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Inefficient Growth Arrest in Response to dNTP Starvation Stimulates Gene Amplification through Bridge-Breakage-Fusion Cycles

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Cells often acquire resistance to the antiproliferative agents methotrexate (MTX) or *N*-phosphonacetyl-L-aspartate (PALA) through amplification of genes encoding the target enzymes dihydrofolate reductase or carbamylphosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD), respectively. We showed previously that Syrian hamster BHK cells resistant to selective concentrations of PALA ($\sim 3 \times ID_{50}$) arise at a rate of $\sim 10^{-4}$ per cell per generation and contain amplifications of the CAD gene as ladder-like structures on one of the two B9 chromosomes, where CAD is normally located. We now find that BHK cells resistant to high concentrations of PALA ($\sim 15 \times ID_{50}$) appear only after prior exposure to selective concentrations of PALA for ~72 h. Furthermore, in contrast to untreated cells, BHK cells pretreated with selective concentrations of MTX give colonies in high concentrations of PALA, and cells pretreated with selective concentrations of PALA give colonies in high concentrations of MTX or 5-fluorouracil. As judged by measuring numbers of cells and metaphase cell pairs, BHK cells do not arrest completely when starved for pyrimidine nucleotides by treatment with selective concentrations of PALA for up to 72 h. We propose that DNA damage, caused when cells fail to stop DNA synthesis promptly under conditions of dNTP starvation, stimulates amplification throughout the genome by mechanisms--such as bridge-breakage-fusion cycles--that are triggered by broken DNA. Amplified CAD genes were analyzed by fluorescence *in situ* hybridization both in cells where amplification was induced by PALA pretreatment and in cells in which the amplification occurred spontaneously, before selection with PALA. The ladder-like structures that result from bridge-breakage-fusion cycles were observed in both cases.

INTRODUCTION

Gene amplification is an important consequence of genomic instability and is likely to be involved both in the generation of tumor cells and in their evolution toward increasingly malignant phenotypes. Cytogenetic and molecular evidence of amplification has been found frequently in tumor cells, and the frequently observed amplification of oncogenes in tumors has led to the postulate that high levels of the encoded proteins contribute to malignancy. In addition, tumor cells can develop resistance to antiproliferative drugs through gene amplification (for reviews, see Stark and Wahl, 1984; Schimke, 1988; Stark *et al.*, 1989; Windle and Wahl, 1992; Stark, 1993).

Although other structures are sometimes seen, newly amplified DNA in drug-resistant rodent cells usually appears as ladder-like repeats containing the

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selected gene, usually located on the same chromosome arm that normally carries a single copy of the gene (Trask and Hamlin, 1989; Smith *et al.*, 1990; Toledo *et al.*, 1992, 1993). Analyses of very early events in *N*-phosphonacetyl-L-aspartate (PALA)[1]-resistant BHK cells and in coformycin-resistant Chinese hamster cells has revealed a high proportion of dicentric chromosomes containing amplifications of the target gene (Smith *et al.*, 1992; Toledo *et al.*, 1992). These findings have been explained in terms of amplification mechanisms that involve bridge-breakage-fusion (BBF) cycles. BBF cycles can start either with a double-strand break in DNA (breakage first) or with a telomere-region fusion (fusion first), leading to dicentric sister chromatids in either case. Although both mechanisms are possible, the evidence for either one is circumstantial at present (reviewed by Stark, 1993). It is clear from fluctuation analyses (Kempe *et al.*, 1976) and other data (Johnston *et al.*, 1983) that amplification events do occur spontaneously at high rates before selection is initiated. Since unperturbed cells are not likely to incur DNA damage often enough to account for the high rates of spontaneous amplification, another explanation has been proposed, namely, telomere-region fusion of sister chromatids (Stark, 1993). On the other hand, previous studies, carried out largely in the laboratory of R.T. Schimke over a period of more than 10 years, have made it clear that treating cells with agents that break DNA can strongly increase the frequency or rate of amplification (for specific references, see reviews by Stark and Wahl, 1984, and Schimke, 1988). However, there has been little analysis of the nature of amplified DNA induced by DNA breakage that would bear on the mechanisms responsible.

The selective agents PALA, methotrexate (MTX), and 5-fluorouracil (5-FU) starve cells for DNA precursors. Treatment of normal cells, which are not permissive for gene amplification (Tlsty, 1990; Wright *et al.*, 1990), with PALA leads to long-term, reversible, p53-dependent growth arrest (Livingstone *et al.*, 1992; Yin *et al.*, 1992), a state similar to that obtained when p53 is overexpressed without starvation or DNA damage (Agarwal *et al.*, 1995). In contrast, treatment of permissive cell lines leads not only to the rapid death of most of the cells, but also to the emergence of rare drug-resistant clones carrying amplified DNA. We now find that pre-exposure to a selective concentration of PALA or MTX leads to resistance to concentrations of the selective drug so high that no colonies are formed without pretreatment. Hence, the amplified DNA in such cells represents the consequences of induced events only. BHK cells were also selected with a protocol that allows identification of colonies arising from spontaneous, rather than induced, events. Analysis of both types of colonies by in situ hybridization reveals similar ladder-like arrays that are likely to result from BBF cycles. Despite this superficial similarity, we propose that BBF cycles are initated differently, by DNA breakage in the induced events and by sister chromatid fusion in the spontaneous events.

[1] Abbreviations used: 5-FU, 5-fluorouracil; BBF, bridge-breakage-fusion; CAD, carbamylphosphate synthetase/aspartate transcarbamylase/dihydroorotase; MTX, methotrexate; PALA, *N*-phosphonacetyl-L-aspartate.

MATERIALS AND METHODS

Cells and Drug Selections

The BHK cells used have been described by Zieg *et al.* (1983). Note that these cells are more sensitive to PALA than the BHK cells used by Kempe *et al.* (1976), who observed an appreciable rate of resistance to 100 μM PALA, in contrast to the results reported here. The HT29 cell line, originally isolated by J. Fogh (Fogh and Trempe, 1975), was obtained from the American Type Culture Collection (Rockville, MD). All cells were grown in DMEM plus 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in an atmosphere of 10% CO_2 at 37°C. For selections, the fetal calf serum was dialyzed against 40 vol of phosphate-buffered saline for a total of 24 h, with two changes of dialysis solution. PALA was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Methotrexate and 5-fluorouracil were obtained from Sigma (St. Louis, MO). To isolate new and independent resistant clones, individual populations (designated as families) of 10^3 cells were grown without selection to 10^6 cells. Multiple samples of 10^5 BHK or 5×10^5 HT29 cells were then seeded in 10-cm dishes for selection. PALA-resistant colonies of 100–500 cells appeared after 18–21 (BHK) or 28–35 days (HT29). The colonies were fixed with 10% formaldehyde and stained with Giemsa. The frequencies reported were not corrected for plating efficiency or for the effect of drug pretreatment on plating efficiency.

Analysis of DHFR Copy Number

Southern analysis was done as described by Sambrook *et al.* (1989). The genomic DNA was cut with *Eco*RI and the DHFR probe was a 0.7-kb insert excised from pSV2dhfr (Sambrook, 1989), which contains mouse cDNA. A human β-actin cDNA probe (from R. Ransohoff, Cleveland Clinic) was used for normalization. The Southern transfers were hybridized at 62°C in 5× SSC buffer for 16 h and washed at 60°C in 1× SSC buffer, three times for 20 min each. The radioactivity in each DHFR or β-actin band was determined by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For each cell line tested, the DHFR/β-actin ratio was determined and the degree of DHFR amplification was calculated by dividing these ratios by the ratio obtained with unselected BHK cells.

Fluorescence In Situ Hybridization

The experiments were performed as described by Smith *et al.* (1990). Briefly, mitotic metaphase spreads were treated with RNAse A in 2× SSC at 37°C for 1 h, dehydrated through an alcohol series, and dried. The chromosomes were denatured in 70% formamide, 2× SSC, pH 7.0, at 76°C for 7.5 min, and dehydrated. An equal mixture of two cosmids (C64 and C81; Guilotto *et al.*, 1986) was used as the probe. The DNA was labeled with biotin-11-dUTP by nick-translation, using the BioNick Labeling System (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. The hybridization mixture contained 5 ng/ml probe, 1 mg/ml sonicated Syrian hamster genomic DNA, 50% formamide, 2× SSC, pH 7.0, 1% Tween-20, and 10% dextran sulfate. After hybridization at 37°C for

16 h, the slides were washed at 42°C three times in 50% formamide, 2× SSC for 5 min each. For immunochemical detection, the slides were first pre-blocked with 5% Carnation dry milk in 4× SSC and 0.05% Tween-20. The biotinylated probe was detected by incubating the slides successively in fluorescein isothiocyanate-avidin (5 mg/ml; Vector Laboratories, Burlingame, CA), anti-avidin (5 mg/ml; Vector Laboratories), and a second layer of fluorescein isothiocyanate-avidin for 20 min each. After each staining step, the slides were washed three times with 4× SSC and 0.05% Tween-20 for 5 min each. The chromosomes were counterstained with 0.1 mg/ml propidium iodide and mounted in Citifluor AF1, an antifade solution (Citifluor, London, UK). Preparations were viewed with a Nikon Microphot FX microscope coupled to an MR-500 laser-scanning confocal imaging system (Bio-Rad Microsciences, Richmond, CA). All photographs were taken directly from the video screen using Kodak Ektar 125 color-print film (Rochester, NY).

RESULTS

Pre-Exposure to a Selective Concentration of PALA Allows Colonies to Form in a High Concentration of PALA

Families of BHK cells were analyzed. Each was derived from an independent 10^3-cell aliquot, which usually does not include a cell with a pre-existing amplification (frequency $\sim 10^{-5}$—see below). After growth from 10^3 to 10^6 cells, multiple 10^5-cell aliquots from each family were replated and exposed to selection. The frequency of resistant cells depends on the PALA concentration, with no colonies obtained at 70 μM or higher (Table 1). Colonies resistant to concentrations of PALA higher than 70 μM, however, can be obtained after exposure to lower selective concentrations for a few days (representative data in Table 2). In most experiments in which transfer from 20 or 30 μM to 100 or 90 μM PALA was employed, resistant colonies were observed. The frequency calculated from 12 such experiments, using 20 and 100 μM PALA, was 5×10^{-5}. Colonies resistant to 270 μM PALA were observed after pre-exposure to 30 μM PALA in one of four experiments.

Table 2. Dependence of the frequency of PALA resistance in BHK cells on the selection protocol

Family	PALA concentration (μM) Day 1	PALA concentration (μM) Day 6	Frequency of resistant cells × 10^5
1	20	20	30
	100	100	0
	0	100	0[a]
	20	100	5
2	30	30	15
	90	90	0
	30	90	6
	30	270	4

Portions of 10^3 BHK cells were expanded to 10^6 cells and multiple 10^5-cell portions were replated and exposed to 20 or 30 μM PALA on day 1. At day 6, the PALA concentration was changed (or not) by replacing the medium, without trypsinizing the cells. After 21 days, the colonies were fixed, stained, and counted.

[a] After 5 days of growth without selection (but with expansion to more plates) 4×10^7 cells were present (the doubling time is about 20 h). All of these cells were dispersed and selected in 100 μM PALA. No colonies were observed.

The dependence of induction of resistance to 90 μM PALA on the time of pre-exposure and the initial PALA concentration was investigated. A small effect was seen after 2 days of pre-exposure to 30 μM PALA, but the frequency of resistant colonies was much higher after 3 days of pre-exposure (Table 3). The dependence on PALA concentration during pre-exposure (Table 4) reveals that selective concentrations are required to see the effect (compare with Table 1). Only a fraction of the colonies selected in 20 μM PALA for 5 days gave rise to colonies that survived subsequent exposure to 100 μM PALA (Table 5). Similarly, using pools of cells exposed to 20 μM PALA for 10 days, the plating efficiency in 20 μM PALA was 10-fold higher

Table 1. Frequency of resistant BHK colonies as a function of PALA concentration

PALA concentration (μM)	Frequency of resistant cells × 10^5
15	19
20	7
25	3.8
30	4.7
45	2.5
70	0
90	0
120	0

Six families of 10^5 cells each were selected at 20, 25, 30, 45, and 70 μM PALA; seven families were selected at 120 μM, and 10 at 90 μM.

Table 3. Dependence of the frequency of resistance to 90 μM PALA on the time of pre-exposure to 30 μM PALA

Hours of pre-exposure to 30 μM PALA	Frequency of cells resistant to 90 μM PALA × 10^5
0	0
7	0
24	0
48	0.3
72	2.0
96	1.5
144	3.0

The protocol is given in Table 2. The frequency in 30 μM PALA alone was 3×10^{-5} in this experiment. Each frequency represents the mean of four to six plates.

Table 4. Effect of PALA concentration during pre-exposure

Concentration of PALA during a 5-day pre-exposure (μM)	Doubling time[a] (h)	Frequency of resistance for cells maintained in the initial concentration of PALA × 10^5	Frequency of cells resistant to 90 μM PALA × 10^5
2	32	0	0
5	38	0	0
10	62	12	4.3
20	[b]	26	5.0
30	[b]	6.5	2.0
40	[b]	2.5	6.0
90	[b]	0	0

3×10^5 cells were tested for each condition.

[a] The doubling time for untreated cells was 20 h.

[b] The number of cells did not double during the course of the experiment.

than in 100 μM PALA. Therefore, not all of the early events that allow growth in 20 μM PALA permit resistance to 100 μM PALA to be acquired quickly. As noted previously (Smith *et al.*, 1990), many of the PALA-resistant cells in a colony eventually do acquire many more copies of CAD than are needed to grow in a low selective concentration of this drug.

Effect of PALA on Cell Growth and Division

BHK cells continued to proliferate and divide in selective concentrations of PALA. Individual cells within small circumscribed areas were observed during the first few days of exposure to 25 μM or 100 μM PALA (Table 6). In both cases, rounded pairs of metaphase cells were observed after 24 h. After 48 or 72 h, many more such pairs of cells were observed in 25 μM than in 100 μM PALA. The total cell number increased during the first 48 h of exposure to 25 μM PALA and then began to decline. No increase was observed during exposure to 100 μM PALA. These observations reveal that BHK cells do not arrest immediately when exposed to a selective concentration of PALA. Instead, they continue to divide slowly and to increase in number for up to 3 days. During this time, DNA is being synthesized, as judged by incorporation of BrUdR. When DNA is made under such poor conditions, damage can occur, triggering amplification events, which can be propagated in a small fraction of the cells (see DISCUSSION).

Table 5. Fraction of colonies at 20 μM PALA that are resistant to 100 μM PALA

	Colonies per dish		Ratio (20 → 100)/20
Family 1	20 = 16 20 → 100 = 17		1.0
Family 2	20 = 26 20 → 100 = 17		0.65
Family 3	20 = 28 20 → 100 = 17		0.61
Family 4	20 = 38 20 → 100 = 10		0.26
Family 5	20 = 17 20 → 100 = 4.5		0.26
Family 6	20 = 34 20 → 100 = 12		0.35
		Average:	0.52

Five dishes were assayed for each family. The protocol was the same as in Table 2. Pretreatment with 20 μM PALA was for 5 days. Note that the ratio will depend on the length of incubation time in 20 μM PALA, since bigger colonies are more likely to contain cells resistant to 100 μM PALA.

In Situ Analysis of Structures Formed in Induced Events

As shown previously (Smith *et al.*, 1990), amplified CAD genes in PALA-resistant BHK cells are found in ladder-like structures in which the individual genes are separated from each other by many megabases of DNA. The ladders are located in expanded regions of the short arm of chromosome B9, which carries a single copy of CAD in wild-type Syrian hamster cells. We analyzed 10 independent colonies selected with 20 μM, and then 100 μM PALA. Eight had ladder-like structures, very similar to the structures seen in single-concentration selections (Figure 1A); two had a much more condensed structure, on the same chromosome arm as the unamplified CAD genes (Figure 1B). Therefore, the two-concentration protocol can sometimes

Table 6. Growth suppression of BHK cells in low and high selective concentrations of PALA

Hours in PALA	Total cells in 25 μM PALA	Percentage of metaphases in 25 μM PALA	Total cells in 100 μM PALA	Percentage of metaphases in 100 μM PALA
2	737	3.5	798	2.3
24	992	8.0	797	6.4
48	926	6.7	649	2.9
72	697	3.3	440	0

10^5-cell populations from four independent families (see above) were plated into 10-cm dishes and exposed to 25 or 100 μM PALA. Each dish was marked with 25 small circles so that the cells within each circle could be observed separately. The total number of flattened cells and the number of rounded metaphase doublets were scored as a function of time for each dish. Frequencies of amplification, determined after staining colonies 28 days later, were 4.3×10^{-5} for 25 μM and 0 for 100 μM.

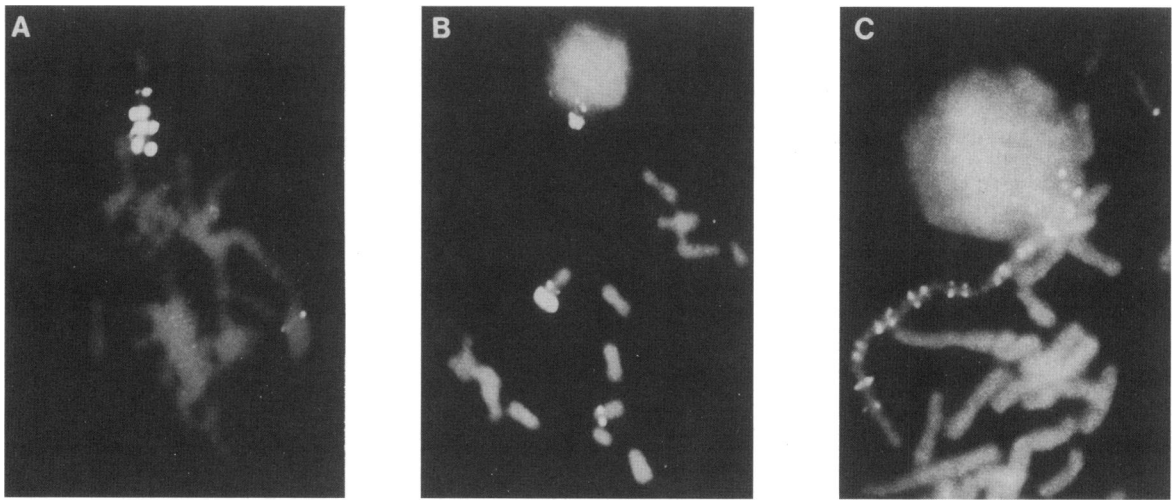


Figure 1. FISH analysis of amplified CAD genes in PALA-resistant BHK cells. (A) A ladder-like structure formed in an induced event. Cells selected in 20 μM PALA for 5 days were transferred to 100 μM PALA. The structure shown was characteristic of 8 of 10 independent colonies that were examined. The normal copies of CAD can be seen at the top of the ladder and in a normal chromosome at bottom right. (B) A condensed structure formed in an induced event. The structure shown was characteristic of 2 of 10 independent colonies from the experiment described in part A. The normal copies of CAD can be seen in the marker chromosome and in a normal chromosome at the bottom. (C) A ladder-like structure formed in a spontaneous event. Cells were selected in 20 μM PALA and plates containing many colonies, representing spontaneous events that occurred early during a period of unselected growth, were identified as described in RESULTS. Ladder-like structures were observed in ~90% of the metaphase cells observed in 10 independent cultures—see the text for details. The example shown is unusually long. The apparent grouping into pairs of doublets was often seen and may be related to the mechanism of amplification shown in Figure 2B.

give rise to structures not seen with single-drug selections. The nature of the condensed structure has not been analyzed further. When colonies from the same family, selected either in 20 μM PALA alone or in 20 μM and then 100 μM PALA, were compared by in situ hybridization, the copy numbers of CAD were similar, consistent with our previous observations that typical amplification sequences eventually give copy numbers and resistance levels far in excess of the minimum levels needed to survive in the concentration of PALA actually used for selection (Smith *et al.*, 1990).

In Situ Analysis of Structures Formed in Spontaneous Events

Previous data have made it clear that spontaneous amplifications of CAD account for a significant fraction of PALA-resistant BHK cells. If there were no spontaneous events, fluctuation analysis would reveal no amplification during the initial period of unselected growth, in contrast to the results observed (Kempe *et al.*, 1976). On the other hand, since exposure of cells to a selective concentration of PALA does induce resistance to a high concentration of PALA (Table 2), it stands to reason that induction of CAD gene amplification must also contribute to resistance when BHK cells are exposed to a single selective concentration of PALA only. Therefore, the colonies obtained in such a straightforward selection arise from both spontaneous and induced events. The data of Figure 1, A and B, represent structures formed in induced events only, since no colonies are formed in a high concentration of PALA without pre-exposure to a selective concentration.

To examine the structures formed in spontaneous amplification events, we used a protocol designed to identify the rare cultures in which spontaneous events predominate, because the first event happened by chance very early during the period of unselected growth. These cultures can be identified readily since the number of resistant colonies they contain is very high. For example, if 100 cells are grown to 10^5 cells before selection, a single spontaneous amplification event at the 1000-cell stage will give rise to 100 resistant colonies, whereas a spontaneous event in the last cell division will give rise to only one colony. A complication is that a culture with many colonies will also result if a pre-existing resistant cell is included in the initial 100-cell aliquot. This undesired outcome can be minimized by making the initial aliquot very small, so that the probability of including a pre-existing mutant is correspondingly low. We can estimate the frequency of pre-existing mutant cells to be $\sim 10^{-5}$ from the following data: 100 plates of 10^5 cells each were prepared from 10^3-cell aliquots and selected in 20 μM PALA; only one plate had ~100 colonies. A

similar experiment with 40 μM PALA also gave only one plate with ~100 colonies.

We used one hundred and twenty 100-cell aliquots, each grown to 10^5 cells, to obtain colonies that arose from spontaneous events. Pre-existing PALA-resistant cells would be expected in about one out of one thousand 100-cell aliquots, and such a rare culture would give rise to 1000 colonies after growth from 100 to 10^5 cells. No such cultures were observed. Eighteen of the 120 cultures had 50–100 colonies each after selection with 20 μM PALA (estimated without fixing and staining) and were judged to result from spontaneous events at the ~1000-cell stage. Ten such independent cultures were examined by in situ hybridization of the pooled cells from each with a CAD probe. In every case, the great majority of the metaphase spreads observed revealed the amplified CAD genes to be organized as ladder-like arrays (Figure 1C). Two hundred of the metaphases observed had such structures, an additional 18 metaphases revealed less well defined extended amplified structures, and only five metaphases showed no amplified CAD genes, possibly due to loss of the marker chromosomes from these spreads. We conclude that spontaneous amplification events generate ladder-like structures and that these are likely to result from BBF cycles.

Cross-stimulation of Amplification

To test the generality of stimulated amplification, BHK cells were challenged with high concentrations of one drug after pre-exposure to selective concentrations of a different drug (Table 7). Pre-exposure to 20 μM PALA for 5 days, followed by selection in 200 nM MTX, gave 34 times more colonies than selection in 200 nM MTX alone, and pre-exposure to 100 nM MTX gave a significant frequency of resistance to 200 μM PALA, a concentration that gave no colonies when used alone. Furthermore, pre-exposure to 20 μM PALA gave an appreciable frequency of resistance to 2 or 4 μM 5-FU, in contrast to the complete absence of colonies when these concentrations of 5-FU were used alone.

Resistance to 5-FUdR is due to high levels of the target enzyme thymidylate synthetase (Rossana *et al.*, 1982), suggesting that amplification of this gene may also account for 5-FU resistance. Because resistance to MTX can arise through several different mechanisms (reviewed by Stark and Wahl, 1984), we assayed for amplification of the DHFR gene. Since there are DHFR pseudogenes, we analyzed genomic DNA by using a quantitative Southern analysis, with an internal standard (see MATERIALS AND METHODS), rather than a whole DNA blotting method. Compared with unselected BHK cells, four independent MTX-resistant colonies gave relative increases in DHFR gene copy number of 2.4-, 2.0-, 1.9-, and 1.2-fold, respectively, and two independent pools of MTX-resistant cells gave relative increases of 1.9- and 3.1-fold. These data indicate that pre-exposure to 20 μM PALA usually stimulates DHFR gene amplification rather than other mechanisms of resistance to MTX.

Table 7. Cross-stimulation of amplification in BHK cells

Pre-treated for 5 days with:	Selected with:	Frequency $\times 10^5$
PALA 20 μM	PALA 20 μM	33
200	200	0
20	200	0.9
20	MTX 100 nM	22
20	200	6.8
MTX 100 nM	100	11.2
200	200	0.2
100	300	2.3
100	PALA 200 μM	0.7
PALA 20 μM	20	16
100	100	0
20	100	4
20	5 FU 1 μM	13
20	2	12
20	4	5.2
20	8	0
5 FU 1 μM	1	30
2	2	0
4	4	0
8	8	0

The frequencies shown are the means from analysis of eight families.

Induced CAD Gene Amplification in Human Cells

To test the effect of pre-exposure to PALA in human cells, we used HT29, a colon adenocarcinoma line. Exposure to 45 μM PALA for 6 or 12 days gave an appreciable frequency of colonies resistant to 450 μM PALA, which were not observed without pre-exposure (Table 8). Although amplification is the predominant mechanism of PALA resistance in HT29 cells

Table 8. Selection of HT29 cells in high PALA after pre-exposure to low PALA

Days of pre-exposure to 45 μM PALA	Frequency of cells resistant to 450 μM PALA $\times 10^5$
0	0
3	0
6	2.2
12	6.2

Ten families of 10^5 cells each were used for each condition. The frequency in 45 μM PALA alone was 6.8×10^{-5}. The cells grow with a doubling time of about 30 h.

and the sole mechanism in rodent cells, recent work (Sharma and Schimke, 1994; Smith and Stark, unpublished data) has made it clear that human cells can achieve resistance to PALA through several distinct mechanisms, some of which do not involve CAD gene amplification.

DISCUSSION

Gene Amplification Is Induced by Agents that Starve Cells for dNTPs

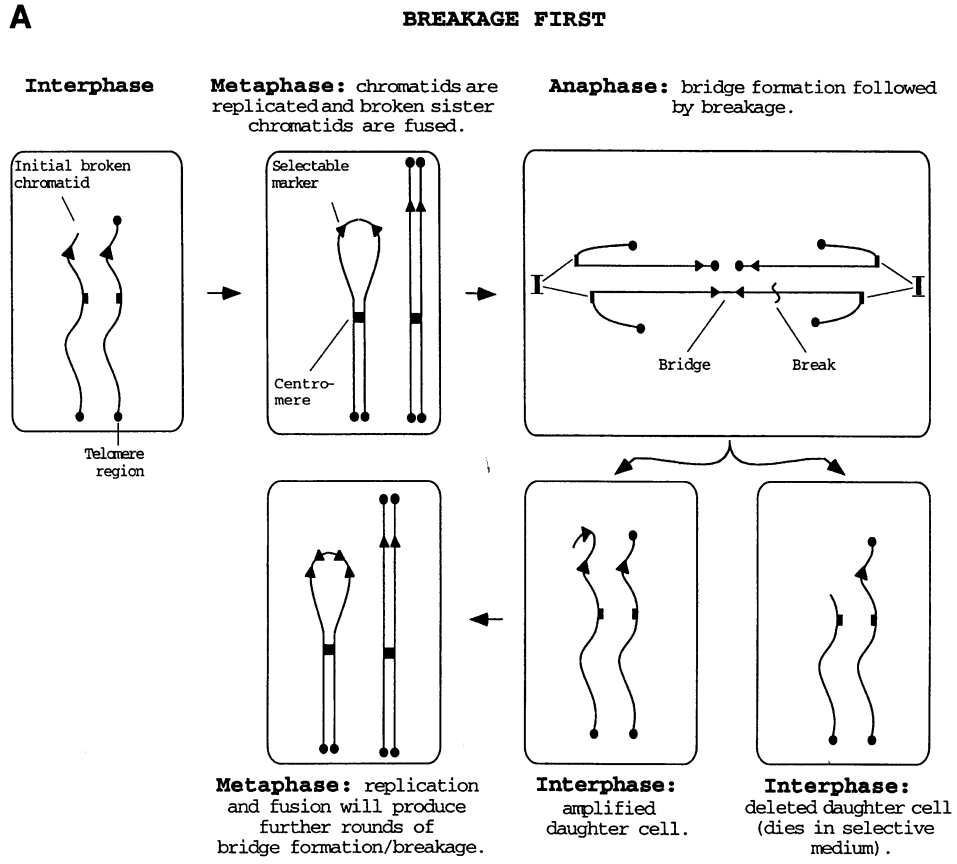
There is ample evidence that agents or treatments that damage DNA (for example, carcinogens, ultraviolet or ionizing radiation) or interfere with DNA synthesis (for example, hypoxia or hydroxyurea) increase the probability of gene amplification (reviewed by Stark and Wahl, 1984; Schimke, 1988). PALA, a specific inhibitor of aspartate transcarbamylase, blocks synthesis of pyrimidine nucleotides, thus inhibiting DNA synthesis by starving cells for dTTP and dCTP. MTX, a specific inhibitor of dihydrofolate reductase, interferes with the one-carbon transfers essential for the synthesis of purine nucleotides and thymidylate, thus inhibiting DNA synthesis by starving cells for dATP, dGTP, and dTTP. Why should exposure to these agents increase amplification frequencies? In 20 μM PALA (3 $\times$ ID_{50}, a selective concentration) BHK cells were not killed immediately. Rather, a significant number of metaphase pairs of rounded cells were still present after 2–3 days, and the total number of cells increased significantly (Table 6). The increase in total cells was not seen and the number of metaphases was much reduced in 100 μM PALA (15 $\times$ ID_{50}, Table 6). We interpret these results to mean that BHK cells manage to make at least some DNA when starved for pyrimidine triphosphates at 3 $\times$ ID_{50} PALA but make much less at 15 $\times$ ID_{50}. A net increase in the amount of DNA in these PALA-treated cells might be due to incomplete inhibition of aspartate transcarbamylase by PALA or to conversion of NTPs, obtained through degradation of rRNA, to dNTPs.

When pools of deoxynucleosidetriphosphates are highly perturbed (as in cells treated with PALA), misincorporation increases dramatically (Reichard, 1988). Excision repair of mismatched bases, in which single-stranded gaps are produced, will only make matters worse, since the gaps cannot be filled appropriately when dTTP and dCTP are limiting. Eventually, double-strand breaks can occur, and these will facilitate amplification. Nelson and Kastan (1994) have shown that treatment of cells with PALA does cause DNA strand breakage. This mechanism should be quite general, so that starvation either for pyrimidine deoxynucleosidetriphosphates (with PALA) or purine deoxynucleosidetriphosphates and dTTP (with MTX) would be expected to increase rates of amplification at many loci, a prediction consistent with the cross-stimulation data of Table 7. As proposed by Windle and Wahl (1992), DNA may also break at stalled replication bubbles when DNA synthesis has been blocked.

Induced and Spontaneous Amplification of the CAD Gene

In a previous study, we examined the amplified structures formed in BHK cells selected with 20 μM PALA and concluded that BBF cycles were a common mechanism (Smith *et al.*, 1990, 1992). From the work presented here, we now appreciate that exposure of cells to 20 μM PALA induces CAD gene amplification. However, previous analyses have also made it clear that at least some of the CAD gene amplification events in BHK cells arise spontaneously, during the period of unselected growth before PALA is added (Kempe *et al.*, 1976). Therefore, the straightforward procedure of selecting BHK cells in 20 μM PALA yields colonies that arise through both spontaneous and induced events. By manipulating the conditions or choosing an appropriate protocol, we have been able to observe the amplified structures formed in induced and spontaneous events separately. Since no spontaneous colonies are observed in selections with 100 μM PALA alone, the colonies that arise in 100 μM PALA after pretreatment with 20 μM PALA must represent induced events only. Induced amplifications appear as ladder-like structures most of the time, and it is likely that BBF cycles account for these structures. The mechanisms giving rise to the more condensed structures observed in 2 of 10 clones are not clear at present. Populations highly enriched for PALA-resistant cells that arose through spontaneous events can be also obtained, as described above. Analyses of these populations also reveals ladder-like structures, again suggesting a BBF mechanism.

Although both induced and spontaneous amplifications of the CAD gene in BHK cells result from BBF cycles, we expect the initiation of these cycles to be different in the two cases. The induced structures are likely to arise through a "breakage-first" mechanism (Figure 2A). However, there is no obvious reason why chromosome B9 of BHK cells should break with high frequency during normal growth in the absence of selection and therefore, as proposed earlier (Smith *et al.*, 1992; Stark, 1993), we favor a "fusion-first" mechanism in which the telomere-proximal regions of two B9 sister chromatids are joined in the initial step of spontaneous amplifications (Figure 2B). Although both the breakage-first and fusion-first mechanisms allow many BBF cycles to follow and give rise to superficially similar structures, as shown in Figure 2, the structures formed in the two events should be distinguishable. When breakage is first, sequences from the telomeric region of the involved chromosome



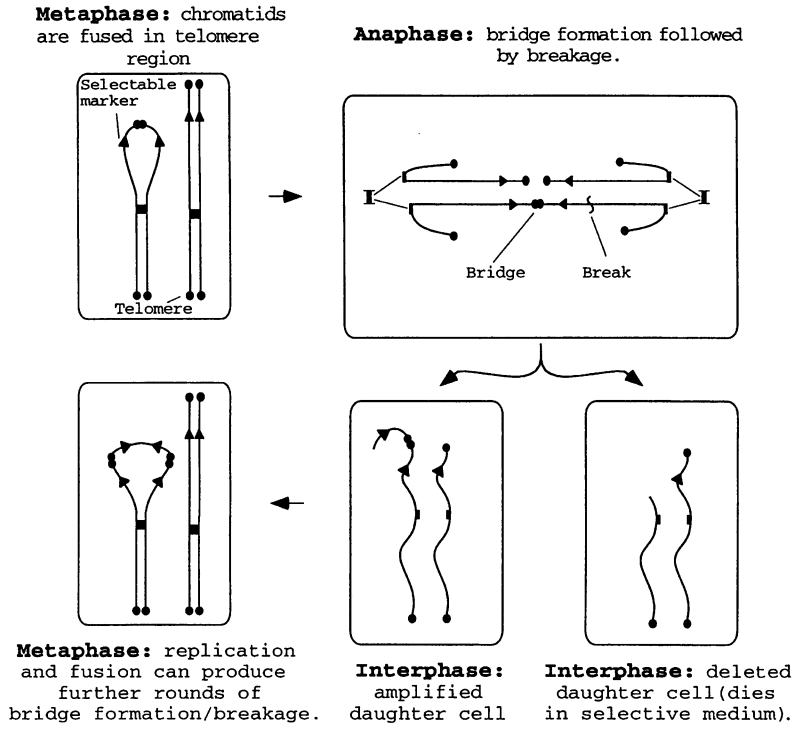


Figure 2. Bridge-breakage-fusion mechanisms for CAD gene amplification in BHK cells. The figure and legend have been adapted from Toledo *et al.* (1993). (A) The breakage-first (induced) mechanism. A chromatid BBF cycle has been initiated by a single break in one of the two chromatid homologues. After replication, the broken ends fuse at the break, generating a monocentric chromosome with fused sister chromatids. After centromere cleavage at anaphase, a dicentric chromatid is formed. The centromeres of this dicentric chromatid move to opposite poles of the mitotic spindle, creating a bridge that is later broken. The break gives rise to one daughter cell with a deleted chromatid, which will die in selective medium, and one daughter cell with an amplified chromatid lacking a telomere, which will undergo further BBF cycles. At metaphase, amplified diploid cells are expected to contain an intact unamplified homlogue and a monocentric amplified chromosome with fused sister chromatids and megabase-long inverted repeats. (B) The fusion-first (spontaneous) mechanism. A chromatid BBF cycle has been initiated by fusion of the telomeric regions of one chromatid pair. The remainder of the process is as described in part A, except that the fused telomeric regions persist in the amplified DNA, as shown in the diagram.

arm will rarely be coamplified with the selected CAD gene, whereas telomeric sequences will usually be coamplified when fusion is first. These predictions will be tested in future experiments. BBF cycles can also account for loss of genetic material (Figure 2). Thus a full understanding of amplification mechanisms may also contribute to our knowledge of at least one way in which heterozygosity can be lost.

Cells resistant to a high concentration of PALA (90–100 μM) are not present or are very rare in populations of BHK cells, suggesting that they form rarely in the absence of induction and perhaps also that they do not grow well in competition with normal cells. Even when induced with a selective concentration of PALA, many of the amplification events that allow cells to grow in the inducing concentration do not give enough copies of CAD quickly enough to allow growth in a high concentration of PALA (Table 5). This result indicates that several BBF cycles are probably needed to build up enough copies of CAD to allow the cell to survive in high PALA (see Figure 2).

The response to PALA of cell lines such as BHK, which are permissive for amplification, is quite different from the response of nonpermissive normal cells, which arrest rapidly when pyrimidine nucleotide pools are depleted, before damage to DNA can occur (reviewed by Chernova *et al.*, 1995). The normal response depends on an intact cell cycle arrest pathway that involves p53, and normal cells lose their ability to prevent amplification when p53 is lost or inactivated (Livingstone *et al.*, 1992; Yin *et al.*, 1992). When the nonpermissive cell line REF52 is forced to grow poorly under conditions of pyrimidine nucleotide starvation by treatment with a low concentration of PALA, the DNA damage that ensues leads to loss of the nonpermissive state, and CAD gene amplification duly follows (Chernova and Stark, unpublished data; discussed in Chernova *et al.*, 1995). Thus, treatment of cancer cells with low concentrations of inhibitors that slow but do not completely arrest DNA synthesis will facilitate DNA breakage even if the cancer cells are not permissive for amplification, and may facilitate the emergence not only of variant cells resistant to the particular drug employed but also of variant cells that have become permissive for DNA damage in general.

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