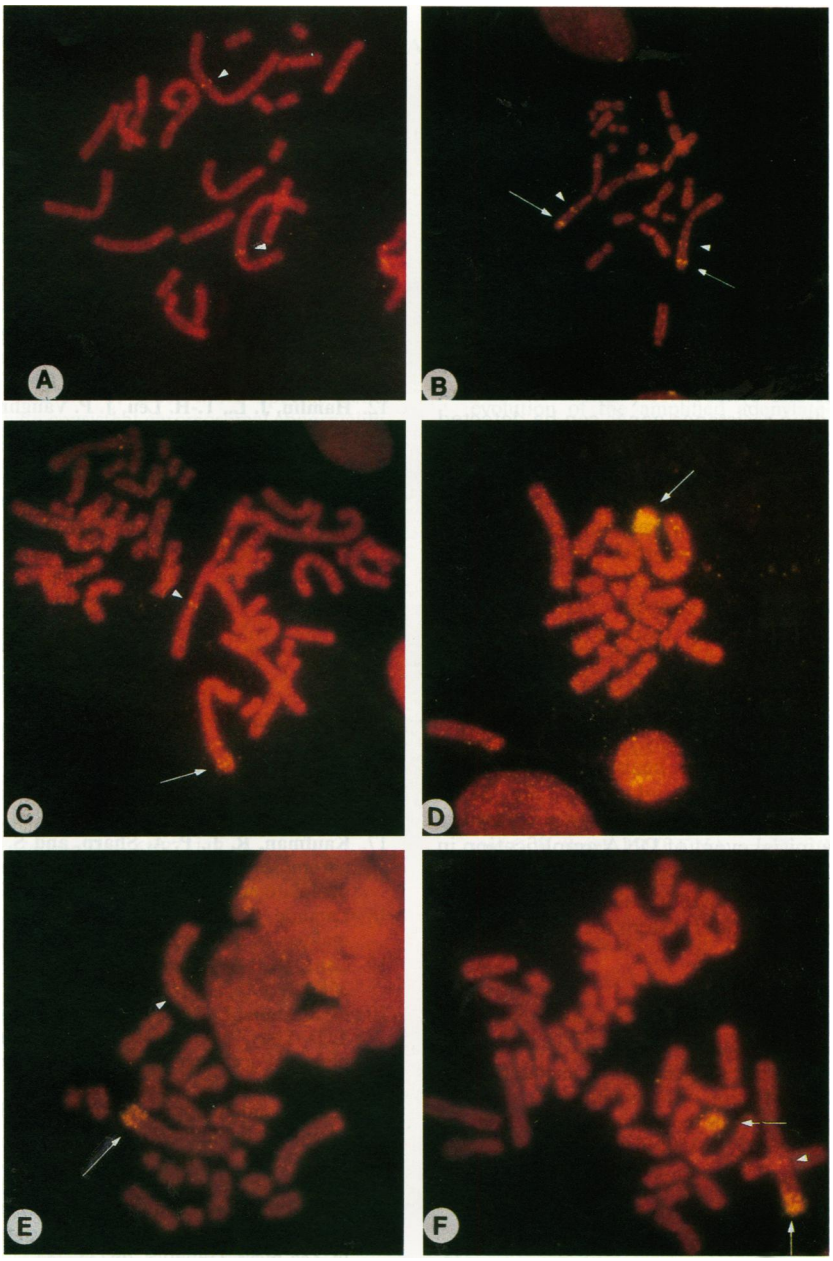

FIG. 7. FISH localization of the *P-gp* gene in VB-resistant CHO cells derived from one-step selection. (A) Drug-sensitive CHO cell (control); (B through F) VB-resistant CHO cells. Arrows point to the amplified loci, and arrowheads point to the single-copy loci. Note that regions highly variable in terms of amplified DNA can be seen in these cells. Also note that the cytogenetic localizations of amplified DNA are distal to the single-copy loci, an observation that is in general agreement with those from the MDR CHO cells shown in Fig. 2.

selection scheme involving progressively increasing drug concentrations, since one can assume that most fragile sites should have responded to the initial drug challenge. Indeed, in the three stepwise selection protocols that yielded VCR15, VBR1.5-1, and ADR10-1 MDR CHO lines used in this study, we previously reported that the levels of *P-gp* amplification did not correlate with the levels of drug resistance. Amplification of the *P-gp* gene, as measured by Southern blot hybridization, occurred only in the early steps of the selection (35, 42). FISH results also showed similarities in cytogenetic characteristics of the amplified DNA between MDR CHO cells developed by one-step selection (Table 1; Fig. 7) and those developed by multistep selection (Fig. 2). These observations, collectively, lend credence to the critical role of fragile sites in the regulation of DNA amplification in hamster cells.

Support for the hypothesis that fragile-site breakage may play an important role in mediating DNA amplification in mammalian cells can be found in observations of tumor cells. Many amplified proto-oncogenes in tumor cells are located near regions known to contain fragile sites. For example, *INT-2*, *HIS*, and *BCL-1* located on 11q13 are amplified in about 10 to 20% of human breast cancers and other malignan-

cies (10, 18, 49). *C-ERB/HER2* on 17q12 is amplified in about 25% of breast cancers (16, 49). All these loci contain fragile sites. Furthermore, using a comparative genomic FISH technique, Kallioniemi et al. (15) have recently identified 16 different chromosome regions that are overrepresented (amplified?) in tumor cell lines and primary tumor biopsies. We found that nine of these amplified regions are consistent with the known chromosome fragile sites (see reference 41 for a list of fragile sites).

The results of our study also support the emerging evidence that genetic instability is a hallmark of multistep carcinogenesis and a prerequisite of DNA amplification (31, 43, 52). Chromosome breaks at some fragile sites are associated with particular human malignancies (10, 24), as well as genetic diseases known as fragile-site syndromes (11). In two recent studies, amplification of drug resistance genes can be detected only in cells that lack wild-type p53 (20, 53), which has been considered guardian of the genome (19). A recent study also demonstrated that expression of oncogene (EIA) and antioncogene (p53) in fibroblasts derived from primary cultures can modulate cellular sensitivities to the cytotoxic effects of ionizing radiation as well as several chemotherapeutic agents (5-fluorouracil, etoposide, and adriamycin) (21), probably through a common cellular pathway leading to cell death. Those findings, together with the ones described in this study, raise the possibility that chromosome fragile sites may be at the receiving end of cellular-damage signals. Damage in these fragile sites, if not properly repaired, may lead to cell death.

Thus, understanding the molecular basis of chromosome fragility should provide important insights into the signal transduction pathway involving cellular responses to cytotoxic agents, as well as into the initial event of DNA amplification in mammalian cells. The fragile-site description given in this study, although it remains at the cytological level with megabase resolution, may serve as a model system for future studies addressing these important issues.

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