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How common are common fragile sites: variation of aphidicolin-induced chromosomal fragile sites in a population of the deer mouse (*Peromyscus maniculatus*)

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Abstract Aphidicolin (APC)-induced chromosomal gaps and breaks were analyzed for ten deer mice (*Peromyscus maniculatus*) from a natural population. The FSM statistical methodology was used to identify fragile sites as chromosomal loci exhibiting significantly non-random numbers of gaps/breaks in each individual and enabled an assessment of variation in fragile sites among the individuals. The individual deer mice exhibited as few as 7 to as many as 19 of the populational total of 34 sites. Two sites were fragile in all individuals and 13 sites were fragile in single individuals only. Defined by populational frequencies of greater than 50%, high-frequency fragile sites constituted 26% of the populational total. Approximately 35% of the total fragile sites were fragile in 20–40% of the population (low-frequency fragile sites) and about 38% were fragile in single individuals only. Analysis of the data pooled over all individuals identified significantly non-random breakage at 80 sites, 47 of which were not identified as fragile in any single individual. It appears, therefore, that fragile site identifications from pooled data have fostered an inflated estimate of the numbers and frequencies of common fragile sites. Comparison of the fragile site and spontaneous breakage (control) data suggest that APC-induced fragile sites represent regions of chromosomes that experience elevated levels of somatic mutation. Additionally, the occurrence of APC-induced fragile sites at or near the interstitial breakpoints of two pericentric-inversion polymorphisms in this population supports the hypothesis that fragile sites experience an increased rate of meiotic chromosomal mutation and are predisposed to undergo phylogenetic rearrangement.

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Introduction

Chromosomal fragility represents a well documented phenomenon about which many questions remain unanswered. Experimental designs aimed at determining the biological relevance of fragile sites require that fragile sites are defined and identified as chromosomal loci which exhibit gaps or breaks at significantly non-random frequencies (Mariani 1989; Jordan et al. 1990; Böhm et al. 1995). Although there has been substantial advancement in the study of the molecular basis of chromosomal fragility, these results have not eliminated the need for cytogenetic-level recognition of which sites are fragile.

Most of the data on chromosomal fragility pertain to the rare fragile sites. Rare fragile sites are typically folate sensitive, appear to be heritable, and occur in relatively few individuals. Sequence data for five rare fragile sites, FRAXA, FRAXE, FRAXF, FRA11B, and FRA16A (Verkerk et al. 1991; Knight et al. 1993; Jones et al. 1994; Nancarrow et al. 1994; Parrish et al. 1994), indicate that each is associated with an expanded region of CGG/CCG trinucleotide repeats. The sequence data further suggest that in the rare fragile sites, regions of long (> 50) pure repeats effect nucleosome formation and that it is the physical characteristics of the repeat that result in the cytogenetic manifestation of fragility (Zhong et al. 1995; Dobkin et al. 1996; Metzenberg 1996). The potential for disease relationship of rare fragile sites is documented in their association with fragile X syndrome (Pieretti et al. 1991), FRAXE mental retardation (Knight et al. 1993), and physical linkage of FRA11B and a Jacobsen syndrome deletion breakpoint (Jones et al. 1994).

Although most of the described fragile sites are classified as common fragile sites, this category is poorly defined, and little is known about the structure or biology of these sites. Chromosomal loci at which metaphase gaps or breaks are induced by aphidicolin (APC) are typically categorized as common fragile sites. However, some common fragile sites are folate sensitive and the clastogens 5-azacytidine and bromodeoxyuridine have also been re-

ported to induce common fragile sites. The fragile sites revealed by these modes of induction are neither unique nor fully coincident (Sutherland and Hecht 1985), and there are likely to be significant differences in the frequencies of specific fragile sites among populations. Although logic argues for a frequency-based classification (Hecht 1986), analytical problems inherent to identifying fragile sites from chromosomal breakage data for single individuals and the coincident lack of data relative to the frequency of fragile sites within or among populations have perpetuated the general categorization of common fragile sites.

Initial data on the molecular structure of common fragile sites support the hypothesis that common fragile sites are fundamentally different from rare fragile sites. Wilke et al. (1996) reported that the sequence of the human common fragile site FRA3B is devoid of large or small nucleotide-repeat sequences that could be potentially involved in fragile site formation. As the molecular basis of common fragile sites is not apparent from the sequence data available, the identification of these sites and studies of their biological and biomedical implications continue to depend upon analysis of the distribution of chromosomal gaps/breaks.

The analytical complexities associated with chromosomal breakage-based identification of fragile sites were summarized by Böhm et al. (1995). Early studies applied various arbitrary criteria for determining which sites were fragile, and initial statistical methods of identifying fragile sites were found to be inappropriate (Smith 1986; Mariani 1989; Jordan et al. 1990; Dahm and Greenbaum 1994) and unsuited to data from single individuals (Dahm and Greenbaum 1994; Böhm et al. 1995). While pooling of data over individuals has been used to circumvent the statistical problems associated with the sparse nature of per-individual chromosomal breakage data, this approach assumes statistical independence of the data and eliminates the ability to assess fragile site variation among individuals. The multinomial model of Böhm et al. (1995) provides an analysis designed to identify fragile sites from chromosomal breakage data for single individuals. A sample application of the FSM methodology (Böhm et al. 1995) led to the suggestion that the analysis of data pooled over individuals results in the false identification of some fragile sites and inaccurate estimates of the populational frequencies of fragile sites.

An additional problem to resolving basic questions concerning fragile sites has been the paucity of information for animal models. Although chromosomal fragility has been documented for a variety of mammalian species (Sanz et al. 1986; Uchida et al. 1986; Robinson and Elder 1987; Tewari et al. 1987; Simi et al. 1990; Smeets and van de Klundert 1990; Poulsen and Ronne 1991; Stone et al. 1991; Ronne 1992; Riggs et al. 1993), there are no available data concerning fragile site variation within or among natural populations. In this study, we apply the statistical methodology of Böhm et al. (1995) to identify and compare APC-induced chromosomal fragile sites among deer mice (*Peromyscus maniculatus*) from a natural population. To test the hypothesis that the identification of common fragile sites is biased by the failure to treat chromosomal breakage data as individually dependent, we compare the results of the per-individual analyses to those from analysis of the data pooled over all individuals. The fragile site identifications for the population studied are also compared to non-random patterns of spontaneous breakage and to the locations and frequencies of three pericentric-inversion polymorphisms in the population studied.

Materials and methods

Specimens of *P. maniculatus* were live-trapped 1 mile south, 2.3 miles west of Hays, Ellis County, Kansas, and maintained alive for periods not exceeding 6 months. Chromosomal preparations were obtained from cells grown using a modification of the spleen lymphocyte culture technique described by Robinson and Elder (1987). Each animal was killed and, under sterile conditions, the spleen removed, minced in collagenase (0.5% in RPMI) solution, and incubated for 30 min. Following centrifugation, the cells were resuspended in T-cell growth medium (Robinson and Elder 1987) and equally distributed into two T-25 tissue culture flasks. The volume of medium in each flask was then brought to 5 ml. After 48-h incubation at 37° C, the culture was centrifuged and the medium decanted. Cells were resuspended in 10 ml of medium and APC (0.2 μM final concentration) was added to one flask; the other flask received no APC and served as the control. Both treatment and control flasks were incubated an additional 18 h at 37° C. Colcemid (0.05 ml of a 10 μg/ml solution) was added to the cultures 20 min prior to harvest. Cells were harvested and metaphases produced using standard cytogenetic techniques.

All individuals were chromosomally characterized from metaphases obtained from the control cultures. G-banding followed a modification of the GTG protocol of Verma and Babu (1989) and C-banding followed a modification of the technique of Sumner (1972). Identification of the chromosomal location of C-band-positive heterochromatin was obtained by sequential G/C-banding. The banded chromosomes were identified and a composite ideogram was constructed according to the standardized karyotype for *Peromyscus* (Greenbaum et al. 1994).

To assure maximum resolution in locating chromatid and chromosome gaps/breaks, metaphases were first non-differentially stained with 2% Giemsa in phosphate buffer. Microscope slide coordinates were recorded for 100 metaphases each from treatment and control cultures. These metaphases were digitized using a Genetiscan A/B workstation (Perceptive Scientific Instruments). To map the breaks to specific chromosomal landmarks, the cells were destained (in Carnoy's fixative) and the chromosomes were G-banded. Metaphases with adequate G-banding were again digitized. The location of chromosomal breaks was determined by direct comparison of the non-differentially stained and G-banded images of the same metaphases and designated according to the standardized G-banded karyotype of *Peromyscus*. Chromatid and chromosomal gaps/breaks were treated equally as representing single chromosomal events.

Fragile sites were identified as chromosomal bands which expressed significantly non-random breakage ($\alpha = 0.05$) as computed using the FSM (version 995) statistical program (Böhm et al. 1995; Greenbaum and Dahm 1995). The standardized X^2 (X^2_s) test statistic was used for all fragile site identifications. As the autosomal heterochromatin of *Peromyscus* has been shown to be highly resistant to APC-induced chromosomal breakage (Dominguez et al. 1995) and as breaks were not observed in the autosomal heterochromatin of the individuals examined, these regions were excluded from the analysis. Each of the G-bands from the included regions was considered to make an equal contribution to the genome. For females, this gave a haploid karyotype containing 331

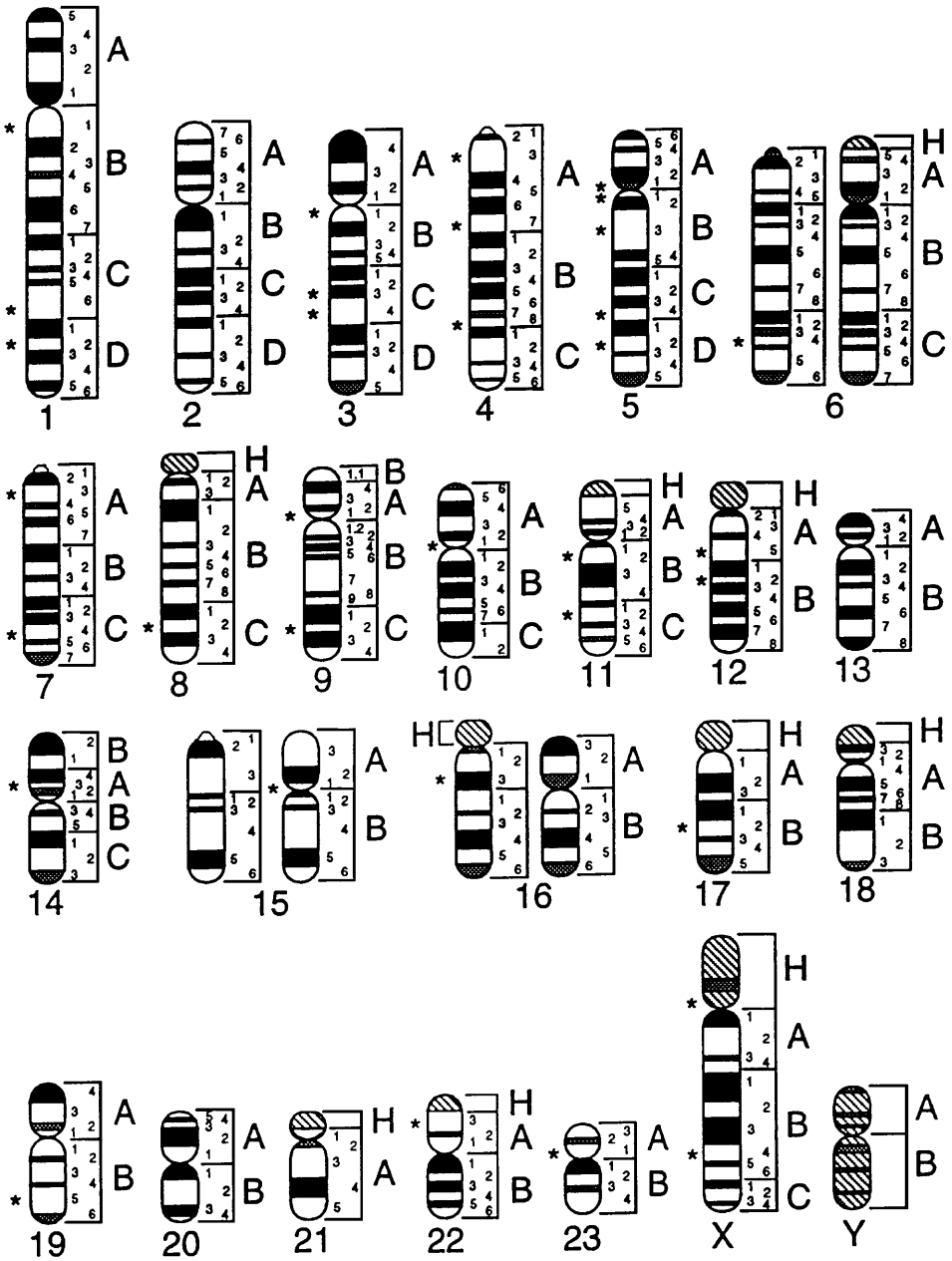

Fig. 1 Ideogram indicating the metaphase chromosomal morphologies, with G-band and non-centromeric C-band distributions, observed in the deer mice examined in this study. Alternative morphologies of the pericentric-inversion polymorphisms of chromosomes 6, 15, and 16 are presented. For presence/absence variations of heterochromatin, only the heterochromatin-present conditions (*H*) are shown. An *asterisk* is placed to the left of each site that was determined (FSM analysis, X^2_s test, $\alpha = 0.05$) to be fragile in one or more individuals

chromosomal bands. For males, the Y chromosome was treated as a single chromosomal band and a total of 332 bands was considered (for a discussion of these assumptions and parameters see Böhm et al. 1995). The identification of fragile sites was computed for each individual and for the data pooled over all individuals. For the analysis of the pooled data, the Y chromosome was eliminated and 331 was used as the haploid number of chromosomal bands.

Results

The diploid number was 48 in all individuals and the observed fundamental numbers ranged from 82 to 85. An ideogram indicating the observed metaphase chromosomal morphologies, with G-band and non-centromeric C-band positions, is presented in Fig. 1. Seventeen of the autosomal pairs (1–5, 7, 9–11, 13, 14, 18–23) were invariant among the individuals. Heterochromatin and pericentric-inversion polymorphisms were observed both as heteromorphisms within individuals and as differences among individuals. Variation involving the presence (Fig. 1) or absence of short arm or distal C-band material was observed for chromosomes 6, 8, 11, 12, 16, and 17. Polymorphism for pericentric inversions was present as alternative submetacentric and acrocentric conditions for chromosomes 6, 15, and 16 (Fig. 1). The acrocentric conditions of chromosomes 6 and 15 were each observed in the heterozygous condition in single individuals (5369 and 5368, respectively). The submetacentric condition of chromosome 16 was heterozygous in two individuals (4497 and 5368) and homozygous in one individual (4489).

Table 1 Numbers of metaphases analyzed, chromosomal gaps/breaks observed, and different bands at which gaps/breaks were observed from aphidicolin (*APC*)-treated and control cultures for the ten *P. maniculatus* examined in this study

Individual	APC-treated				Controls			
	Metaphases	Breaks	Mean (SD)	Bands	Metaphases	Breaks	Mean (SD)	Bands
4483	61	175	2.9 (1.5)	73	105	16	0.2 (0.6)	14
4484[a]	71	138	1.9 (0.8)	78	–	–	–	–
4487	85	216	2.5 (1.3)	76	101	10	0.1 (0.3)	10
4489	93	190	2.0 (1.0)	81	111	28	0.3 (0.8)	25
4497	60	121	2.0 (0.9)	68	105	15	0.1 (0.5)	11
5368	67	139	2.1 (1.1)	67	102	11	0.1 (0.4)	11
5369	56	132	2.4 (1.3)	62	71	8	0.1 (0.4)	8
5372	92	239	2.6 (1.2)	82	97	27	0.3 (0.8)	22
5374	81	173	2.1 (1.1)	82	101	27	0.3 (0.7)	23
5375	78	203	2.6 (1.4)	69	112	8	0.1 (0.3)	7
Total	744	1726	2.4 (0.1)	208	905	150	0.2 (0.1)	83

[a] The control culture for individual 4484 yielded an insufficient number of metaphases for analysis

The chromosomal breakage data are summarized in Table 1. In the nine individuals for which a sufficient number of metaphases was obtained from the control cultures, the number of gaps/breaks ranged from 8 to 28 and averaged from 0.1 to 0.3 per metaphase; the overall mean was 0.2 breaks per metaphase. The 150 total spontaneous breaks mapped to 83 different bands in the 905 metaphases analyzed from the control cultures. The numbers of spontaneous breaks were considered insufficient to justify analysis for the identification of fragile sites on a per-individual basis (Greenbaum and Dahm 1995). FSM analysis of the spontaneous breakage data pooled over the nine individuals identified six sites (1C6, 3C4, 5B3, 6C2, 19B5, and XB4) with statistically significant breakage frequencies.

For the APC-treated cultures, the number of gaps/breaks per individual ranged from 121 to 239 and averaged from 1.9 to 2.9 per metaphase (Table 1). The total of 1726 APC-induced gaps/breaks mapped to 208 different G-bands from 744 metaphases. A summary of the fragile sites identified by FSM analysis and the distribution of the chromosomal gaps/breaks at these sites is presented in Table 2. For each data set analyzed, FSM provides a "critical value" (C_α) corresponding to the minimum number of breaks necessary for any particular site to be declared as fragile for that data set (Böhm et al. 1995). For the distributions of the APC-induced breakage, C_α ranged from 3 to 4 for individuals and was 5 for the data pooled over all ten individuals. Among the individuals, the number of fragile sites ranged from 7 to 19 (Table 3) and a total of 34 different sites was identified as fragile. Analysis of the chromosomal distribution of the per-individual breakage indicated that between 33 and 68% (mean = 49.7 ± 11.3%) of the breakage in an individual occurred at sites identified as fragile in that individual (Table 2). The locations of the sites identified as fragile in one or more individuals are indicated in Fig. 1. Of the sites identified as fragile in one or more individuals, two (19B5 and XB4) were fragile in all ten individuals and 13 were fragile in single individuals only. The remaining 19 loci were fragile in two to nine of the ten individuals.

FSM analysis of the APC-induced breakage pooled over all ten individuals identified a total of 80 fragile sites. Of the sites identified as fragile in the per-individual analyses, only one site (a single-individual occurrence at 3C3) was not identified as fragile in the pooled data analysis (Table 2). However, analysis of the pooled data identified significantly non-random breakage at 47 sites which were not identified as fragile in any single individual. Of the 1726 total gaps/breaks examined, 1451 (84%) occurred at sites identified as fragile in the pooled data analysis.

Discussion

The data presented here clearly indicate that the presence or absence of specific APC-induced fragile sites varies among individuals. The individual deer mice exhibited significantly non-random breakage in as few as seven to as many as 19 of the populational total of 34 sites identified as fragile by FSM analysis of the per-individual chromosomal breakage data (Table 3). Only two of the 34 fragile sites were fragile in all ten of the deer mice examined. Grouped to generally conform to Hecht's (1986) frequency-based classification of fragile sites, high-frequency (= common) fragile sites (defined by populational frequencies > 50%) constituted 26% of the total fragile sites in the population (Table 2). Approximately 35% of the total fragile sites were fragile in 20–50% of the individuals and were considered to represent low-frequency fragile sites. As only ten individuals were examined in this study, we were constrained to distinguish the category of rare fragile sites as comprised by the 38% of fragile sites which were fragile in single individuals only.

Considered as the average proportion of the fragile sites in an individual (Table 3), high-frequency sites constituted 65 (41–100)%, low-frequency fragile sites 24 (0–

Table 2 Observed chromosomal gaps/breaks at sites determined to be fragile (FSM analysis, X^2_s test, $\alpha = 0.05$) in one or more of the *P. maniculatus*, examined in this study. The sites are arranged by frequency of occurrence. (C_α Critical value, *n* number of individuals fragile at that site, *P* proportion of total breakage which occurred at sites identified as fragile in that individual, *asterisk* significantly non-random breakage)

Band	Individual										*n*	Pooled
	4483	4484	4487	4489	4497	5368	5369	5372	5374	5375		
19B5	19*	8*	14*	12*	6*	8*	9*	5*	17*	7*	10	105*
XB4	9*	5*	15*	18*	9*	13*	9*	22*	6*	9*	10	115*
6C2	10*	5*	32*	13*	8*	1	4*	13*	9*	3*	9	98*
9A1	8*	4*	10*	4*	3	3	9*	19*	4*	14*	8	78*
3C4	19*	8*	13*	5*	3	3	4*	2	6*	9*	7	72*
5B3	10*	0	2	4*	2	6*	6*	4*	5*	8*	7	47*
14A3	12*	0	1	1	5*	10*	10*	10*	5*	21*	7	75*
16A3	2	1	11*	2	5*	7*	8*	15*	4*	14*	7	69*
1C6	5*	4*	3	5*	3	1	3*	10*	2	6*	6	42*
5C4	3	4*	2	2	0	4*	1	6*	3	6*	4	31*
7C4	0	1	3	3*	3	4*	3*	3*	1	0	4	21*
12B2	3	0	4*	3*	2	5*	1	8*	3	1	4	30*
23A1	1	2	13*	1	0	4*	2	4*	2	4*	4	33*
3B1	1	1	2	4*	1	2	2	2	6*	12*	3	33*
4A7	0	1	1	2	5*	3	1	6*	2	5*	3	26*
4A3	1	0	0	0	0	1	0	5*	1	3*	2	11*
5A1	2	2	1	0	0	0	0	13*	1	3*	2	22*
5B1	1	0	2	3*	1	2	1	3*	3	1	2	17*
5D2	1	0	0	4*	1	0	0	3*	0	1	2	10*
7A3	1	3	3	6*	2	1	1	3*	3	2	2	25*
XH1	0	0	3	5*	1	1	2	4*	1	1	2	18*
1B1	1	2	1	0	0	0	0	2	1	4*	1	11*
1D2	3	0	0	1	0	1	0	1	9*	1	1	16*
3C3	0	0	0	0	0	0	0	0	0	3*	1	3
4B8	0	1	1	4*	1	1	1	0	1	1	1	11*
8C2	1	3	2	4*	0	1	2	2	3	2	1	20*
9C2	1	4*	2	0	0	2	2	2	2	0	1	15*
10A1	1	1	0	0	2	1	0	1	4*	0	1	10*
11B2	1	1	2	3*	2	1	1	0	1	2	1	14*
11C2	0	2	6*	1	1	0	0	0	2	1	1	13*
12A5	1	4*	1	0	0	0	1	0	2	0	1	9*
15A1	1	1	5*	0	0	0	0	0	0	1	1	8*
17B2	0	0	0	0	1	0	0	1	0	4*	1	6*
22A3	0	2	0	2	5*	1	2	0	0	0	1	12*
C_α	4	4	4	3	4	4	3	3	4	3	–	5
P	0.53	0.33	0.57	0.51	0.36	0.43	0.49	0.64	0.43	0.68	–	–

58)%, and rare fragile sites 11 (0–22)%. However, the greatest contributor to variation among the individuals was the number of low-frequency sites. For the sample of deer mice examined in this study, the variance in the number of low-frequency sites was more than 5 times greater than the variance in the number of either high-frequency or rare fragile sites. The individuals with the three highest numbers of total fragile sites exhibited the highest numbers of low-frequency fragile sites.

As suggested by Böhm et al. (1995), it appears that the analysis of chromosomal breakage data pooled over individuals causes a large number of sites to be inaccurately identified as fragile and that this has probably resulted in inflated estimates of the number and frequencies of common fragile sites. Of the 80 sites identified as fragile in our pooled data analysis, 47 (59%) were not fragile in any individual deer mouse and an additional 12 (15%) were fragile in only one of the ten mice examined. From the pooled data analysis, we would have concluded that the population contained more than twice the number of fragile sites justified from the analysis of individuals and that the individuals uniformly expressed more than 4 times the highest number of sites present in any of the individuals studied.

Extremely sparse data sets, such as those obtained for the spontaneous chromosomal breakage in control cultures (Table 1), are not reliable for per-individual FSM identification of fragile sites (Greenbaum and Dahm 1995; unpublished data). The application of FSM analysis to the pooled spontaneous breakage data for our sample of

Table 3 The number and categorical distribution of APC-induced chromosomal fragile sites (FSM analysis, X^2_s test, $\alpha = 0.05$) in each of the ten *P. maniculatus* examined in this study. (FS_T total number of fragile sites identified in each individual, *HFFS* number of fragile sites which were present in more than half of the individuals examined, *LFFS* number of fragile sites which were present in more than one but half or fewer of the individuals examined, *RFS* number of fragile sites that were present in single individuals only)

Individual	FS_T	HFFS (%)	LFFS (%)	RFS (%)
4483	8	8 (100)	0 (0)	0 (0)
4484	9	6 (67)	1 (11)	2 (22)
4487	10	6 (60)	2 (20)	2 (20)
4489	17	7 (41)	7 (41)	3 (18)
4497	7	5 (71)	1 (14)	1 (14)
5368	9	5 (56)	4 (44)	0 (0)
5369	10	9 (90)	1 (10)	0 (0)
5372	19	8 (42)	11 (58)	0 (0)
5374	11	8 (73)	1 (9)	2 (18)
5375	18	9 (50)	6 (33)	3 (17)
Range	7–19	5–9 (41–100)	0–11 (0–58)	0–3 (0–22)
Mean	10.1	7.1 (65)	3.4 (24)	1.3 (11)

deer mice, however, provided evidence of a fragile site-related pattern in these data. When the mapped breakage from the control cultures was treated as being from a single individual, the sites identified as having significantly non-random breakage (1C6, 3C4, 5B3, 6C2, 19B5, and XB4) were all among the group of high-frequency fragile sites in this population and included the two loci which were determined to be fragile in all individuals. Coincidences between fragile sites and sites of spontaneous breakage have been previously reported by Hecht et al. (1988) and Austin et al. (1992). These results support the hypothesis that APC-inducible fragile sites are more than artifacts of APC clastogenicity and support the contention that fragile sites represent regions of chromosomes that experience elevated levels of somatic mutation.

Comparisons of fragile sites and chromosomal rearrangements between humans and non-human primates (Miro et al. 1987; Smeets and van de Klundert 1990) have been interpreted as indicating the existence of a positive relationship between fragile sites and chromosomal evolution. Our data provide population-level support for the hypothesis that fragile sites experience an increased rate of meiotic chromosomal mutation and are, therefore, predisposed to undergo phylogenetic rearrangement. The population of deer mice examined harbors a low (0.05) frequency of pericentric inversions of chromosomes 6 and 15 and a considerably higher (0.20) frequency of an inversion of chromosome 16. The fragile site analysis revealed the presence of APC-sensitive fragile sites (15A1 and 16A3) at or near the interstitial breakpoints of the inversions of chromosomes 15 and 16 (Fig. 1, Table 2). As might be expected under conditions of a direct relationship between numbers of fragile sites and frequency of chromosomal mutation, the fragile site at 16A3 was present in high frequency in this population and the fragile site at 15A1 was detected in only a single individual (Table 2). There was no obvious correspondence between the individuals carrying the inversions and fragility at 15A1 and 16A3. However, fragility at 15A1 and 16A3 was restricted to the wild-type chromosomal morphology in both cases; breaks were not observed at these loci on acrocentric chromosomes 15 or submetacentric chromosomes 16. This latter observation suggests that chromosomal rearrangement at a fragile site can result in the loss of fragility at that site in the rearranged chromosome.

The maintenance of chromosomal polymorphism in natural populations remains an intriguing genetic problem, particularly in mammals. The widespread maintenance of pericentric-inversion polymorphisms within and among populations of *P. maniculatus* and other species of this genus (reviewed in Greenbaum et al. 1994), convergent evolution for chromosomally indistinguishable pericentric inversions among species of *Peromyscus* (Greenbaum and Baker 1978; Robbins and Baker 1981; Rogers et al. 1984; Stangl and Baker 1984; Greenbaum et al. 1994), and various population-genetic factors pertaining to the dynamics of the process of chromosomal evolution (for discussion and references see Sites and Reed 1994) lead to the expectation of chromosomally localized levels of mutation far in excess of that typically cited for single-gene mutations. The interaction of hypermutagenic fragile sites with meiotic factors favoring the propagation of rearrangements located in the late-synapsing portions of the chromosomes (Greenbaum et al. 1986; Hale 1986; Hale and Greenbaum 1988) provides a viable hypothesis for the major processes governing chromosomal evolution in the genus *Peromyscus.*

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