Hum Genet (1984) 67 : 136–142

Human Genetics

DNA polymerase α inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes

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Summary. Aphidicolin, a specific inhibitor of DNA polymerase α, is known to induce chromosomal aberrations. At concentrations that did not greatly affect mitotic index, aphidicolin induced a striking number of chromosome gaps and breaks distributed in a highly nonrandom manner in cultured human lymphocytes. Specific chromosome bands, especially 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22 were preferentially damaged in lymphocytes from each of 12 subjects studied. Total and site-specific damage was dose dependent and greatly increased when folic acid was removed from the medium. The sites most sensitive to aphidicolin damage include the "hot spots" seen under conditions of thymidylate stress and in studies of spontaneous chromosomal damage. The fragile X site, which can also be induced by thymidylate stress, was not induced by aphidicolin in lymphocytes, suggesting a separate mechanism for its induction. Aphidicolin represents a novel tool for detection of hot spots on human chromosomes through the mechanism of DNA polymerase α inhibition. The hot spots induced by aphidicolin represent a new class of fragile sites which we term common fragile sites.

Introduction

Seventeen heritable fragile sites on human chromosomes are presently known (Sutherland 1983). Fourteen of these, including that at Xq27 (fragile X), are induced by culturing cells under conditions of thymidylate stress created by folate deprivation (Sutherland 1979), inhibition of thymidylate synthetase (Glover 1981; Tommerup 1981), or inhibition of dihydrofolate reductase (Sutherland 1979; Mattei et al. 1981). In addition to allowing expression of these fragile sites, cell growth under thymidylate stress often produces lesions at "hot spots" which appear to occur nonrandomly in the genome (Sutherland 1983).

The fragile X is associated with one form of X-linked mental retardation. The significance of the 16 remaining fragile sites and the hot spots is not known, although associations with mental retardation (Sutherland 1982) and cancer chromosome breakpoints have been suggested (Yunis 1983; Hecht and Sutherland 1983).

Most fragile sites and hot spots share one common mechanism of induction, that is, thymidylate stress. How thymidylate stress causes these lesions is not clear. One possibility is that the rate or fidelity of DNA synthesis is preferentially affected at fragile sites and hot spots. As a test of this hypothesis, aphidicolin, a diterpenoid mycotoxin, was tested for its effects on chromosomes from normal and fragile X individuals. Aphidicolin is a specific inhibitor of eukaryotic DNA polymerase α which is primarily associated with chromosomal DNA replication (Ikegami et al. 1978). It does not inhibit polymerases β or γ which are associated with DNA repair and mitochondrial DNA synthesis, respectively (Ikegami et al. 1978; Hanoka et al. 1979).

Aphidicolin was found to induce chromosomal gaps and breaks in a highly nonrandom manner in cultured lymphocytes. A small number of specific sites in the human genome are preferentially damaged by low doses of aphidicolin. These sites include the hot spots seen with thymidylate stress but not the fragile X.

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Materials and methods

Subjects

Heparinized venous blood was obtained from eight males and four females. Subjects 7757 and 7758 were mentally retarded brothers known to express the fragile X when their cells are cultured under conditions of thymidylate stress. Subject 8148 was their mother in whom the fragile X has not been demonstrated despite repeated trials. Subjects 9263 and 9264 were also mentally retarded brothers known to express the fragile X, and 9265 was their fragile X negative father. Subjects 8213 and 8214 were a healthy couple with a history of recurrent spontaneous abortions. The remaining subjects were healthy adult volunteers. Apart from the two sets of fragile X brothers, all subjects had normal karyotypes.

Cell culture

Blood was cultured within 24 h of venipuncture by the conventional whole blood microculture technique. Culture medium (Irvine Scientific) was RPMI 1640 (FA^+) containing 10% fetal bovine serum (Hy-Clone, Sterile Systems Inc.) or RPMI 1640 without folic acid (FA^-) with 5% fetal bovine serum. All medium was additionally supplemented with 2 m*M* glutamine, phytohemagglutinin, and penicillin/streptomycin. Cells were cultured at 37°C for a total of 96 h before harvesting.

Chemicals

Aphidicolin was dissolved in dimethyl sulfoxide (DMSO) or in ethanol (EtOH) for one experiment (Table 2) and added

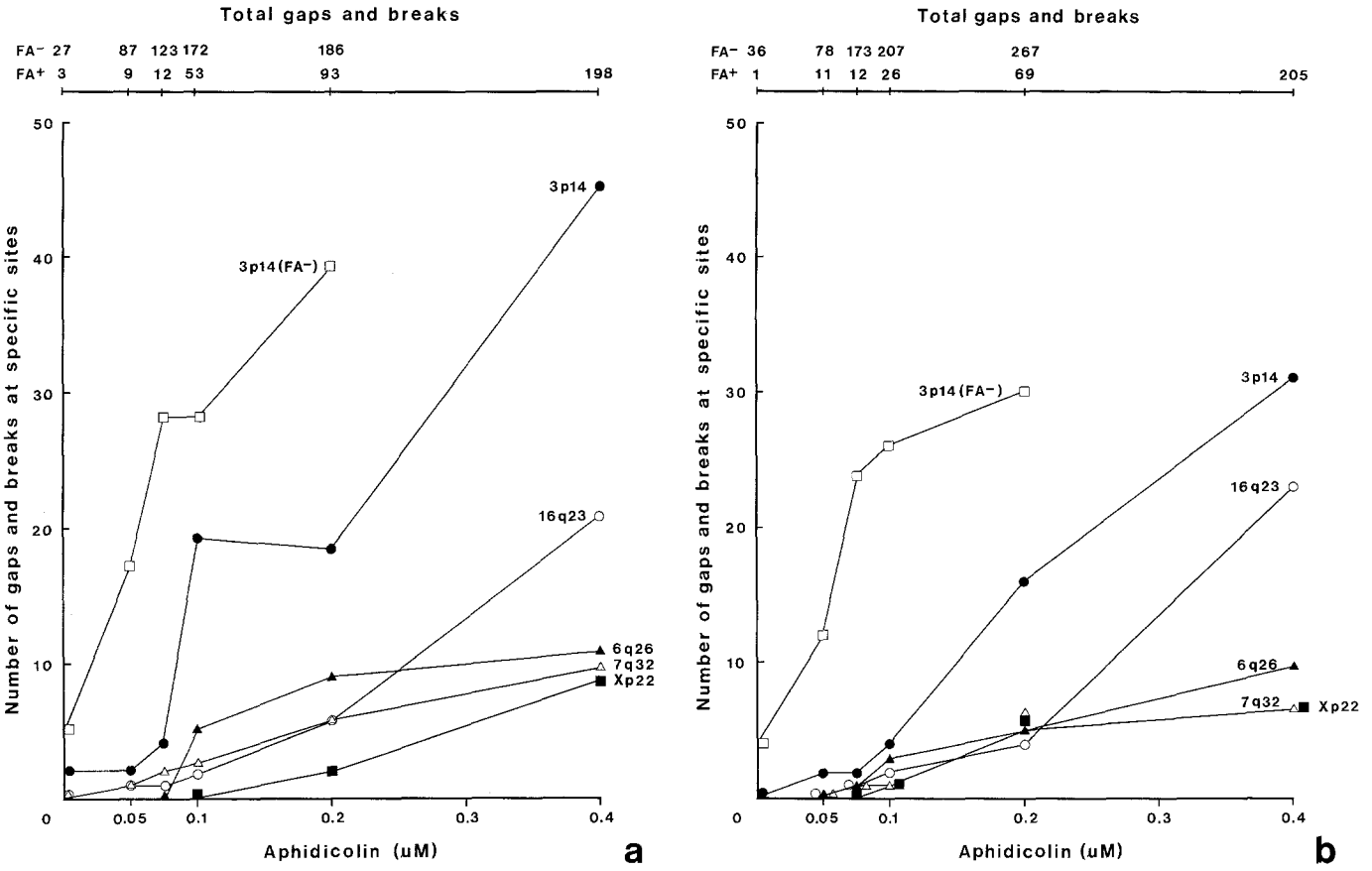

Fig. 1. Dose response for induction by aphidicolin of total chromosomal gaps and breaks *(top)*, and gaps and breaks at five sensitive sites *(bottom)*. Fifty metaphases were analyzed per aphidicolin concentration used. (FA^-) indicates that cells were cultured in folic acid-deficient medium. **a** Subject 8370; **b** Subject 8371

directly to the cultures 26 h prior to harvest. The final concentration of vehicle in the medium was never greater than 0.2%. Control cultures were established with and without 0.2% of DMSO or EtOH added. No increases in chromosome aberrations were seen in control cultures with DMSO or EtOH.

Scoring

All cell cultures were coded before slide preparation. Thus, cells were scored blindly for chromosome aberrations without knowledge of treatment. Air dried slides were Q-banded in [N,N*bis*-(6-chloro-2-methoxyacridin-9-yl)spermine] [$(CMA)_2S$] (Deugau et al. 1978) which has a very slow quenching rate and allows ample time for specific location of breakpoints. Scoring of aberrations was performed independently by two observers with each scoring 25 cells per culture when possible.

Results

Aphidicolin did not affect cell growth greatly or diminish the mitotic index significantly at the concentrations and times chosen in these experiments. Thus, DNA polymerase α was only partially inhibited and not totally blocked, since DNA replication did not cease. Aphidicolin concentrations above 0.2 μM in FA^- or 0.4 μM in FA^+ medium induced so many breaks and gaps that cells could not be accurately scored and sites of sensitivity at lower doses were overshadowed. The concentrations and duration of aphidicolin exposure utilized here were selected so as not to produce too many unscorable cells that could create a scoring bias.

Dose response for the induction of total gaps and breaks by aphidicolin in lymphocytes from two normal individuals is shown in Fig. 1 *(top)*. The induction of gaps and breaks was clearly dependent on the concentration of aphidicolin. The frequency of lesions was much greater with FA^- medium than with FA^+ medium and, since the difference is greater than with FA^- medium alone, the data suggest a possible synergistic action of the two variables.

The distribution of breakpoints in aphidicolin-treated cells was highly nonrandom (see below). Dose response for the induction of gaps and breaks at five of the sites most sensitive to damage by aphidicolin is also shown in Fig. 1. Band 3p14 is the most frequently damaged site. At 0.4 μM aphidicolin in FA^+ medium, 45 and 31 breaks were seen at 3p14 in 50 cells from Subjects 8370 and 8371, respectively. With this treatment four cells from Subject 8370 and two cells from Subject 8371 had a break or gap at 3p14 in both homologues. Thus, 82% and 58% of metaphases from the two subjects had at least one break at 3p14. Over the range 0.05 μM to 0.4 μM aphidicolin in complete FA^+ medium, 36%–58% of all breaks and gaps were seen at these five sites (Fig. 1).

As was the case with total gaps and breaks, the site-specific aberrations were greater in FA^- than FA^+ medium. The induction of gaps and breaks at 3p14 in FA^- medium is shown as an example in Fig. 1. The data suggest a synergistic effect between aphidicolin and folate deficiency for this site-specific aberration as well as for total gaps and breaks.

Table 1. Chromosomal lesions induced by aphidicolin and growth in folate-deficient medium in cells from nine subjects[a]

Medium	Aphidicolin (μM)[c]	Total gaps[b] and breaks in 50 cells	Gaps and breaks in band					
			3p14	16q23	7q32	Xp22	6q26	2q31
FA^+	0.0	0.7 ± 1.0	0.3 ± 0.7	0.0	0.0	0.0	0.0	0.0
FA^+	0.2	58.7 ± 20.1	11.8 ± 3.2	5.8 ± 1.8	4.1 ± 2.7	2.3 ± 1.9	4.6 ± 3.0	3.0 ± 1.6
FA^-	0.0	17.8 ± 11.8	3.1 ± 1.4	1.6 ± 1.0	1.2 ± 1.2	0.6 ± 0.9	1.4 ± 1.3	0.8 ± 1.6
FA^-	0.2	131.0 ± 63.4	22.6 ± 10.4	12.7 ± 4.0	9.8 ± 6.4	6.9 ± 5.0	4.9 ± 2.0	3.8 ± 1.5

[a] Data pooled from four separate experiments and expressed as mean ± standard deviation
[b] Does not include gaps and breaks at Xq27 in two fragile X subjects
[c] DMSO (0.2%) added 26 h prior to harvest in cultures without aphidicolin

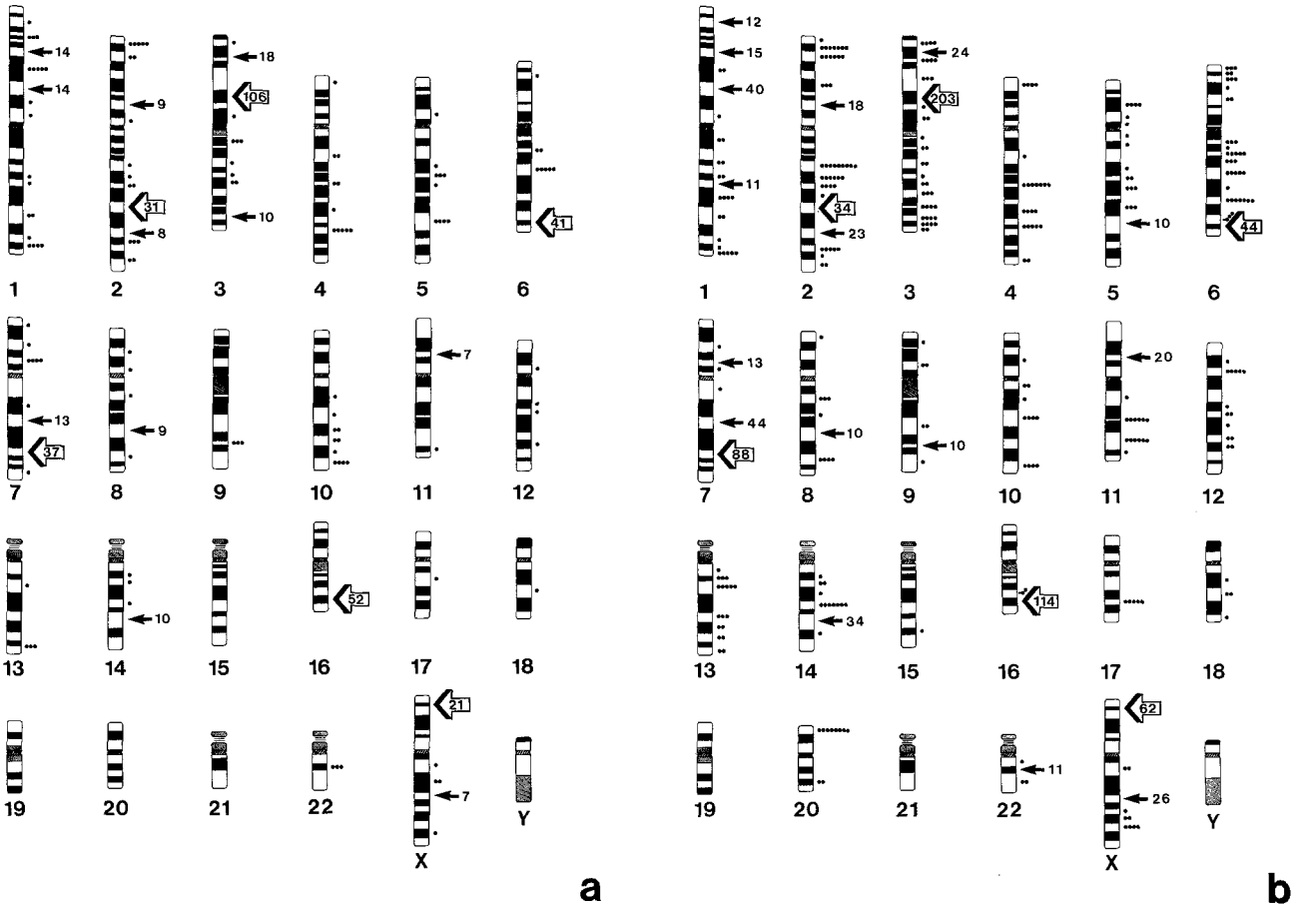

Fig. 2. Position of all chromosomal lesions induced by 0.2 μM aphidicolin in cultured lymphocytes (50 cells scored from each of nine subjects). **a** Cells cultured in complete (FA^+) medium; **b** cells cultured in folic acid-deficient (FA^-) medium. *Arrows* indicate bands with a significant excess of lesions (see text for details). *Large arrows* indicate sites with the greatest excess of lesions in both media

The effects of growth in FA^- medium and of 0.2 μM aphidicolin treatment in both FA^+ and FA^- medium on chromosome damage in cells from nine subjects are summarized in Table 1. All nine subjects showed the greatest number of lesions at 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22. Breaks or gaps at these sites were induced by aphidicolin in all nine subjects. Lesions at these sites were also often seen in untreated FA^- cultures, but not in FA^+ (complete medium) cultures (Table 1).

The total distribution of the 528 breaks and gaps induced by 0.2 μM aphidicolin in FA^+ medium is shown in Fig. 2a. A chi square analysis of this distribution with respect to chromosome regions was made. This analysis of distribution by region overcomes any potential scoring bias of assigning a break or gap to a specific band within a region. Expected values for lesions per region were based on the relative length measurements of 79 regions published by Koskull and Aula (1973). The distribution of breaks and gaps is highly nonrandom ($\chi^2_{78\ d.f.} = 2052$, $P \ll 0.001$).

A more critical analysis of the distribution of lesions can be made with regard to bands with the reservation that the expected value for lesions within each band is somewhat low as estimated from the available data. Based on a haploid karyotype of 400 bands (ISCN 1981) and assuming each band to have an equal probability of breakage, the expected number of breaks per band from the 528 lesions induced by 0.2 μM aphidicolin in FA^+ medium (Fig. 2a) is 1.3 lesions per band. A chi square analysis shows that any band with six or more lesions is nonrandomly damaged significantly in excess ($\chi^2_{1\ d.f.} \geq 13.6$, with Yates correction; $P < 0.001$). Thus, in addition to the obvious excess of lesions at 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22, an

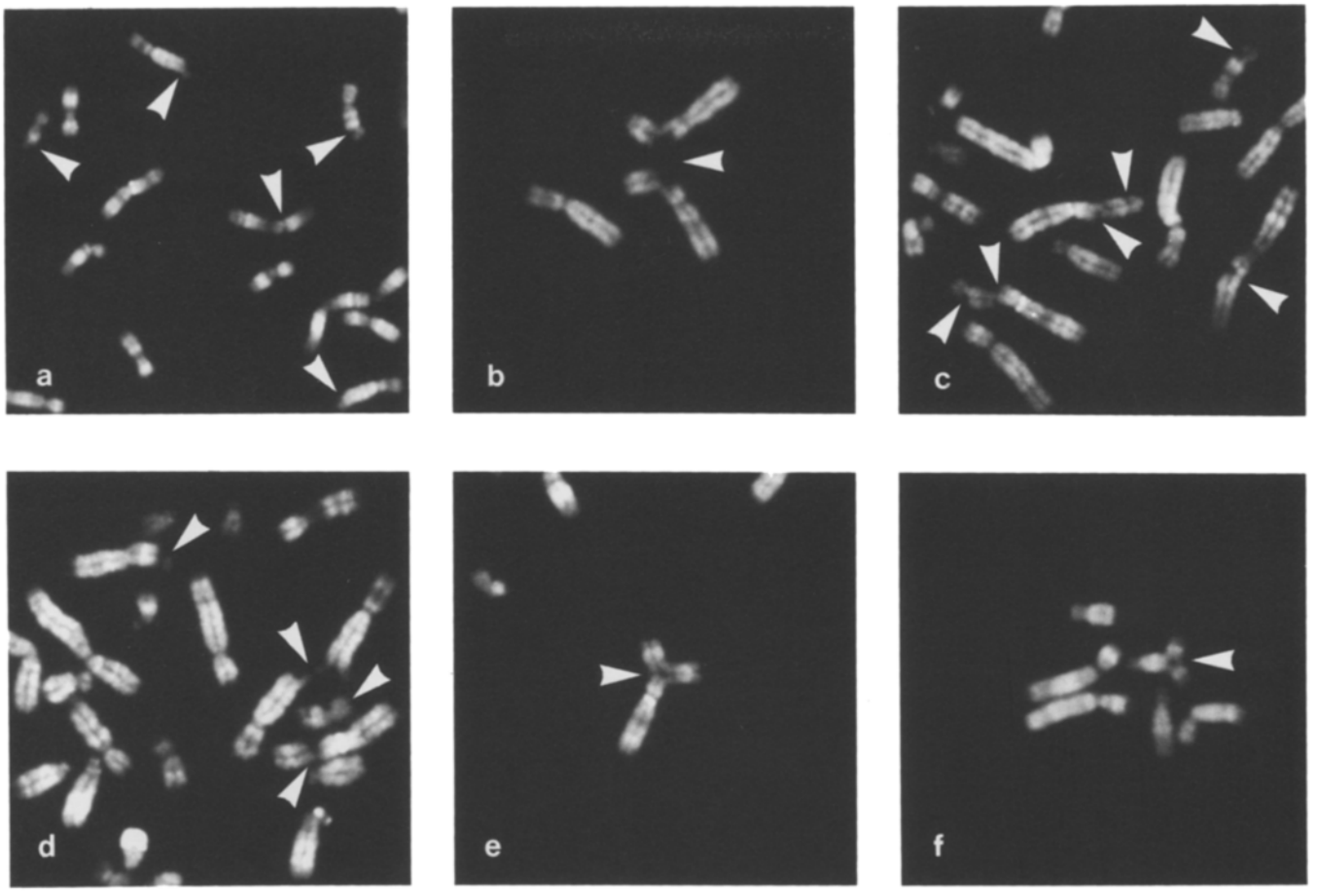

Fig. 3a–f. Partial metaphases with chromosomal aberrations induced by aphidicolin. **a** 1p22, 6q26 (both homologues), 7q32, 16q23; **b** 3p14 (both homologues); **c** 1p22, 3p14, and 3p24 (both homologues), 16q23; **d** 3p14, 16q23, 6q26 (both homologues, in association), Xp22; **e** triradial 3p14; **f** triradial 14q24

excess of breaks and gaps was induced at bands 1p22, 1p32, 2p13, 2q33, 3p24, 3q27, 7q22, 8q22, 11p13, 14q24, and Xq22.

The same type of analysis for the 1179 breaks and gaps induced by 0.2 μM aphidicolin in FA^- medium was performed for comparison. The total distribution is shown in Fig. 2b. Chi square analysis shows that any band with 10 or more lesions is significantly in excess ($\chi^2_{1\ d.f.} \geq 14.7$, with Yates correction; $P < 0.001$). An excess of lesions was induced at all of the sites as in FA^+ medium with the exception of band 3q27. In addition, bands 1p36, 1q25, 5q31, 7p13, 9q32, and 22q12 showed significant non-random damage.

Examples of site-specific aberrations scored are shown in Fig. 3. Aberrations were usually scored as chromosome gaps, but chromosome breaks, chromatid gaps, and chromatid breaks were also seen at lower frequencies. A comparison of the distribution of type of aberration is meaningless with regard to the time of damage since aphidicolin was present throughout the entire cell cycle prior to harvest. Rare triradial configurations were seen for most of the common lesions. Examples of triradials at 3p14 and 14q24 are shown in Fig. 3.

Under the same conditions at which the common fragile sites were induced in cultured lymphocytes, aphidicolin was not effective in inducing the fragile X (Table 2). Aphidicolin and DMSO appeared to reduce the number of cells expressing the fragile X in Subjects 7757 and 7758. To rule out the possible effects of DMSO, an experiment was performed in which aphidicolin was dissolved in DMSO and dissolved alternatively in ethanol. Aphidicolin decreased the number of cells expressing the fragile X in Subject 9263 regardless of the vehicle in which the aphidicolin was dissolved. There was no apparent effect of aphidicolin on the fragile X in Subject 9264. Thus in three of four fragile X males studied, aphidicolin decreased expression of the fragile X under the set of experimental conditions used to induce the common fragile sites.

Discussion

The objective of this study was to determine the effect of DNA polymerase α inhibition on human chromosomes. Aphidicolin is clearly clastogenic to human chromosomes. Induction of chromosome breakage in human cells by aphidicolin has previously been reported (Bender and Preston 1981; van Zeeland et al. 1982), but no site-specific breakage was described. Our data clearly show that chromosomal lesions induced by aphidicolin are nonrandom and that the greatest breakage occurred at "hot spots" induced by thymidylate stress. Interestingly, the fragile X was not induced by aphidicolin under the same conditions in cultured lymphocytes.

Bands 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22 were consistently most sensitive to breakage by aphidicolin. Gaps and breaks at these sites, especially 3p14, 6q26, and 16q23, are frequently encountered when cells are grown in folate-deficient medium (Sutherland 1983) or with FUdR (Glover, un-

Table 2. Effect of aphidicolin on expression of the fragile X

Experiment	Subject	Medium	Aphidicolin[b] (μM)	Fra(X)/cells	Number of other aberrations
1	7757	FA^-	0	19/50	8
		FA^- + DMSO[a]	0	11/50	3
		FA^-	0.2	4/50	79
		FA^+	0.2	0/50	40
1	7758	FA^-	0	22/50	13
		FA^- + DMSO	0	5/50	4
		FA^-	0.2	4/50	71
		FA^+	0.2	0/50	43
2	8148	FA^-	0	0/50	7
		FA^- + DMSO	0	0/50	7
		FA^-	0.2	0/50	71
		FA^+	0.2	0/50	64
3	9263	FA^-	0	21/50	nq[c]
		FA^- + DMSO	0	16/50	nq
		FA^-	0.2	0/50	nq
		FA^+	0.2	1/50	nq
		FA^- + EtOH	0	25/50	nq
		FA^-	0.2 in EtOH	2/50	nq
		FA^+	0.2 in EtOH	0/50	nq
3	9264	FA^-	0	7/50	nq
		FA^- + DMSO	0	4/50	nq
		FA^-	0.2	6/50	nq
		FA^+	0.2	1/50	nq
		FA^- + EtOH	0	4/50	nq
		FA^-	0.2 in EtOH	6/50	nq
		FA^+	0.2 in EtOH	1/50	nq
3	9265	FA^-	0	0/50	nq
		FA^- + DMSO	0	0/50	nq
		FA^-	0.2	0/50	nq
		FA^+	0.2	0/50	nq
		FA^- + EtOH	0	0/50	nq
		FA^-	0.2 in EtOH	0/50	nq
		FA^+	0.2 in EtOH	0/50	nq

[a] DMSO (0.2%) or EtOH (0.2%) added 26 h prior to harvest
[b] Aphidicolin dissolved in DMSO unless otherwise stated and added 26 h prior to harvest
[c] Not quantitated. The common fragile sites were, however, noted in all family members (see text)

published work) to induce the fragile X. In the present study, damage at these six sites accounted for about 40% of all lesions induced by aphidicolin and about 40% of all lesions seen in cells cultured in folate-deficient medium without aphidicolin. However, the percentage of all chromosomes with these lesions was much greater with aphidicolin.

Nine other bands also showed a significant excess of gaps and breaks both in FA^+ and in FA^- medium with aphidicolin (0.2 μM). Seven additional bands showed excess breakage only in FA^- cultures wherein damage by aphidicolin was enhanced. The distinction between breakage at the six bands mentioned above and these sensitive sites is arbitrary. It is based only on the obvious excess of lesions occurring at the six more sensitive sites and the fact that damage at these sites is frequently seen when cells are grown in FA^- medium for expression of the fragile X.

Excess breakage at some of the sites most sensitive to aphidicolin or growth in FA^- medium has been frequently reported in studies of spontaneous and induced chromosome damage in man (Lubs and Samuelson 1967; Brøgger 1975; Ayme et al. 1976; Aula et al. 1976). In at least two (Brøgger 1975, Lubs, personal communication 1983), if not all of these studies, folic acid-deficient medium 199 was used without the knowledge that it induces chromosomal lesions. It is very likely that folate deficiency contributed to the excess breakage at some sites via the mechanism shared with aphidicolin. In view of this, these data should be reexamined to distinguish lesions induced in vitro by folate-deficient growth from "hot spots" occurring by other mechanisms.

Heritable fragility at bands 3p14 and 16q23 has recently been reported in individuals undergoing genetic evaluation for various disorders (Rudduck and Franzen 1983; Wegner 1983; Shabtai et al. 1983) and the question of disease association has been raised. We have seen lesions at 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22 in all of our subjects after aphidicolin treatment, including a mother and her two sons, and a father and his two sons. Lesions have occasionally been observed in both homologues in cells from normal subjects. While the number of individuals studied was small, it appears that these sensitive sites are extremely common, and are heritable. Any associa-

tion with inherited disease analogous to fragile-X-linked mental retardation appears fortuitous.

Sutherland has referred to lesions at 3p14, 6q26, and 16q23 as autosomal lesions or hot spots (Sutherland 1983). Because at least one site sensitive to aphidicolin occurs on the X chromosome, and because all criteria for fragile sites are met, we suggest that the term aphidicolin induced "common fragile sites" be used to describe bands 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22. The other chromosomal sites damaged nonrandomly by aphidicolin may be regarded as possible common fragile sites whose confirmation requires further study. The 17 fragile sites listed by Sutherland (1983) should be termed "rare fragile sites".

A comparison of the common fragile sites with sites of frequent breakage occurring in chromosome rearrangements ascertained through chromosomally abnormal probands (Palmer 1981) shows no sites in common. However, inherent biases exist in this type of comparison due to the sample population used (Palmer 1981; Jacobs 1981), and determination of sites of breakage and exchange should ideally be made on mutant germ cells to eliminate biases due to selection (Jacobs 1981). Thus, the possibility that the common fragile sites are related to breakage and exchange in either germ cells or somatic cells in vivo cannot be ruled out. A striking coincidence exists between the breakpoints at 3p14 and 3p24 and the somatic cell breakpoints involved in del(3p) seen in some cases of small cell carcinoma of the lung (Whang-Peng et al. 1982).

Unexpectedly, aphidicolin induced the common fragile sites in cultured lymphocytes without inducing the fragile X under the same experimental conditions. Both the folate-sensitive rare fragile sites and the common fragile sites are induced under conditions of folate or thymidylate stress. This observation lead to the earlier suggestion that they share a common mechanism of induction (Glover 1981). It now appears that separate but overlapping mechanisms may account for the appearance of the fragile X and the common fragile sites. We can better understand the mechanism of induction of the common fragile sites in light of the results of these experiments.

Aphidicolin inhibits DNA polymerase α (Ikegami et al. 1978) and thus semi-conservative DNA synthesis by blocking progression of the replication fork (Lonn and Lonn 1983) and interfering with the joining of adjacent DNA intermediates (Yagura et al. 1982). It has no effect on polymerases β or γ, on the synthesis of protein or RNA (Ikegami et al. 1978), on the synthesis of deoxyribonucleotides (Pedrali-Noy et al. 1980), or on DNA ligase activity (DePamphilis et al. 1980). It thus appears that the common fragile sites are induced by inhibition of replication fork progression or joining of DNA intermediates preferentially at these sites and that thymidylate stress conditions may partially inhibit polymerase α.

Two alternative hypotheses can be considered. First, inhibition of polymerase α by aphidicolin has been shown to indirectly inhibit thymidylate synthetase in intact Chinese hamster fibroblasts, possibly through an "allosteric" interaction between enzymes of a DNA synthesizing complex (Reddy and Pardee 1983). Thus aphidicolin may induce the common fragile sites by indirectly affecting other enzymes involved in DNA synthesis, such as thymidylate synthetase. However, it seems unlikely that an indirect effect on thymidylate synthetase is the cause per se of common fragile site induction since the fragile X, which is induced by inhibition of thymidylate synthetase (Glover 1981; Tommerup 1981), is not readily induced by aphidicolin in cultured lymphocytes.

Second, there are conflicting reports on the role of polymerase α and aphidicolin on DNA repair in mammalian cells (Ciarrocchi et al. 1979; Hanoka et al. 1979; Pedrali-Noy and Spodari 1980; van Zeeland et al. 1982; Smith and Patterson 1983; Snyder and Regan 1981). An effect on repair of lesions might explain the synergistic effect observed between aphidicolin and growth in folate-deficient medium on the induction of lesions. However, it is difficult to explain how an effect on repair of lesions could account for the induction of the same site-specific aberrations by aphidicolin in complete medium. The synergistic effect could equally be explained by inhibition of replication synthesis. Experiments to test the effects of aphidicolin during different stages of the cell cycle may help to clarify the role of DNA repair on the induction of fragile sites.

If the hypothesis is correct that the common fragile sites are induced by partial inhibition of polymerase α and progression of DNA replication preferentially at these sites, this predicts that any agent or condition that directly or indirectly affects DNA synthesis via polymerase α will give rise to these specific lesions. Thymidylate stress appears to share this ability with aphidicolin.

Thymidylate stress has many other effects on eukaryotic cells (for review see Kunz 1982), including perturbation of deoxyribonucleotide pools (increased dCTP, dUTP pools and decreased dTTP, dGTP pools) in a manner quite different from aphidicolin (decreased dCTP pools; Nicander and Richard 1981). This difference should be viewed with some caution, however, since the effect of aphidicolin on pool changes was measured in mouse cells and may be different in human cells. Aphidicolin does appear to have variable effects on different species or cell types. For example it induces endoreduplication in Chinese hamster cells (Huang et al. 1983), an effect not seen under the conditions of our experiments. One effect of pool perturbation is misincorporation of bases, including uracil, into the DNA. The suggestion that base misincorporation due to pool imbalances may relate to induction of the fragile X has been made (Glover 1981; Krumdieck and Howard-Peebles 1983), although no direct evidence to support this suggestion presently exists.

These other effects of thymidylate stress may explain why aphidicolin does not induce the fragile X while both aphidicolin and thymidylate stress induce the common fragile sites. The mechanisms for induction, however, do not explain why the lesions are site specific. The answer to this question will require analysis on the molecular level and is a current challenge in human genetics.

Acknowledgements. We thank Dr. Chia-cheng Chang and Dr. Stephen Warren for helpful discussion. Aphidicolin was a gift from Dr. Chang and from the Developmental Therapeutics Program, National Cancer Institute. We are also grateful to the staff of Southwest Biomedical Research Institute for their assistance and to Dr. Rod Norman for advice on statistical analyses. This work was supported by New Investigator Research Award HD16850 from the National Institutes of Child Health and Human Development to T.W.G. and by grant CA25055 from the National Cancer Institute.

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Received November 9, 1983 / Accepted January 4, 1984