Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6657–6661, August 1991
Medical Sciences

Preferential integration of marker DNA into the chromosomal fragile site at 3p14: An approach to cloning fragile sites

(fluorescence *in situ* hybridization/DNA transfection/pSV2Neo/chromosome aberrations)

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Communicated by Janet D. Rowley, April 22, 1991

ABSTRACT **Fragile sites are specific regions of chromosomes that are prone to breakage. In cells cultured under conditions that induce fragile site expression, high levels of inter- and intrachromosomal recombination have been observed involving chromosomal bands containing fragile sites. To determine whether expression of specific fragile sites would facilitate preferential integration of exogenous DNA at these recombination hot spots, the vector pSV2Neo was transfected into a Chinese hamster–human somatic cell hybrid containing a derivative chromosome 3 as its only human component. Chromosome 3 contains a common fragile site at band 3p14.2 (FRA3B) that is induced by aphidicolin. Both cells induced to express FRA3B and the uninduced control cells were transfected with the pSV2Neo selectable plasmid. *In situ* hybridization of a biotin-labeled pSV2Neo probe to metaphase chromosomes revealed one to three integration sites in each stably transfected clone. Four of 13 clones transfected under conditions of FRA3B induction showed integration of pSV2Neo at 3p14; these clones also showed specific integration into hamster chromosome 1 and a rearranged chromosome characteristic of CHO cells (mar2). The 7 control clones, however, showed an apparently random pattern of pSV2Neo integration. Significant hybridization of pSV2Neo to both FRA3B and Chinese hamster chromosomes 1 and mar2 was seen in 100 cells from pooled colonies transfected after treatment with aphidicolin. These results suggest that preferential integration of marker DNA into human and Chinese hamster fragile sites occurs with exposure to aphidicolin. The nature of the DNA sequences at fragile sites is unknown and, despite a number of approaches, these sequences have not yet been isolated; our procedure may represent an approach to the cloning of fragile sites.**

Chromosomal fragile sites are characterized cytolologically as distinct chromosomal regions that exhibit gaps or breaks when cells are cultured under specific conditions (1). Fragile sites have been examined extensively in human cells; however, recent studies suggest that chromosomal fragile sites also exist in other mammals (2). The recognized fragile sites have been grouped according to the culture conditions necessary for their expression. The 26 "rare" fragile sites are caused by infrequent alleles segregating in the population and are inherited codominantly: normal and gapped homologues are observed in the same cell. They consist of 18 folate-sensitive fragile sites, 5 distamycin-inducible fragile sites, 2 bromodeoxyuridine-inducible fragile sites, and 1 unclassified site. The 87 "common" fragile sites that have been observed in all individuals examined to date are induced by chemicals such as aphidicolin and caffeine and are enhanced by low folate levels (3).

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With the exception of the fragile X chromosome (FRAXA) that is associated with a common form of mental retardation, no pathological role for any other fragile site has been identified (4). However, a number of investigators including Yunis and Soreng (5) and Le Beau and Rowley (6) identified a remarkable concordance between the chromosomal location of some fragile sites and the breakpoints in nonrandom abnormalities observed in human tumors.

The genetic and molecular basis for chromosome fragility at these sites is not known nor are the biological consequences of fragile site expression fully understood. Tommerup *et al.* (7) have shown that fragile sites predispose to intrachromosomal recombination as measured by sister-chromatid exchanges. Yunis *et al.* (8) and Glover and Stein (9) reported that fragile sites can also predispose to deletions and interchromosomal recombination (translocations). Thus these observations suggested that DNA strand breakage occurs frequently, if not always, during fragile site expression and that these sites are highly recombinogenic.

The nature of the DNA sequences at fragile sites and their position relative to cancer-specific break points occurring in the same band is unknown. A number of groups have begun work to characterize fragile sites at the molecular level; however, to our knowledge, no fragile site has been cloned. Warren *et al.* (10) examined chromosomal rearrangements involving the region of the FRAXA. They used somatic cell hybrids, containing a single human X chromosome; upon fragile site induction, chromosome breakage occurred at Xq27.3 and the distal chromosomal segment was translocated to a hamster chromosome. The isolation of these hybrid cells suggested a means for the molecular cloning of fragile sites. To characterize the FRA3B, Wang *et al.* (11) have isolated somatic cell hybrids containing rearranged chromosome 3 homologues resulting from breakage near the fragile site. Cosmid probes derived from 3p14.1–p21.2 were mapped relative to the break points on chromosome 3 in these hybrid cells (11). Despite the development of physical maps surrounding the FRAXA and FRA3B, these studies have not yet resulted in the cloning of the corresponding fragile site.

We present data suggesting an alternative and potentially more direct approach to cloning fragile site sequences. We show that, upon FRA3B induction, transfected marker DNA sequences preferentially integrate into this fragile site. Cells containing pSV2Neo integrated into FRA3B provide a means by which flanking fragile site sequences may be cloned. Isolation of these DNA sequences will facilitate studies that address the structure and function of the fragile site region.

MATERIALS AND METHODS

Cell Culture. The Chinese hamster ovary (CHO)–human somatic cell hybrid H3-4, containing a rearranged human

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chromosome 3, der(3)(pter → q21::q26.2 → qter), was kindly provided by Harry Drabkin (University of Colorado). This cell line was established by fusion of human acute myeloid leukemia cells characterized by a t(3;3)(q21;q26.2) and the mutant CHO cell line Urd-C by using UV-inactivated Sendai virus (12). Hybrid cells selectively retain human chromosome 3, which contains the uridine monophosphate synthetase gene. The short arm (p) of the human der(3) was normal whereas the long (q) arm was rearranged. Cytogenetic analysis of the hybrid cells was performed using Chinese hamster karyotype guidelines described by Siciliano *et al.* (13). The commonly occurring "Z" marker chromosomes were analyzed with assistance from the publication of Deaven and Peterson (14).

Vectors and Probes. The 5.7-kilobase pSV2Neo vector, containing the neomycin-resistance gene, was used as a marker for transfection into H3-4 cells (15). To study the pattern of marker integration, concatemers of pSV2Neo transfected into H3-4 cells, were used to amplify signal intensity in fluorescence *in situ* hybridization studies, and were prepared as follows: The plasmid (100 μg) was digested to completion with *Bgl* I and then ligated for 3 days at 4°C in 50 μl containing 19 Weiss units of T4 ligase. *Bgl* I digestion yields a variable 3-nucleotide overhang. Religation results in head-to-tail concatemers. Nonligated pSV2Neo was used in transfections where single colonies were isolated for further analysis. pSV2Neo, total placental DNA (Sigma), and a 2-kilobase α-satellite fragment, specific for the centromeric region of chromosome 3 (ONCOR), were used for the subsequent analysis of stable transfectants by fluorescence *in situ* hybridization.

Induction of Breakage and Recombination at Fragile Sites. Expression of FRA3B was induced as follows: the H3-4 hybrid was seeded at 5×10^5 cells per 10-cm plate in folic acid-deficient minimum essential medium (MEM; GIBCO) and treated with aphidicolin (0.4 μM; Sigma) for 24 hr at 37°C. Metaphase cells were prepared using standard techniques and the cells were banded using a trypsin/Giemsa staining technique as described (16). Metaphase cells were scored for the presence of breaks and gaps. Recombinations and deletions in H3-4 cells were induced as follows: Hybrid cells were seeded at a density of 1×10^5 cells per 10-cm plate and grown in the presence of 0.4 μM aphidicolin in folic acid-deficient MEM for 5 days. The cells were then subcultured by seeding at 1×10^5 cells per 10-cm plate in medium without drugs. The cultures were incubated for 24 hr to allow the cells to "recover" and were harvested. Twenty-five trypsin/Giemsa-banded metaphase cells were analyzed to establish the relative frequency of chromosome rearrangements in the cell population.

Transfection of Fragile-Site-Induced Cells. The H3-4 cell line was transfected with the selectable plasmid pSV2Neo using a modification of the calcium-phosphate precipitation technique described by Chen and Okayama (17). Exponentially growing H3-4 cells were treated with trypsin and seeded at 5×10^5 cells per 10-cm plate. Experimental plates were placed in 10 ml of folic acid-deficient MEM and incubated overnight at 37°C in a 5% CO_2/95% air atmosphere. Control plates, grown in MEM, were set up in parallel. Aphidicolin (0.4 μM) was added dropwise to the cells grown in folic acid-deficient medium and the plates were incubated for 30 min at 37°C. Vector DNA (25 μg) was mixed with 0.5 ml of 0.25 M $CaCl_2$ in 0.5 ml of 2× BBS [50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid/280 mM NaCl/1.5 mM Na_2HPO_4; Calbiochem–Behring] and incubated for 10–20 min at room temperature. This calcium phosphate–DNA solution was then added dropwise to both experimental and control cultures, and the plates were incubated for 15–24 hr at 35°C in a 2–4% CO_2/96–98% atmosphere. The precipitate formed gradually during the overnight incubation. Thereafter, cells were rinsed twice with MEM, refed, and incubated for 24 hr at 35–37°C in 5% CO_2 95% air. The cells were subcultured (>1:10 ratio) and incubated for an additional 24 hr. Cells were grown in selective growth medium, containing G418 (400 μg/ml), for 2–3 weeks. Colonies that survived and proliferated during this period were usually transfectants containing stably integrated pSV2Neo. Well-separated colonies (>15 days old), consisting of >100 cells, were isolated with metal cloning cylinders according to methods described by Reid (18). Southern blot analysis was performed as described (19).

Fluorescence *in Situ* Hybridization. The procedure used for *in situ* hybridization is a modification (20) of the method described by Lichter *et al.* (21). Probes were prepared by nick-translation using biotin-labeled (Bio-11-dUTP; Enzo Diagnostics) or digoxigenin-labeled (digoxigenin-11-dUTP; Boehringer Mannheim) dUTP. Hybridization of biotin-labeled probes was detected with fluorescein isothiocyanate-conjugated avidin. Digoxigenin-labeled probes were detected by incubation with rhodamine-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim). Metaphase chromosomes were identified by 4′,6-diamidino-2-phenylindole dihydrochloride staining.

RESULTS

H3-4 Cell Line. Cytogenetic analysis of H3-4 cells using trypsin-Giemsa banding and fluorescence *in situ* hybridization of biotinylated total human placenta DNA revealed that the der(3)t(3;3) was the only human chromosome retained in H3-4 cells (92% of the 25 metaphase cells examined had this chromosome) (Fig. 1). The chromosome number ranged from 20 to 50, and 44% of these cells were hypotetraploid. The composite karyotype of the H3-4 cell line is 23,XX,−2,−3,−4,−6,−7,−8,−10,+Z2,+Z4,+Z8,+Z12,+Z13,+mar1,+mar2,+mar3,t(5;?)(q37;?),t(6;?)(p11;?),del(7)(p11p13),+human der(3)t(3;3)(q21;q26.2).

Induction of Fragile Sites. Chromosomal aberrations were observed in the human der(3) in H3-4 cells exposed to aphidicolin (Table 1). After 24 hr of aphidicolin treatment, 19% of all chromosome aberrations occurred at band p14 of the human der(3); of these, three (8%) were gaps and four (11%) were breaks (Fig. 2). After 5 days of aphidicolin treatment, 18% of all chromosome aberrations occurred at band p14 of the der(3). Of six aberrations in the FRA3B region, one gap, three breaks, one deletion, and one translocation (involving a hamster chromosome) were observed. These results indicate that the FRA3B is induced under these culture conditions.

Aberrations of hamster chromosomes occurred with a high frequency in two specific regions in metaphase cells examined. After 24 hr of aphidicolin treatment, 13% of chromosome aberrations occurred at hamster chromosome 1, bands q26–31, of which three (8%) were gaps and two (5%) were breaks, and 11% occurred in mar2, bands q11–13, of which three (8%) were gaps and one (2%) was a break. After 5 days of aphidicolin treatment, 10% of chromosome aberrations occurred at hamster chromosome 1, bands q26–31, and 16% were observed at bands q11–13 of hamster mar2. The observation of chromosomal aberrations at two specific regions in hamster chromosomes (1q26–31) and mar2 (q11–13) suggests that these bands may represent common fragile sites in the hamster genome.

Analysis of Transfected Clones. Calcium phosphate-mediated transfection of pSV2Neo was performed on H3-4 cells with aphidicolin treatment (experimental) or without aphidicolin treatment (control). A total of 20 clones containing stably integrated pSV2Neo were isolated from two experimental and two control transfections. Cytogenetic anal-

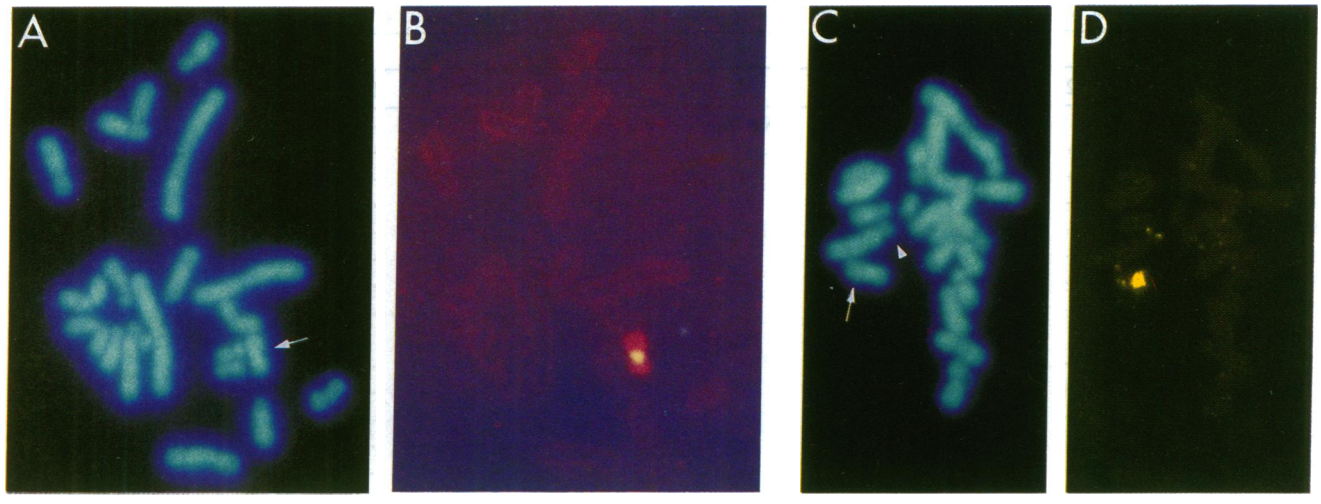

FIG. 1. (*A* and *B*) Fluorescence *in situ* hybridization of total human placental DNA and a chromosome 3 α-satellite centromere-specific probe to H3-4 metaphase cells. (*A*) Counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. (*B*) Detection of hybridized probes. Digoxigenin-labeled human placental DNA, detected with anti-digoxigenin-rhodamine, identifies a single human chromosome in H3-4 cells (red signal). This chromosome was unequivocally identified as human chromosome 3 by cohybridization of the biotin-labeled chromosome 3-specific probe detected with fluorescein isothiocyanate-conjugated avidin (yellow signal at the centromere). (*C* and *D*) Fluorescence *in situ* hybridization of pSV2Neo and chromosome 3 centromere probes to H3-4 cells (TI16b) transfected with pSV2Neo after aphidicolin treatment. (*C*) Metaphase cell counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. (*D*) Hybridization of biotinylated probes was detected with fluorescein isothiocyanate-conjugated avidin. The chromosome 3 α-satellite probe hybridizes to the centromere of the human der(3) and the pSV2Neo probe hybridizes to both chromatids at band p14 (yellow dots in *D* or arrow in *C*). Hybridization of pSV2Neo was also detected at band p13 of a rearranged hamster chromosome t(5;?)(p13;?) (arrowhead).

ysis of trypsin/Giemsa-banded metaphase cells from transfected clones revealed that these cells retained the der(3).

To identify the specific site of pSV2Neo integration in these clones, fluorescence *in situ* hybridization was performed using biotinylated pSV2Neo, total human placental DNA, and a human chromosome 3 centromeric probe (Table 2). Between 5 and 10 metaphase cells were analyzed for each clone. Fluorescent signals from the biotin-labeled pSV2Neo probe were visualized as yellow dots on chromatids. Chromosomal bands were scored as sites of integration only if both chromatids were labeled in at least 2 cells. There were one to three integration sites in each clone. This pattern of integration of pSV2Neo was consistent with Southern blot analysis (data not shown). In 4 of the 13 experimental clones, pSV2Neo was localized to the FRA3B region (Fig. 1). Specific integration into two hamster chromosomes was also observed. pSV2Neo was localized to hamster chromosome 1, bands q26–31, in 9 clones, and to hamster mar2, bands q11–13, in 4 clones. The 7 control clones, however, showed an apparently random pattern of pSV2Neo integration.

In two other control transfections performed without fragile site induction, 24 clones were isolated and analyzed as described above. One and occasionally two or three integration sites were observed in each clone by fluorescence *in situ* hybridization. Twenty-two clones exhibited a random pattern of integration, but 2 clones exhibited pSV2Neo integration in the same region of hamster chromosome 10 (bands q11–13). No integration was seen in the human der(3) or hamster chromosome 1, q26–31, and mar2, q11–13.

Because detailed analysis of the observed pattern of pSV2Neo integration in large numbers of colonies requires independent colony isolation and, thus, would be impractical

Table 1. Aphidicolin-induced chromosome aberrations in the H3-4 somatic cell hybrid and pSV2Neo-transfected clones

Aberration	Chromosome aberration(s)*, no.	Recurring chromosome aberration(s), no.					
		Human 3p14		1q26–31		mar2q11–13	
		No.	%	No.	%	No.	%
Aphidicolin (24 hr)							
Gap	23	3	8.3	3	8.3	3	8.3
Break	13	4	11.1	2	5.5	1	2.7
Deletion	0	0	0	0	0	0	0
Rearrangement	0	0	0	0	0	0	0
Total	36	7		5		4	
Aphidicolin (5 days)							
Gap	9	1	3.0	0	0	1	3.0
Break	21	3	9.0	2	6.0	4	12.1
Deletion	1	1	3.0	0	3.0	0	0
Rearrangement	2	1	3.0	1	0	0	0
Total	33	6		3		5	

Values in % columns refer to percent of total aberrations.
*Twenty-five cells were examined from each experiment.

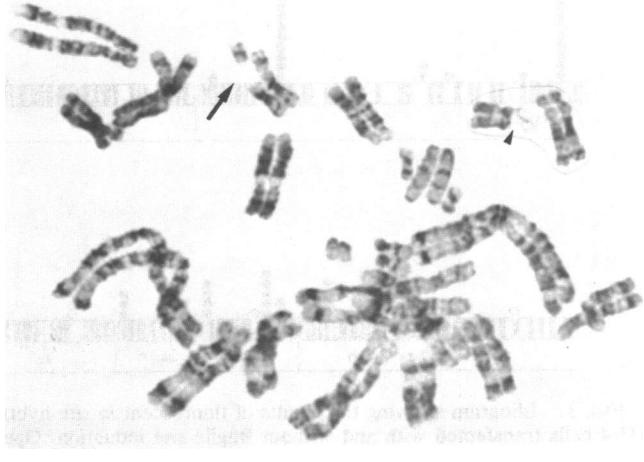

FIG. 2. Trypsin/Giemsa-banded metaphase from the hybrid cell line H3-4 showing chromosomal breaks in the human der(3) at band p14 (arrow) and in hamster chromosome 8 at band p16 (arrowhead).

Table 2. Localization of pSV2Neo in clones from the H3-4 somatic cell hybrid transfected with or without induction of fragile sites

Clone	Chromosome																				
	der(3)	1	2	3	4	5	8	9	10	X	t(5;?)	t(6;?)	del(7)	Z2	Z4	Z8	Z12	Z13	mar1	mar2	mar3
No fragile site induction																					
TII4a	–	–	–	–	–	–	–	q26	–	–	–	–	–	–	–	–	–	–	–	–	–
TII4b	–	–	–	–	–	–	–	–	–	–	q12	–	–	–	–	–	–	–	–	–	–
TII4c	–	–	–	–	–	–	–	–	–	–	–	p13	–	–	–	–	–	–	–	–	–
TII4d	–	–	–	–	–	–	–	q11–13	–	–	–	–	–	–	–	–	–	–	–	–	–
TII4e	–	q46–ter	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII3a	–	q36–42	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII3b	–	q14–22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fragile site induction																					
TI6d	p14	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TI6e	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TI8e	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	q11–13	–
TI8f	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII6d	p14	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII6b	p14	–	–	–	–	–	–		–	–	p13	–	–	–	–	–	–	–	–	q11–13	–
TII6e	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	q11–13	–
TII7b	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII7e	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII7f	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII8d	p14	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TII8e	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	q11–13	–
TII8f	–	q26–31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Analysis of 24 clones from two other transfection experiments performed without aphidicolin treatment showed an essentially random pattern of pSV2Neo integration. There were no integrations in the der(3) or hamster chromosome 1 (q26–31) or mar2 (q11–13). There was, however, integration of pSV2Neo in hamster chromosome 10, bands q11–12, in two clones.

and time consuming, pSV2Neo transfected independent H3-4 colonies were pooled and were analyzed by fluorescence *in situ* hybridization. To facilitate the rapid analysis of large numbers of metaphase cells, a stronger hybridization signal was achieved by transfecting concatemers of pSV2Neo into H3-4 cells (Fig. 3). One hundred cells from 501 pooled colonies (>100 cells in size), transfected under conditions of FRA3B induction, were analyzed and 80% of these cells gave positive signals. A total of 91 signals were observed; of these 14 (15.3%) were identified on the der(3), 15 (16.4%) were localized to hamster chromosome 1, q26–31, and 20 signals (21.9%) were localized to hamster mar2, q11–13. Hybridization to these three regions was highly significant ($P < 0.0001$). No other significant cluster of hybridization was observed. In 100 cells analyzed from 800 pooled colonies transfected without aphidicolin treatment, 51% of cells gave positive signals. No significant hybridization was observed on the der(3) or on hamster chromosomes 1 and mar2, but signal (26%) was observed on hamster chromosome 10, bands q11–12. The fact that signal was not detected in every cell indicates that some integrations of pSV2Neo were below the level of "consistent" detection by fluorescence *in situ* hybridization.

DISCUSSION

Our studies demonstrate that the induction of a fragile site leads to the preferential integration of marker DNA sequences into that fragile site. Thus the pSV2Neo selectable marker was integrated into the FRA3B region in 4 of 13 clones transfected with aphidicolin treatment. This is in marked contrast to our results in control experiments in which none of 31 clones transfected without aphidicolin induction showed integration into the fragile site at 3p14.2. An unex-

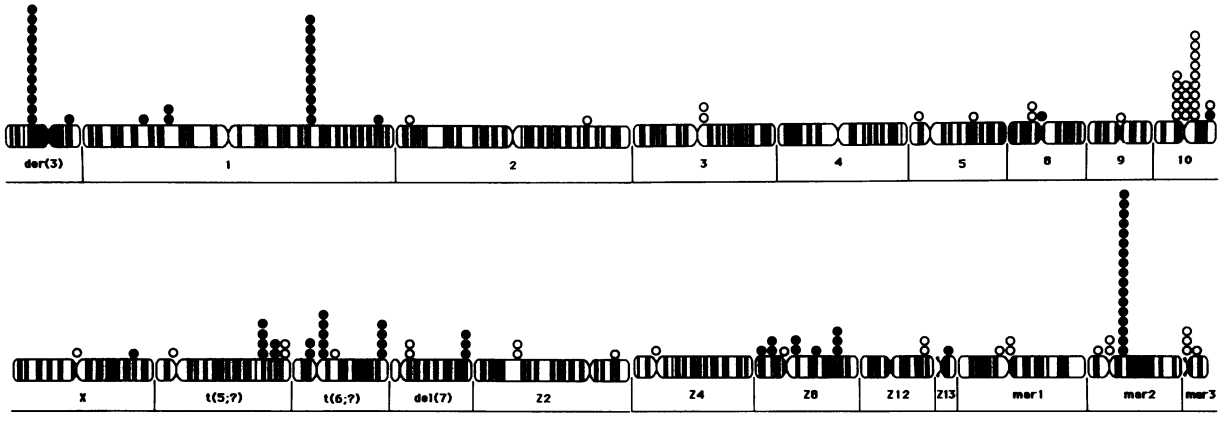

FIG. 3. Idiogram showing the results of fluorescent *in situ* hybridization analysis of pSV2Neo integration in cells from pooled colonies of H3-4 cells transfected with and without fragile site induction. Open circles show the location of pSV2Neo in 100 cells transfected without aphidicolin treatment; significant hybridization was observed at hamster chromosome 10, bands q11–12 ($P < 0.0001$). No significant hybridization was observed at human FRA3B, at hamster chromosome 1, bands q26–31, and mar2, bands q11–13. Solid circles show pSV2Neo localization in 100 cells transfected with aphidicolin treatment; significant hybridization was observed at 3p14 of the human der(3) as well as hamster chromosome 1, bands q26–31, and mar2, bands q11–13.

pected finding was the preferential integration of the vector into two locations in the hamster genome under conditions of fragile site induction, namely, chromosome 1, q26–31 (9 clones), and mar2, q11–13 (4 clones). Our data thus confirm that fragile sites are highly recombinogenic and suggest that these sites may also occur in other mammalian cells.

To validate our findings of a nonrandom pattern of pSV2Neo integration with aphidicolin treatment, we examined 100 cells from the pooled colonies of stable transfectants. Significant integration was observed at FRA3B and hamster chromosome 1 and mar2 ($P < 0.0001$). Initial analysis of 7 control clones suggested that vector integration was random in cells transfected without fragile site induction. To substantiate these findings, a larger number of clones were isolated from two other control transfections. In 24 clones, 22 clones exhibited a random pattern of integration, but 2 clones exhibited pSV2Neo integration in the same region of hamster chromosome 10 (bands q11–13). No integration was seen in the human der(3) or hamster chromosome 1, q26–31, and mar2, q11–13 (see Table 2). Examination of 100 cells from pooled colonies transfected without aphidicolin treatment showed that no signal was present at human 3p14 or hamster chromosome 1 and mar2, but there was significant hybridization at q11–12 of hamster chromosome 10. Integration of pSV2Neo in hamster chromosome 10, in control but not in experimental transfections, may represent a naturally occurring hamster fragile site that may have been "masked" by treatment with aphidicolin. Spontaneous expression of fragile sites has been documented in humans [e.g., the distamycin-inducible site at 16q22 (22)]; whether there are counterparts in the Chinese hamster is not known. These observations suggest the existence of aphidicolin-inducible fragile sites in the Chinese hamster that, to our knowledge, have not been characterized. Identification of sites of fragility in the hamster genome make it possible to examine the evolution and divergence of fragile sites from hamster to man.

We have demonstrated that upon exposure to aphidicolin for 24 hr, H3-4 cells showed a tendency to form chromosomal gaps and breaks at FRA3B (20%). Upon prolonged exposure to aphidicolin (5 days), more breaks than gaps are observed at 3p14 and deletions and rearrangements are also evident. The two sites of specific breakage in the hamster genome (chromosome 1, q26–31, and mar2, q11–13) are also sites of pSV2Neo integration. Warren *et al.* (10) and Glover and Stein (9) reported induction of chromosomal rearrangements at the rare fragile site FRAXA and the common fragile site FRA3B in Chinese hamster × human hybrids containing a single human chromosome. Our results and those of earlier studies (8–10) support the hypothesis that fragile sites result from, or lead to, DNA breakage and that, under appropriate culture conditions, fragile sites can predispose to chromosomal aberrations including deletions and interchromosomal recombination. Demonstration that these sites of fragility preferentially facilitate integration of exogenous DNA challenges assumptions that transfected DNA integrates in a random fashion (23). Knowledge of these integration hot spots may provide important information in gene targeting and viral integration studies.

At present, to our knowledge, there are no data on the structural features of the DNA sequences responsible for specific breakage and recombination at fragile sites; however, a number of theories have been proposed to explain this cytogenetic phenomenon. These include the hypotheses that fragile sites result from unequal crossing-over of pyrimidine-rich DNA sequences (24) or from incomplete chromatin condensation caused by late and incomplete replication (25). Other investigators have suggested that fragile site DNA sequences may be related to chromosomal telomeres (26). To date, to our knowledge, the most direct means of elucidating the nature of fragile sites (i.e., to clone and sequence these regions) has not yet been achieved. The preferential integration of pSV2Neo in the fragile site region can be used to isolate the DNA sequences flanking the fragile site and, thus, represents an approach to cloning chromosomal fragile sites.

Note Added in Proof. Subsequent to submission of this paper several groups have reported cloning of the FRAXA in YAC vectors (27–29).

We thank Drs. Janet D. Rowley and Manuel O. Diaz and Noel Bouck for valuable comments, Dr. Harry Drabkin for providing the H3-4 cell line, and Yogesh Patel and Anthony Fernald for technical assistance. This research was supported by National Institutes of Health Grant CA41644 (M.M.L. and T.W.M.). M.M.L. and T.W.M. are Scholars of the Leukemia Society of America. F.V.R. was supported by Grant CA42557 (J. D. Rowley) from the National Institutes of Health.

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