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FRAGILE X CHROMOSOME ASSOCIATED WITH FAMILIAL SEX-LINKED MENTAL RETARDATION: EXPRESSION IN FIBROBLAST CULTURE

by

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ABSTRACT

A form of familial sex-linked mental retardation has been associated with the expression of a fragile site near the terminal end of the long arm of the X chromosome. Previous reports on the fragile X chromosome showed expression of the fragile site to be limited to chromosome preparations from peripheral blood lymphocytes of mentally retarded males and their female relatives in families in which the disorder was segregating. Fragile site expression has also been shown to be a function of the medium employed in cell culture. The fragile X chromosome could only be demonstrated in lymphocytes cultured in medium 199 or media deprived of folic acid.

This study was undertaken to develop a method for demonstrating the fragile X chromosome in cultured skin fibroblasts. Fibroblast cell lines from five patients (two mentally retarded males, two obligate carrier females, and a potential carrier female) from a family in which familial sex-linked mental retardation was known to be segregating were established and routinely maintained in a complete culture medium. Forty-three hours prior to chromosome harvest, cells from each patient were transferred to media deficient in folic acid. Under conditions of folic acid deprivation, it was possible to elicit expression of the fragile X chromosome in skin fibroblasts from all five patients studied. No fragile X chromosomes were detected in fibroblasts from three normal control subjects.

In a preliminary assessment of the reliability of the fibroblast method, three patients (two mentally retarded males and a potential carrier female) from a second unrelated family in which the disorder is known to be segregating were studied with this method. The fragile X chromosome could be demonstrated in fibroblasts from both of the retarded male patients but could not be demonstrated in fibroblast chromosome preparations from the potential carrier female.

Lymphocytes for all patients studied were grown under similar folate deprived conditions for the purpose of comparing the effectiveness of fibroblast culture with lymphocyte culture in demonstrating the expression of the fragile X chromosome. Neither tissue was shown to consistently provide a higher frequency of expression of the fragile X chromosome.

In addition to folate deprivation, it was shown that two other features of the fibroblast method influenced the frequency of expression of the fragile X chromosome. The fragile site was expressed at a significantly higher frequency in chromosome preparations in which the chromosomes were not severely contracted. The frequency of expression in fibroblasts was also shown to be significantly higher with a hypotonic treatment at chromosome harvest using 1% NaCitrate rather than 0.075M KCl.

Because fragile site expression was shown to be a function of the degree of chromosome condensation, two agents, 5-BrdU and actinomycin-D, were studied to examine their

decondensation effects on the frequency of expression. Neither BrdU nor actinomycin D proved effective in accentuating the frequency of expression.

Since fibroblasts behave much like amniocytes in terms of cell culture and chromosome harvest, the development of a method for demonstrating the fragile X chromosome in cultured skin fibroblasts is a step toward the prospect of reliable antenatal diagnosis of familial sex-linked mental retardation associated with a fragile X chromosome.

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ABBREVIATIONS

Tissue culture media:

MEM -Eagle's Minimal Essential Medium. Complete tissue culture medium containing 15% fetal calf serum.

M199 -Medium 199. Tissue culture medium containing 5% fetal calf serum and low in folic acid (0.01 mg/L).

MEM-FA -Eagle's Minimal Essential Medium without folic acid. Tissue culture medium containing 5% fetal calf serum and no folic acid.

MEM-Ad -Eagle's Minimal Essential Medium without adenine. Tissue culture medium containing 5% fetal calf serum and deficient in adenine.

A detailed description of the various tissue culture media is presented in section 2.12 and Appendix II.

Chromosome banding methods:

AgNOR-banding -Sat-banding or silver nucleolar organizer-banding. Ammoniacal-silver staining of the satellite regions of the D and G group acrocentric chromosomes, and generally thought to be specific for the rDNA sequences associated with nucleolar organizer activity.

C-banding -Constitutive heterochromatin banding. A chromosome banding method employing giemsa staining following denaturation pretreatments in acid and alkaline and a period of renaturation in 2xSSC. Specifically stains centromeric heterochromatin and the paracentromeric heterochromatic regions on chromosomes 1, 9, 16, and the long arm of the Y chromosome.

G-banding -Giemsa banding. Chromosome banding accomplished with a variety of methods, belonging to two major technical categories. 1. GTG G-banding, giemsa staining after pretreatment with a proteolytic enzyme (pronase or trypsin). 2. ASG G-banding, giemsa staining after pretreatment with acid or alkaline followed by saline.

R-banding -Reverse banding. A chromosome banding method employing giemsa staining following denaturation pretreatment with heat and weak acid, or acridine orange staining following the incorporation of BrdU during cell culture. Banding pattern is essentially the reverse of the G-banding pattern.

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Other abbreviations:

BrdU 5-Bromo-2'-Deoxyuridine. A base analog of thymidine.

EDTA Ethylene-Diamine-Tetra-Acetic acid (disodium salt). A chelating agent of calcium (Ca^{+2}) and magnesium (Mg^{+2}).

EGTA Ethylene Glycol-bis-(β-amino-ethyl ether)N,N'-Tetra-Acetic acid. A chelating agent useful in the determination of calcium (Ca^{+2}) in the presense of magnesium (Mg^{+2}).

FCS Fetal Calf Serum.

HBSS Hank's Balanced Salt Solution. A standard saline solution.

PHA Phytohemoglutinin (M Form). Plant lectin used for mitogenic stimulation of lymphycytes in cell culture.

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CHAPTER 1

INTRODUCTION

1.1 Familial sex-linked mental retardation.

Mental retardation is included in the diagnosis of some 40 different sex-linked genetic diseases (McKusick, 1978; Jennings et al., 1980). Many of these disorders are distinguishable on the basis of physical and biochemical abnormalities. However, until recently, one of the most common forms of familial sex-linked mental retardation, the Martin-Bell syndrome (Martin and Bell, 1943), has lacked any clearly definable physical or biochemical abnormality. Since 1943, several large pedigrees have been reported documenting the segregation of this non-specific form of familial sex-linked sex-linked mental retardation (Renpenning et al., 1962; Dunn et al., 1963; Opitz et al., 1965; Lehrke, 1974; Deroover et al., 1977; Ruvalcaba et al., 1977).

1.11 Prevalence. Non-specific sex-linked mental retardation represents about 70% of all diagnosed sex-linked mental retardation and is thought to be responsible for the documented excess of males in the mentally retarded population (reviewed by Herbst, 1980). Turner and Turner (1974) proposed that this non-specific form of familial sex-linked mental retardation probably accounts for one in five institutionalized mantally retarded males with IQ's between 30 and 55. On an overall population base, they calculated a frequency of 0.74/1000 males. A recent study has indicated that the prevalence of this disorder may be higher; using

data from the British Columbia Health Surveillance Registry, Herbst (1980) calculated a prevalence of 1.83/1000 males.

1.12 Association with a fragile X chromosome. Lubs (1969) first reported the presence of a fragile X chromosome in a family in which non-specific familial sex-linked mental retardation was segregating. He observed the fragile X chromosome in four mentally retarded males and two normal females in three generations of the family. The fragile X chromosome appeared to have a constriction, or fragile site, near the terminal end of the long arm of the chromosome. (Fig. 1) and was expressed in only a portion of the cells examined from a patient. Since the first report, the association of a fragile X chromosome with familial sex-linked mental retardation has been documented by a number of other investigators (Giraud et al., 1976; Harvey et al., 1977; Howard-Peebles et al., 1979; Sutherland and Ashforth, 1979; Turner et al., 1980).

In 1977, Sutherland showed that expression of the fragile site on the X chromosome in peripheral blood lymphocytes was dependent on the medium used in cell culture. Only in Medium 199, of the tissue culture media examined, was the demonstration of the fragile X chromosome possible. This finding prompted a number of investigators to re-study the chromosomes of members of several families in which the disorder was known to be segregating in order to look for the presence of the fragile X chromosome. In several of these families the fragile X chromosome was found (Dunn et al., 1963, re-

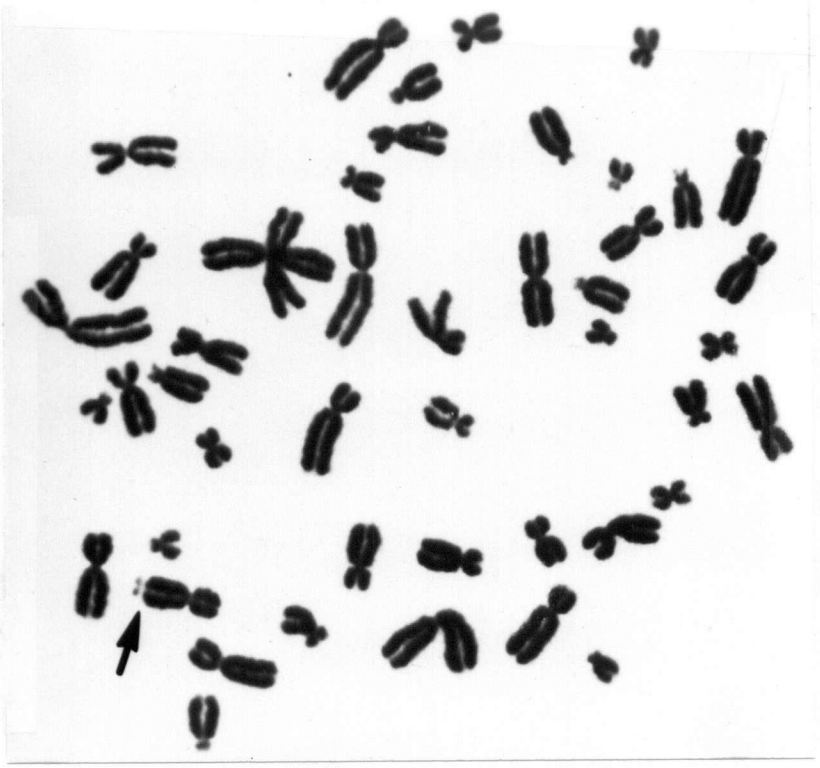

FIGURE 1

Fragile X chromosome (arrow) of a mentally retarded male patient from a family in which familial sex-linked mental retardation is segregating.

studied by Dunn et al., 1980, and Jacobs et al., 1980; Ruvalcaba et al., 1977, restudied by Jennings et al., 1980; Bowen et al., 1978, restudied by Martin et al., 1980).

Sutherland's discovery also encouraged a number of investigators to re-examine their clinical files for case reports of non-specific familial sex-linked mental retardation, and to study the chromosomes of these families using the appropriate culturing conditions. Soudek et al. (1980) found that 10 of 22 families restudied exhibited the fragile X chromosome. Turner et al. (1980) found the fragile X chromosome to be segregating in seven of 23 families, and, in another recent study, Jacobs et al. (1980) found the fragile X in six of seven families. The one family that did not exhibit the marker in the Jacobs et al. study was the family originally reported by Renpenning et al. (1962). Because non-specific familial sex-linked mental retardation has frequently been referred to as "Renpenning syndrome" and taken as synonymous with "Martin-Bell syndrome", it is important now that the distinction be made between Renpenning syndrome and "familial sex-linked mental retardation associated with the fragile X chromosome".

1.13 Clinical manifestations of familial sex-linked mental retardation. The need for a distinction between familial sex-linked mental retardation associated with the fragile X chromosome and other forms of non-specific sex-linked mental retardation has been underscored by the recent association of some phenotypic abnormalities with the fragile X chromosome. In general, these phenotypic abnormalities occur

irregularly among patients. While no clinical feature can as yet be considered pathognomonic for sex-linked mental retardation associated with the fragile X chromosome, some characteristics have been noted by an increasing number of investigators and are clinically significant. Among them are macroörchidism, prognathism and prominent ears, high arched palate, and speech impairment incompatible with the degree of intellectual deficit (Herbst, 1980; Jacobs et al., 1980; Jennings et al., 1980; Soudek et al., 1980). Other features, occurring less frequently, include dermatoglyphic and CNS irregularities and forms of psychosis.

Macroörchidism, or megalotestes, associated with the segregation of familial sex-linked mental retardation has received the attention of a number of investigators (Ruvalcaba et al., 1977; Bowen et al., 1978; Cantu et al., 1978; Turner et al., 1978; Howard-Peebles and Stoddard, 1979, 1980; Jacobs et al., 1979, 1980; Sutherland and Ashforth, 1979). While it appears that patients suffering from familial sex-linked mental retardation associated with the fragile X chromosome may or may not exhibit macroörchidism, very few, if any, mentally retarded patients with macroörchidism have been reported in whom the fragile X chromosome is not present (Sutherland and Ashforth, 1979; Howard-Peebles and Stoddard, 1980; Jacobs et al., 1980; Jennings et al., 1980). Further documentation will be necessary before macroörchidism can be used as a consistent morphological indicator for chromosome studies, but at present it is being used successfully as a pre-screening test

for re-examining institutionalized mentally retarded males for the fragile X chromosome (Howard-Peebles, personal communication; Soudek and Partington, personal communication).

On the basis of the re-examination of clinical case files and previously reported families, with familial sex-linked mental retardation, for the presence of the fragile X chromosome, 52 of 86 families have demonstrated the marker X chromosome (Table I). Although this frequency may reflect some ascertainment bias, it appears that the fragile X chromosome is segregating in at least 50% of cases originally categorized as non-specific familial sex-linked mental retardation. This frequency suggests that familial sex-linked mental retardation associated with the fragile X chromosome may be second only to Down syndrome as a form of mental retardation associated with a specific chromosome abnormality.

1.14 Fragile X expression in mentally retarded males and their female relatives. Table I shows the number of mentally retarded males, obligate carrier females, and potential carrier females, having a 50% risk of carrying the disorder, who have been reported in the literature as demonstrating the fragile X chromosome in chromosome studies of cultured peripheral blood lymphocytes. While it appears that analysis of peripheral blood lymphocytes cultured in M199 provides a reliable and satisfactory method for determining if a mentally retarded male is carrying the fragile X chromosome, it has proven less satisfactory in determining female carrier status. In the original report

TABLE I

Expression of the fragile X chromosome in members of families reported in the literature.

No. Families Marker Positive / No. Families Studied	No. Patients Marker Positive / No. Patients Studied (Frequency Range)			Reference
	Mentally Retarded Males	Obligate Carrier Females	Potential Carrier Females (50% risk)	
6/6	5/5 (18-50%)	2/2 (?)	1/1 (20%)	Giraud et al. (1976)
4/4	14/14 (8-41%)	4/6 (2-24%)	2/2 (7%,17%)	Harvey et al. (1977)
3/6	9/9 (4-33%)	1/2 (15%)	—	Howard-Peebles et al. (1979) Howard-Peebles & Stoddard (1979, 1980)
6/7	36/36 (7-50%)	4/17 (3-12.9%)	6/20 (1.5-21.4%)	Jacobs et al. (1980)
2/3	8/8 (1.5-18%)	1+3?/6 (0.5-4.5%)	2/3?+ (1.5%,8.5%)	Jennings et al. (1980)

continued...

No. Families Marker Positive / No. Families Studied	No. Patients Marker Positive / No. Patients Studied (Frequency Range)			Reference
	Mentally Retarded Males	Obligate Carrier Females	Potential Carrier Females (50% risk)	
1/1	4/4 (12-33%)	1/2 (28%)	1/5 (3%)	Lubs (1969)
1/1	2/2 (3.0%,15.5%)	3/3 (0.5-1.5%)	3/3 (0.5-6.5%)	Martin et al. (1980)
1/1	1/1 (7%)	0/1	0/1	Turleau et al. (1979)
7/23	25/25 (24-45%)	4/12 (?)*	8/20 (?)*	Turner et al. (1980)
9/22	27/29 (2-29%)	2/4 (?)*	2/5 (?)*	Soudek et al. (1980)
12/12	21/21 (4-44%)	5/13 (2.5-19.5%)	9/17 (6-26%)	Sutherland (1979c)
52/86	152/154	29/68	34/77	TOTALS

\+ unclear status in manuscript
* frequencies not given in manuscript

of the fragile X chromosome by Lubs (1969), all four affected males expressed the fragile X chromosome, at frequencies of 12 - 33% of the cells examined. Lymphocytes from a 59 year old obligate carrier female did not exhibit the fragile X chromosome, while those from another carrier, aged 24, exhibited the marker in 28% of the cells. In the 22 year old sister of the latter patient, the fragile X chromosome was present in only 3% of the cells.

In the study of lymphocytes from six obligate carrier females from three families, Harvey et al., (1977) were able to demonstrate the marker in all but two at frequencies ranging from 2% to 24%. All 14 mentally retarded males studied demonstrated the fragile X at frequencies between 8 and 41%.

In the 11 studies of 86 families reported in Table I, virtually all of the affected males studied demonstrated the fragile X chromosome. The frequency of expression fell within a wide range: 1.5 - 50%. The fragile X chromosome has not been demonstrated in any normal related male controls (brothers and uncles) when they have been studied (Jacobs et al., 1980; Soudek et al., 1980; Turner et al., 1980).

However, it seems that reliable determination of female carrier status by the use of the fragile X chromosome is not possible. Less than half of the older age obligate carrier females demonstrated the fragile X chromosome in the studies reported in Table I. About half of the potential carrier females demonstrated it. The latter observation is consis-

tent with the proportion expected from Mendelian transmission of a sex-linked abnormality to daughters of obligate carrier females. The frequencies of expression of the fragile X chromosome in obligate and potential carrier females are generally much lower than those obtained in related mentally retarded males. Fragile X expression in females also appears to show an age dependency. In his study of 30 carrier females, Sutherland (1979c) concluded that the frequency of marker expression in carriers rapidly declines in females over the age of 25 yrs., and that the marker is frequently not observed in older obligate carrier females (30+ yrs.).

One interesting aspect of these families is that occasionally the pedigrees will contain a female who is of low normal intelligence, and who also carries the fragile X chromosome (Giraud et al., 1976; Howard-Peebles et al., 1979; Sutherland, 1979c; Jacobs et al., 1980). Only a few of these females have been studied in detail, but the limited evidence suggests that there may be a positive correlation between the frequency of expression of the marker chromosome and the degree of their intellectual deficit; the lower the intelligence of the heterozygous female, the greater is the frequency of expression of the fragile X chromosome observed (Jacobs et al., 1980; Soudek et al., personal communication).

An explanation for the occurrence of heterozygous females with low intelligence may be that these particular individuals represent an extreme in the distribution of

paternal and maternal X chromosome inactivation. In these females, the X chromosome carrying the fragile site, and therefore the the abnormal genes responsible for mental retardation, may be active in a high proportion of cells.

In an attempt to determine if the pattern of X chromosome inactivation in such females was unusual, Uchida (personal communication) examined the late replication patterns of X chromosomes in two carrier females with normal intelligence who exhibited the marker and compared them to the patterns in two low intelligence carrier females who also exhibited the marker. She determined that both the normal X and the fragile X were late replicating in an equal proportion of cells in the normal female carriers. [This pattern of random inactivation has also been reported by Martin et al. (1980).] In the two dull carrier females, the normal X was preferentially inactivated about three times more frequently than the fragile X.

While low intelligence carrier females have been reported infrequently in the literature, and the precise etiology of their low intelligence remains obscure, further studies may clarify the relation between the expression of the fragile X chromosome in carrier females, X chromosome inactivation, and the infrequent manifestation in some female patients of a degree of mental deficiency.

1.2 Heritable fragile sites.

1.21 Distribution. In addition to the fragile X chromo-

some, heritable fragile sites on human chromosomes 2, 9, 10, 11, 12, 16, 17, and 20 have been reported (reviewed by Sutherland, 1979b). The fragile sites on chromosome No.'s 2, 10, 16, and 20 have been shown to be inherited in simple Mendelian fashion. The autosomal fragile sites have been demonstrated in phenotypically normal individuals as well as abnormal individuals, but they have not been associated solely with any specific phenotypic disorder (Schmid and Vischer, 1969, Bühler et al., 1970; Magenis et al., 1970; Reeves and Lawler, 1970; Fraccaro et al., 1972; Ferguson-Smith, 1973; Oliver et al., 1978; Sørensen et al., 1979). Therefore, they have generally been regarded as normal chromosome variants. The estimated frequency of occurrence of heritable fragile sites in the population is 1 in 444 or 0.2% (Hecht and Kaiser-McCaw, 1979).

These fragile sites usually appear as achromatic discontinuities traversing both chromatids of one of the arms of a metaphase chromosome. They are generally located at a median interstitial position on the chromosome arm and appear very much like secondary constrictions that have been associated with nucleolar organizer activity in classical plant and animal cytogenetics. Fragile sites, as distinct from secondary constrictions, have not been described in any other mammalian species (Sutherland, 1979b).

1.22 Cytogenetics. While research on heritable fragile sites other than the one on the X have not yielded any information of direct practical applicability in genetic or reproductive counseling, it has resulted in better under-

standing of the fragile site in relation to irregular chromosome structure and behavior.

Heritable fragile sites occur, not randomly, but at particular regions on chromosomes (Giraud et al., 1976; Sutherland, 1979b). Chromosome banding studies have permitted assignment of each of the fragile sites to a particular region on a chromosome arm. The reported sites are: 2q11, 9q12, 10q23, 11q23, 12q13, 16q22, 17p12, 20p11, and Xq27-28. The best documented of these are the sites at 2q11, 10q23, 16q22, and Xq27-28 (Sutherland, 1979b).

Minor discrepancies in the precise location of some fragile sites have been reported. For instance, Sutherland (1979b) reported the locus of the fragile site on chromosome No. 10 as 10q23 on the basis of G-banding. Using R-banding, Giraud et al. (1976) reported the locus as 10q242. Discrepancies in the location of a discontinuity seem to be a function of the different banding methods employed (Savage, 1977). Precise location of the fragile site on the X chromosome has been impeded by the fact that the fragile site is located very near the terminal end of the long arm of the chromosome, and conventional G-banding does not clearly distinguish the G-negative band at Xq28 from the G-positive band at Xq27. R-banding results seem to indicate that the fragile site is located in the proximal portion of band q28 (Turleau et al., 1979).

There is evidence to suggest that expression of the

fragile site on the X chromosome is due in part to an abnormality in the chromosome condensation process at mitosis and not due to a structural alteration of the X chromosome such as a translocation. Banding studies of chromosomes with fragile sites have shown that the banding patterns of the chromosome arms adjacent to the fragile sites are consistent with the normal banding patterns for that chromosome. R and G-banding studies have shown that the banding pattern of the fragile X chromosome is consistent with the banding pattern of the normal X (Harvey et al., 1977; Sutherland, 1979a; Turleau et al., 1979; Howard-Peebles and Stoddard, 1980). Because of the striking similarity in appearance between the satellites on the terminal end of the fragile X chromosome and the short arm satellites on the D and G group chromosomes, it could be suggested that the satellite is translocated from one of these chromosomes. However, a study by Turleau et al. (1979) showed the Xq27-28 fragile site to be C-band negative, or lacking any translocated centromeric heterochromatin. In addition, studies using silver staining, which is specific for nucleolar regions (AgNOR banding), did not reveal any nucleolar organizer activity at the Xq27-28 fragile site (Sutherland and Leonard, 1979; Howard-Peebles and Howell, 1979; Turleau et al., 1979). The fact that satellite stalks from acrocentric chromosomes known to be involved in translocation are still stainable with this technique (Neu et al., 1976), suggests that it is unlikely that the fragile X includes material translocated from

one of the D or G group chromosomes.

DNA replication studies have provided more direct evidence that the constriction in the chromosome arm characteristic of a fragile site may indeed be the result of abnormal chromosome condensation. All of the known heritable fragile sites occur in regions that are normally late replicating, i.e., replication occurs within the last five hours of DNA synthesis (Kondra and Ray, 1978). Abnormally late replicative behavior has been reported in connection with the expression of the 2q12 fragile site. In experiments using tritiated thymidine, Bühler et al. (1970) found that the 2q12 fragile region was replicated later than normal. The 9q12 fragile region has also been shown to be abnormally late replicating (Schmid and Visher, 1969). Since incomplete condensation has been shown to parallel late replication (Stubblefield, 1964; Zakharov and Egolina, 1968), it can be concluded that defective condensation is responsible, at least in part, for the expression of some of the heritable fragile sites.

Recent work by Sutherland (1979a) on specific tissue culture requirements for heritable fragile site expression suggests that the commitment to fragile site expression is more likely due to cellular events prior to, or early in, the replicative period of the cell cycle. This will be discussed further in the following section.

1.23 Tissue origin and culture conditions. Virtually all observations of heritable fragile sites have been confined

to studies of peripheral blood lymphocytes. At the inception of this study, none of the heritable fragile sites had been demonstrated in cultured skin fibroblasts, with the exception of the fragile site at 2q12 (Fraccaro et al., 1972; Ferguson-Smith, 1973; Sutherland, 1979b). In the few instances where bone marrow cells have been studied, fragile sites were demonstrable only at 16q22 and Xq27-28 (Magenis et al., 1970; Sutherland, 1979b). However, the frequencies of expression of these fragile sites in fibroblasts and bone marrow preparations were frequently lower than those found in lymphocyte culture for the same patient (Sutherland, 1979b).

Some significant work on the influence of different tissue culture media on the expression of fragile sites in peripheral blood lymphocytes has recently been reported by Sutherland (1977, 1979a). Preliminary studies (Sutherland, 1977) had shown that the frequency of expression of the fragile sites at 2q21, 10q23, and Xq27-28 was substantially higher if the cells were cultured in Medium 199 rather than other tissue culture media such as Eagle's MEM, RPMI 1640, Ham's F10, or CMRL 1969. The Xq27-28 fragile site could only be demonstrated in M199. More recently, Sutherland (1979a) suggested that it is the relatively low level (0.01 mg/L) of folic acid in Medium 199 that is responsible for the increase in the frequency of expression of some fragile sites. He showed that if parallel lymphocyte cultures were established in media containing graduated concentrations of folic acid (0.0 to 0.5 mg/L folic acid), the frequency of

expression rapidly decreased as the concentration of folic acid increased. Some fragile sites, namely 2q12, 10q23, and Xq27-28, were particularly sensitive to altered concentrations of folate, while the frequency of expression of the fragile site at 16q22 was not affected at all.

Increasing concentrations of thymidine or 5-BrdU, a base analog of thymidine, (0.0 to 3.0 mg/L) produced results comparable to those observed with increasing concentrations of folate for the fragile sites at 2q12, 10q23, and Xq27-28. Thymidine and BrdU were, however, less effective than folic acid at inhibiting expression of the fragile sites.

Further evidence in support of a hypothesis that it is the low folate level in M199 that is responsible for the increased frequency of fragile site expression came from experiments in which lymphocytes were cultured in media containing normal levels of folate plus the folic acid antagonist, methotrexate (Sutherland, 1979a). Under these culture conditions the frequency of expression increased.

Sutherland postulated that the metabolic mode of action of folate deprivation on fragile site expression was through pyrimidine biosynthesis, specifically in the conversion of dUMP to dTMP mediated by the coenzyme 5,10-meTHFA: uridine monophosphate (dUMP)+5,10-methylene tetrahydrofolate (5,10-meTHFA)→thymidine monophosphate (dTMP)+dihydrofolate (DHFA). If this reaction is inhibited mildly, the resulting deficiency in dTMP, thymidylate, would restrict DNA synthesis and potentially lead to lesions in the chromosomes. That this

reaction may be the area of metabolism involved in fragile site expression has been supported by the expression enhancing effects of the folate antagonist methotrexate (Sutherland, 1979a). Methotrexate blocks the conversion reaction of DHFA to THFA by inhibition of dihydrofolate reductase (Erbe, 1975). This reaction is tightly integrated with the production of the coenzyme, 5,10-meTHFA.

Sutherland (1979a) showed that the time of adding either folic acid or thymidine to the lymphocyte cultures also affected the frequency of fragile site expression. Both folic acid and thymidine were most effective in inhibiting expression if they were added at least 24 hrs. prior to chromosome harvest. The inhibitory effects substantially decreased when either factor was added closer to chromosome harvest. These findings seem to indicate that culture media influences on fragile site expression are most effective prior to, or early in, the S-phase of the cell cycle preceeding chromosome harvest. This timing effect is consistent with the postulated mode of action of folate deprivation, namely, reduced pyrimidine biosynthesis. Low folate levels could lead to substantially reduced levels of thymidylate available for DNA synthesis.

Sutherland further suggested that extending the culture period in media low in folic acid from 72 to 96 hrs. for those fragile sites sensitive to folate deprivation (2q12, 10q23, Xq27-28) improves the frequency of expression of the marker chromosomes, and that reducing the concentration of fetal calf serum (FCS) to 5% from the 10 - 20% FCS levels used

conventionally in lymphocyte culture, also improved the frequency of marker chromosome expression. With particular reference to the Xq27-28 fragile site, other investigators (Howard-Peebles and Pryor, 1979; Gerrard and Fox, personal communication) have also suggested that reduced levels (0 - 5%) of FCS improve the frequency of expression. Some investigators have cautioned, however, that extremely low levels of FCS can substantially impair cell growth and lead to a virtually unscorable mitotic index (Turleau et al., 1979).

Sutherland also showed that the frequencies of expression of the fragile sites at 2q12 and Xq27-28 were sensitive to the pH of the culture media with optimal expression between pH 7.4 and 7.6. This is slightly more alkaline than the normal culturing pH of 6.8 to 7.2. Fragile sites at 10q23 and 16q22 were not sensitive to alterations in pH. Based on response by the various fragile sites to folate, and thymidine, deprived culturing conditions, as well as an optimal expression of some fragile sites at a particular pH, Sutherland (1979a) proposed a classification of fragile sites: those that are sensitive to folate deprivation (e.g. 2q21, 10q23, and Xq27-28), those that are resistant to folate deprivation (16q22), those that are pH dependent (2q21 and Xq27-28), and those that are not pH dependent (10q23 and 16q22). Accordingly, the fragile site at Xq27-28 would be classified as both folate and pH dependent.

1.3 Rationale for the present study.

While earlier studies of the fragile X chromosome in

families in which familial sex-linked mental retardation was segregating established that the prevalence of the disorder was high, they also defined what appeared to be severe limitations to the diagnostic usefulness of the fragile X chromosome. For instance, the fragile X chromosome, while specific for the disorder, was found to be generally expressed only in a portion of the metaphases scored from cultured peripheral blood lymphocytes from affected males. The presence of the marker chromosome in many obligate or potential carrier females, especially those over 25 yrs. of age, frequently proved difficult or impossible to show. Furthermore, the expression of the fragile X chromosome in lymphocytes could be seen only under certain culturing conditions. Finally, expression of the marker X chromosome could not be demonstrated in skin fibroblast metaphases (Hecht and Kaiser-McCaw, 1979; Sutherland, 1979c).

The failure to find the fragile X chromosome in cultured skin fibroblasts implied that its demonstration in cultured amniocytes would unlikely, and the fragile X chromosome would therefore not be a useful chromosome marker in antenatal diagnosis of familial sex-linked mental retardation.

This study was undertaken to: 1) develop a method for demonstrating the fragile X chromosome in cultured skin fibroblasts, examining some of the factors that influence a reliable determination of the frequency of expression in this tissue, and 2) examine the effects in both cultured lympho-

cytes and fibroblasts of specific supplemental media factors which, because of their abilities to interact with chromosome components, might be useful in accentuating the frequency of expression of the fragile X chromosome in tissue culture. In addition, it was hoped that the results from this study would provide further information on the structural and functional properties of fragile chromosomes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sources of tissue samples and tissue culture media.

2.11 Subjects. Blood and skin biopsy specimens were obtained from eight individuals from two unrelated families in both of which there is clinical and genetics evidence for familial sex-linked mental retardation. For convenience these individuals are referred to as Patients A, B, C, D, E, F, G, and H. Family No. 1 is part of a large pedigree originally reported by Dunn et al., (1963). Family No. 2 is a case on file with the Department of Medical Genetics, University of British Columbia (file #02438). Pedigrees of Families No. 1 and 2, including the identities of the members studied are presented in Figure 2.

Family No. 1 is of German Catholic background. The parents of Patient A immigrated to North America from the Ukraine in the late 19th century. She is considered to be an obligate carrier female of normal intelligence, who has had three mentally retarded sons and five normal daughters, three of whom gave birth to retarded sons. Patient C is one of the mentally retarded sons of Patient A, and Patient B is one of her obligate carrier daughters. Patient D is a mentally retarded son of Patient B. Patient E is a daughter of Patient B and has a 50% risk of being a carrier female.

Family No. 2 is of Dutch Mennonite background and is also descended from immigrants from the Ukraine. Patients F and G

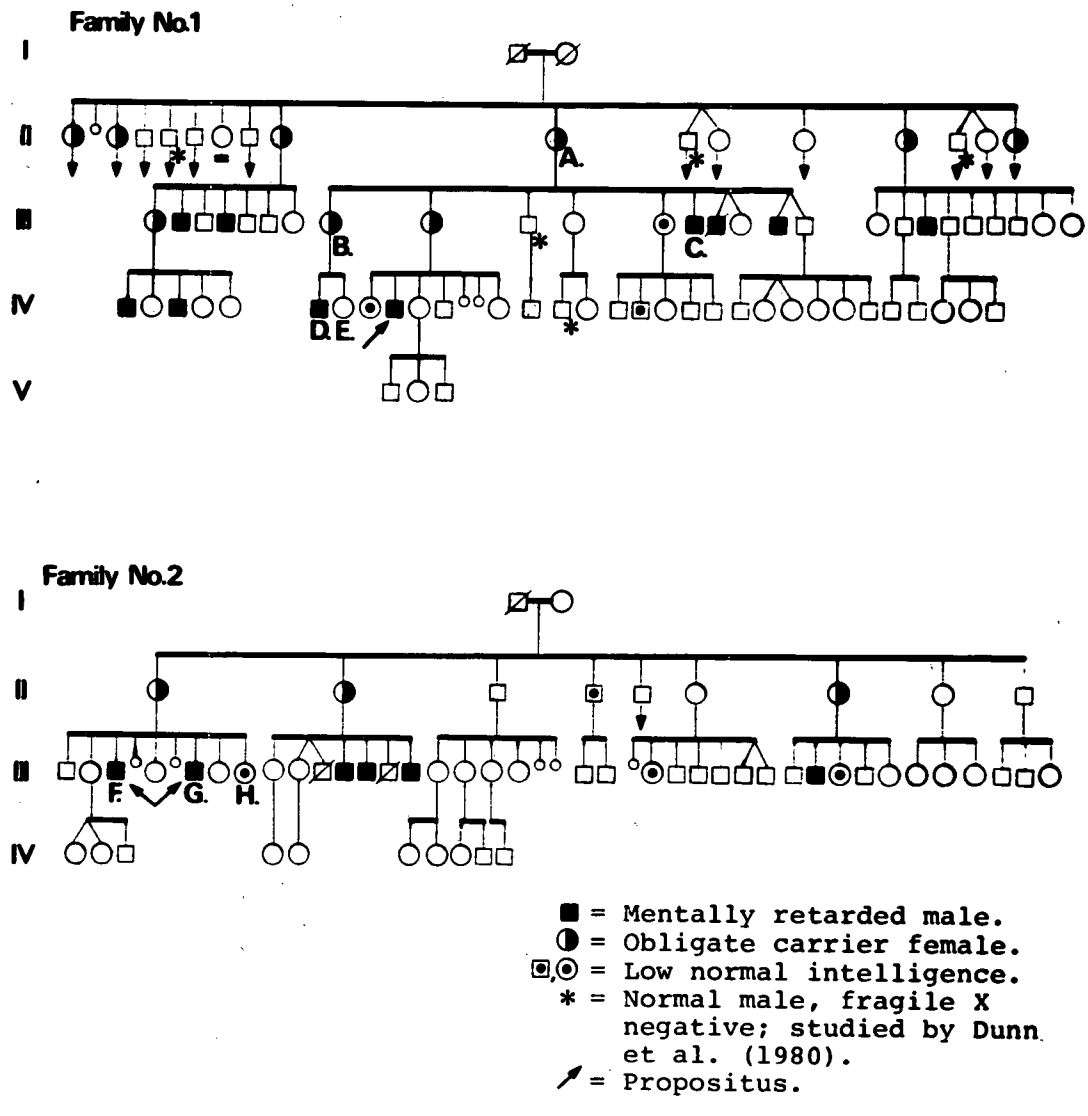

FIGURE 2

Pedigrees of Family No. 1 (Patients A - E) and Family No. 2 (Patients F - H) showing segregation of familial sex-linked mental retardation, e.g. transmission of the disorder through normal heterozygous females to affected sons. The pedigree presented for Family No. 1 is reduced in part from the original report on the family (Dunn et al., 1963).

are mentally retarded brothers, and Patient H is their sister. She is a potential carrier female who is probably of low normal intelligence (personal communication from mother of Patient H and case file).

Control specimens were obtained from three individuals unrelated to either family studied. They were matched for sex and age to the two younger affected males (Patients D and G) and to a potential carrier female (Patient E). The individual ages of patients and controls are presented in Table II.

Blood samples were obtained by venepucture, and skin specimens were obtained by scissor snip biopsy in all cases with informed consent (Appendix A).

2.12 Tissue culture media. Four different tissue culture media were used in experiments to be reported on peripheral blood lymphocytes and established fibroblast cell lines. These were:

MEM: Eagle's Minimal Essential Medium with Earle's Salts & L-glutamine (Gibco, Lot No. R892106) supplemented with 15% FCS buffered with sodium bicarbonate (folic acid at 0.01 mg/L).

M199: Medium 199 with 25 mM HEPES buffer, Hank's Salts & L-glutamine (Gibco, Lot No. A891012), supplemented with 5% FCS and adjusted to pH 7.6 with 7.5% $NaHCO_3$ (folic acid at 0.01 mg/L).

MEM-FA: MEM without folic acid supplemented with 5% FCS and adjusted to pH 7.6 with 7.5% $NaHCO_3$ (folic acid at 0.00 mg/L).

MEM-Ad: MEM deficient in adenine, made by supplementing MEM with 0.3 mg/L guanine and adding 0.2 mg/L azaserine to block purine biosynthesis, supplemented with 5% FCS and adjusted to pH 7.6 with 7.5% $NaHCO_3$ (folic acid at 0.01 mg/L).

MEM and M199 were purchased directly from the Grand Island

TABLE II

Patients studied from Family No. 1
and Family No. 2 and matched controls.

Family No. 1		Status	Age	Pedigree Number
Patient	A	Obligate Carrier Female	74	II-10
	B	Obligate Carrier Female	55	III-58
	C	Affected Male	49	II-63
	D	Affected Male	31	IV-144
	E	Potential Carrier Female	25	IV-145
Family No. 2				
Patient	F	Affected Male	33	III-3
	G	Affected Male	29	III-7
	H	Potential Carrier Female	26	III-9
Controls				
	I	Normal Male	29	
	J	Normal Male	31	
	K	Normal Female	25	

Biological Company (Gibco). MEM-FA and MEM-Ad were made up in the laboratory. A detailed listing of components of each of the four media is given in Appendix B. Aliquots of 100 ml of each medium were supplemented with 1 ml of an antibiotic-antimycotic solution (100X) containing 10,000 U/ml penicillin, 25 mcg/ml Fungizone®, and 10,000 mcg/ml streptomycin (Gibco Lot No. A990311). Any media used to culture lymphocytes was also supplemented with PHA (Gibco), 0.2 ml per culture, for mitogenic stimulation at culture initiation. All Fetal Calf Serum (FCS) media supplements were taken from Lot No. 29101111, Flow Laboratories, which contained a low level of folic acid relative to several other laboratory lots of serum (Appendix C).

2.2 Demonstration of the fragile X chromosome in cultured skin fibroblasts.

2.21 Rationale for the procedure. The work of Sutherland (1979a) had suggested that the reported lack of expression of the fragile X chromosome in cultured skin fibroblasts might be overcome by appropriate manipulation of the culture conditions, particularly those affecting folate metabolism. Since it was determined that it would be difficult if not impossible to obtain chromosome preparations from fibroblasts maintained for a long term in media low in, or entirely lacking folic acid (Erbe, 1975, 1979), a method was adopted for this study whereby skin fibroblast cell lines would be established and routinely maintained in medium containing normal levels of folate, and then the cells would be transferred to media

deficient in folic acid a brief period prior to chromosome harvest. To facilitate this transfer to folate deficient culturing conditions, a fibroblast culturing and harvest protocol originally described by Kajii et al. (1973) was adopted with some modification. At the time of transfer to folate deprivation, fibroblasts would be removed from flask culture in medium containing normal levels of folate, rinsed of any residual medium, and then transferred to medium low in, or entirely lacking, folic acid. From the time of transfer to folate deficient media, cells would be grown directly on slides that would be studied on the microscope.

This method would have two advantages that could be important in eliciting fragile site expression in fibroblasts. First, fibroblast cells from a single tissue culture flask could be split and transferred to different tissue culture media with varying concentrations of folic acid. This would permit comparison of the effects of different levels of folate on the frequency of expression in a relatively uniform population of cells. Second, the hypotonic and fixation treatments at chromosome harvest would be done on cells adhering to a slide. This would provide a relatively gentle harvest procedure and eliminate any satellite loss that might occur with more rigorous harvest techniques.

2.22 Procedure for establishing fibroblast cell lines. Fibroblast cell lines were initiated from skin biopsies by the standard explant procedure (Fraccaro et al., 1960; Hamerton, 1971) and routinely maintained in Eagle's MEM (Gibco) supple-

mented with 15% FCS. A 3mm skin snip was taken from the forearm from patients and controls and transported to the laboratory in cold saline. The skin biopsy was minced, and the pieces were equally distributed into three 35 x 10 mm (Falcon) petri dishes. The explants were covered with a No. 2: 22 mm square sterile cover slip, and 3 ml of fibroblast maintenance medium (MEM) was added to each dish. The explants were then cultured at 37°C in a 5% CO_2 atmosphere. The medium was changed weekly, and confluent fibroblast cultures were routinely obtained within three to five weeks. At confluence, the fibroblasts were trypsinized (1:20 Difco trypsin: HBSS) from each culture dish and grown to confluence in T75 flasks (Lux). Aliquots of fibroblasts from all individuals were frozen after the third passage and stored in liquid nitrogen.

2.23 Procedure for chromosome preparation from fibroblasts. All fibroblast experiments were done on cells in the third to ninth passage in flask culture; each passage represented a 1:4 split of a confluent T75 (Lux) flask, or approximately 2.3 cell generations.

Forty-three hours prior to chromosome harvest, cells grown to confluence in T75 (Lux) flasks in medium containing normal levels of folic acid were removed following incubation for 15-25 minutes at 37°C with 2 mls of 1:20 trypsin (Difco). They were resuspended in MEM-FA, 3 mls per flask. A 1 ml aliquot of this cell suspension was diluted 1:10 in medium low in (M199) or entirely lacking (MEM-FA) folic acid. At this step in the procedure, 1 ml aliquots of fibroblasts

from three patients, Patients B, C, and D, were also resuspended in MEM as well as MEM-FA and M199. Aliquots of 1.0 - 1.5 mls of these individual suspensions were separately layered onto sterile microscope slides held in a 100 x 15 mm square petri dish (3 slides per dish) and then incubated 30 to 45 min. at 37°C. After the cells had settled, the petri dish was filled with 15 ml of warm medium and allowed to incubate 24 hrs., at which time the medium was changed, and the cells were incubated an additional 19 hrs. Three hrs. prior to chromosome harvest, 0.3 ml Colcemid (10 mcg/ml) was added to each culture dish.

Chromosome harvest was initiated by gently aspirating off the culture medium and then covering the slides with 15 mls of warm 1% NaCitrate. Cells were exposed to this hypotonic condition for 15 min. at 37°C, after which 15 ml of cold 3:1 methanol:acetic acid was gently pipetted over the NaCitrate and allowed to fix the cells for 5 min. The slides were removed from the petri dishes and taken through two additional 5 min. fixes in Coplin jars. The slides were removed from the Coplin jar, briskly blown on, and briefly warmed over a bunsen burner. This was immediately followed by a 10 to 15 second dip in 1:50 ethanol:75% acetic acid. Each slide was finally rewarmed briefly and air-dried.

2.3 Procedure for peripheral blood lymphocyte culture and chromosome preparation.

Lymphocyte cultures were established according to the recommended protocol of Sutherland (1979c) with minor modifi-

cation in order to provide a standard frequency of marker expression for each patient, against which results from fibroblast cultures and results from further modification of the lymphocyte culturing procedure could be compared.

A single sample of blood was obtained from each individual with the exception of Patient D from whom two samples were obtained on separate occasions. Lymphocyte cultures were initiated within 3 to 5 hrs. of obtaining the blood sample.

A lymphocyte enrichment method (Moorhead et al., 1960) was adopted rather than the whole blood technique currently recommended for lymphocyte culture, in anticipation of the possibility that the use of low levels of Fetal Calf Serum (5%) in the culture media might lower the mitotic index (Turleau et al., 1979). Prior to initiating lymphocyte cultures, a 10 ml blood sample was allowed to separate for one to two hours at room temperature. The leukocyte-rich plasma fraction, including buffycoat and 5 to 6 drops of the red cell fraction, was then carefully drawn off and mixed thoroughly. A 0.25 to 0.5 ml volume of this suspension (approximately 2 X 10^3 cells) was added to aliquots of culture medium which were placed in 25 cm^2 Falcon tissue culture flasks. Culture media consisted of M199 buffered with 25 mM HEPES, adjusted to pH 7.6 with 7.5% $NaHCO_3$ and supplemented with 5% FCS. A 0.2 ml aliquot of PHA (Gibco) was added to each flask just prior to incubation, and the cultures were capped tightly and maintained at 37^{o}C for 96 hrs. The

culture medium and the period of incubation followed the recommended protocol of Sutherland (1979c) for expression of the fragile X chromosome in peripheral blood lymphocytes. Colcemid (Gibco, 10 mcg/ml), 0.075 ml per 5 ml culture, was added 50 min. prior to chromosome harvest.

At chromosome harvest, the lymphocyte suspension cultures were transferred to siliconized 15 ml glass conical centrifuge tubes and spun for 9 min. at 150xg. The cells were washed once in HBSS, and the supernatant was discarded. For hypotonic treatment, the cell pellet was then gently resuspended in pre-warmed 0.075 M KCl, and incubated for 15 min. at 37°C.

The suspension was again centrifuged, 9 min. at 150xg, and all but 1 ml of supernatant over the pellet was aspirated-off. For the purposes of fixation, 1 to 2 ml of cold 3:1 methanol:acetic acid was gently layered over the remaining supernatant. The pellet was then gently resuspended, and more cold fixative was added in 2 ml aliquots to a final volume of 7 ml with gentle agitation. There were 3 additional washes in fresh cold fix. The pellet was finally resuspended in 1 ml of fresh cold fix, and the cells were dropped on cold, wet slides. The slides were then briefly warmed over a bunsen burner and allowed to air-dry.

2.4 Examination of supplemental media factors in tissue culture.

2.41 Rationale for the procedure. Evidence from the

study of heritable fragile sites has suggested that fragile site expression is associated with the chromosome replication process. While the evidence from the study of the replicative behavior of heritable fragile sites is somewhat contradictory with respect to precisely when in DNA replication there is a commitment to fragile site expression, i.e. the abnormally late replicative behavior of the 2q12 fragile site reported by Bühler et al. (1970), while the expression of the 2q12 and Xq27-28 fragile sites were reported by Sutherland (1979a) to require a commitment prior to, or in the earlier stages of, DNA replication, there is agreement that expression is at least in part a function of the replicative process.

Sutherland (1979a) proposed that the mode of action of a folate deficiency on fragile site expression was mediated through pyrimidine biosynthesis, specifically in the reduced levels of thymidylate available for de novo DNA synthesis. If pyrimidine biosynthesis is indeed the metabolic area associated with the expression of the fragile site on the X chromosome, the Xq27-28 region may be an AT-rich DNA sequence very sensitive to altered concentrations of thymidylate.

In an attempt to test the notion that both available thymidylate and DNA replication are at least in part responsible for expression of the Xq27-28 fragile site, and that the Xq27-28 fragile site is an AT-rich DNA sequence particularly sensitive to altered concentrations of nucleotides, this study examined the effects of a medium deficient in thymi-

dine's DNA base pair, adenine (MEM-Ad). A deficiency in adenine should effectively mimic the effects of a thymidine deficiency on fragile site expression.

The relationship between impaired chromosome replication and the actual expression of a chromosome lesion or fragile site at mitosis is not known. The lack of chromosome condensation, evident when a fragile site is expressed, suggests that agents that are known to interfere with the normal condensation process might be useful in eliciting the expression of the fragile X chromosome in fibroblasts or, perhaps, in improving the frequency of expression in either lymphocyte or fibroblast cultre. The chromosome decondensation effects of 5-BrdU (Palmer, 1970; Zakharov et al., 1974) and actinomycin-D (Yunis and Chandler, 1977) have been well documented. BrdU has also been shown to specifically effect telomeric decondensation on some chromosomes (Hsu and Somers, 1961). Sutherland et al. (1980) have also recently defined a new category of fragile sites that require BrdU for expression. BrdU and actinomycin-D may therefore be useful in accentuating the frequency of expression of the fragile X chromosome in tissue culture.

2.42 Modification of the fibroblast culture procedure. At the time of transfer to folate deficient media, fibroblasts from Patient D were also transferred and maintained to chromosome harvest in MEM-Ad. To examine the influence of 5-BrdU and actinomycin-D on the frequency of expression, BrdU (10^{-5}M) was added 3½ hrs. prior to chromosome harvest to

parallel cultures of MEM-FA for two of the carrier females studied (Patients B and E), and actinomycin-D (Cosmegen, Merck Sharp & Dohme) was added at a final concentration of 5 mcg/ml to a parallel culture of MEM-FA for Patient E, 45 min. prior to chromosome harvest. Chromosome harvest for cells cultured in MEM-Ad and those incorporating BrdU and actinomycin-D followed the standard protocol outlined in section 2.23.

2.43 Modification of the lymphocyte culture procedure. Lymphocyte cultures for Patients B and D were established in MEM-Ad. These parallel cultures followed the general culture initiation and chromosome harvest protocol outlined for M199.

To examine the influence of 5-BrdU and actinomycin-D on the frequency of expression of the fragile X chromosome in lymphocytes, modifications to the standard lymphocyte technique were as follows. In experiments using BrdU, parallel lymphocyte cultures for Patients B and E were established in MEM-FA and MEM-FA plus 5-BrdU (10^{-5}M) added 3 to 5 hrs. prior to chromosome harvest. In experiments with actinomycin-D, lymphocytes from Patients B and D were cultured initially in M199 at 37°C as usual. After 72 hrs. incubation, methotrexate (Lederle)[1] was added to give a final concentration of 10^{-7}M.

[1]Methotrexate is a folate antagonist and at low concentrations will effectively synchronize the cell population in culture (Yunis, 1976), so that many of the metaphase cells at chromosome harvest will be at the same relative degree of chromosome condensation.

After 17½ hrs. additional incubation, the cells were released from the methotrexate block by transfer to fresh medium and allowed to continue incubating 5 hrs. to chromosome harvest. Actinomycin-D was added 45 min. prior to chromosome harvest to give a final concentration of 5 mcg/ml. Fifteen minutes prior to chromosome harvest 0.075 cc. Colcemid (10 mcg/ml) was added to the culture.

2.5 Chromosome staining procedure.

On a routine basis, slides that had aged at least one week were stained with 2.0% aceto-orcein (Gibco) according to the following protocol. Three drops of stain were put on the slide which was then covered with a cover slip. The cover slip was allowed to sit for 10 min. and was then removed. The slide was taken through three changes of 100% ethanol and then air-dried. For permanent mounting, slides were dipped briefly in xylene, and a cover slip was mounted with Eukitt.

G-banding of the chromosome preparations followed the technique originally described by Seabright (1971) with some modification. Prior to staining, a slide was heated 3 to 5 hrs. at 60°C. The slide was treated in trypsin (3cc of reconstituted Difco trypsin in 60 mls of 0.9% NaCl) for 20 to 30 sec.; the treatment was extended for older slide material. The slide was then passed through 1% $CaCl_2$ (1 min.), 2 washes in distilled water, and stained 45-60 sec. in Giemsa (2 cc Gurr R66 Giemsa stain, in 20 cc phosphate buffer,

pH 6.8, plus 30 cc distilled water). The slide was rinsed in distilled water, lightly blotted, and allowed to air dry. G-banded slides were permanently mounted by the method described above.

2.6 Methods of observation and tabulation of data.

All metaphases scored fell within a condensation criterion in which the No. 2 chromosome used as an index measured 9 to 15 μm in length. The studies which led to the application of this condensation criterion will be discussed more comprehensively in the Results, section 3.21. These revealed clearly that the fragile X chromosome was more frequently detectable in metaphases where the chromosomes were not severely contracted.

Well spread metaphases that met the established condensation criterion were scored for the fragile X chromosome and recorded as either positive or negative and, occasionally, as questionable. In this questionable category were those metaphases in either a satellite-like piece of chromatin was detached and not clearly associated with the long arm of the X chromosome. Also included in this category were metaphases where there was some ambiguity in the expression of the satellite, for example chromosome overlap or the possible involvement of a D or G group satellite. Cells scored in this questionable category were not used in the determination of the frequency of the expression of the fragile X chromosome. The frequency of expression of the fragile X chromosome for any given patient under a particular culturing condition was expressed

as the number of cells expressing the fragile X chromosome as a proportion of the total number of cells scored.

Scoring for the fragile X chromosome was done directly under the microscope. All observations of the fragile X chromosome were made on a Zeiss Photomicroscope under oil-immersion phase-contrast at 1250X. Photographs were taken on Kodak High Contrast Copy Film 1276.

To determine if differences between any two frequencies of expression were statistically significant, Chi square (χ^2) analysis for independence of the two nominal variables was employed using a contingency table. For expected values of the frequency of expression of the fragile X chromosome that were low (< 5) and therefore inappropriate for analysis using the Chi square test, Fisher's Exact Probability Test was applied (Sokal and Rohlf, 1969).

CHAPTER 3

RESULTS

3.1 Demonstration of the fragile X chromosome in cultured skin fibroblasts.

Initial observations of fibroblast metaphases utilizing the culturing and harvesting procedure developed for this study indicated that the fragile X chromosome was expressed. It was also evident that there was a considerable amount of variation in the frequency of expression of the fragile X chromosome, and that this variability was in part related to the degree of chromosome condensation in metaphases being scored. Therefore, an analysis of the frequency of expression of the fragile X chromosome with relation to the degree of condensation was conducted.

The results of this analysis are presented below in section 3.21, Table VI, and will be described in detail in that section. Since comparisons of frequencies of expression between subjects were to be made, a scoring criterion based on this analysis was established and imposed on cells to be scored.

3.11 Expression in fibroblasts from patients from Family No. 1. The fragile X chromosome was demonstrated in cultured skin fibroblasts from all five patients (A-E) from Family No. 1. Cell lines from three patients (B,C, and D) were transferred and maintained to chromosome harvest in medium containing normal levels of folic acid (MEM) as well

as in media deficient in folic acid (M199 and MEM-FA). The fragile X chromosome was expressed in fibroblasts transferred to MEM, albeit at very low frequencies (1.2 - 6.3%). There were significantly higher frequencies of expression in fibroblasts transferred to media deficient in folic acid (Table III).

In Family No. 1, the highest frequencies of expression of a fragile X chromosome (5.9 - 35.0%) were found in fibroblasts, cultured in media deficient in folic acid, from the two mentally retarded males, Patients C and D. Frequencies of expression for the obligate and potential carrier females, Patients A,B, and E, ranged from 9.2 to 19.3% in the two media deficient in folic acid.

Parallel blind studies on fibroblasts from two patients (D and E) and normal controls (J and K) matched for age, sex, and fibroblast passage number were done to determine whether folate deprivation could elicit expression of the fragile X chromosome in control fibroblasts. Folate deprivation did not lead to detectable fragile X chromosomes in either male or female control fibroblasts.

Table III details the frequencies of expression of a fragile X chromosome in fibroblast metaphases for Patients A-E and normal controls, J and K, for medium containing normal levels of folic acid (MEM) and media low in (M199) or entirely lacking (MEM-FA) folic acid. The data in Table III indicate that, while there was a significantly higher frequency of expression of the fragile X chromosome in the folate deficient media than in the medium containing normal

TABLE III

Expression of the fragile X chromosome in cultured skin fibroblasts from patients from Family No. 1 and normal controls: comparison of various growth media.

Patient	Fibroblast Passage No.	Media	No. Cells Expressing Marker / No. Cells Scored‡	Frequency in %
A	3	M199	9/93	9.7
Obligate	3	MEM-FA	—	—
Carrier, 74 yrs.	5	MEM-FA	—	—
B	3	MEM	5/115	4.3
Obligate	3	M199	25/129+	19.3
Carrier, 55 yrs.	3	MEM-FA	9/49	18.3
C	3	MEM	2/173	1.2
Affected Male	3	M199	11/179+	5.9
49 yrs.	3	MEM-FA	43/168*	25.6
	5		35/155	22.6
D	3	MEM	9/143	6.3
Affected Male	3	M199	39/202+	19.3
31 yrs.	3	MEM-FA	43/168	25.6
	5		19/54*	35.0
	9	M199	19/109	17.4
	9	MEM-FA	36/155	23.2
Control J	9	M199	1?/133	<1
Normal Male, 31 yrs.	9	MEM-FA	0/103	<1
E	3	M199	19/107	17.8
Potential	3	MEM-FA	11/119*	9.2
Carrier, 25 yrs.				
Control K	3	M199	0/65	<1
Normal Female	3	MEM-FA	0/85	<1
25 yrs.				

‡ all metaphases scored fell within the condensation criterion described in the text.

\+ significantly different when compared to the frequency of expression in MEM: B_3: $\chi^2_1=12.74$, $p<.001$; C_3: $\chi^2_1=6.16$, $p<.05$; D_3: $\chi^2_1=11.85$, $p<.001$.

* significantly different when compared to the frequency of expression in M199: C_3: $\chi^2_1=24.95$, $p<.001$; D_5: $\chi^2_1=6.13$, $p<.05$; E_3: $\chi^2_1=3.55$, $p<.05$.

levels of folic acid, there was no consistency in the comparative frequencies of expression between the two folate deficient media, M199 and MEM-FA.

For Patient C, the frequency of expression of the fragile X chromosome was significantly higher in MEM-FA than M199; for Patient E the opposite was true. Patient B showed no significant difference in the frequency of expression between M199 and MEM-FA, while Patient D showed a significantly higher frequency of expression of the fragile X chromosome in MEM-FA than in M199 in fibroblasts at passage 5, but no difference in frequencies between the two folate deficient media at passage 3 and passage 9. The data in Table III also indicate that duplicate experiments, using the same culture medium conditions but later passage fibroblast cell lines on a patient, showed no significant change in the frequency of expression between early and later passage cells (Patient C, MEM-FA; Patient D, M199 and MEM-FA).

3.12 Expression in fibroblasts from patients from Family No. 2. In order to determine if the success in demonstrating the fragile X chromosome in fibroblasts from individuals in Family No. 1 was due to behavior unique to the fragile X chromosome in that family, the fibroblast method was applied to established cell lines from three individuals from a second unrelated family. The fragile X chromosome was demonstrated in fibroblast metaphases from both of the mentally retarded males studied in Family No. 2. The frequencies of expression of a fragile X chromosome in fibroblasts cultured in M199

for Patients F, G, and H and Control I are detailed in Table IV. The frequencies observed for Patients F and G were 17.2% and 14.5% respectively. Thus far, a fragile X chromosome has not been seen in fibroblast preparations from Patient H, a potential carrier female of low normal intelligence who does exhibit the fragile X chromosome in lymphocytes (following section). Two separate experiments have proved unsuccessful in demonstrating the fragile X chromosome in fibroblasts from this patient; the fibroblast cell lines established for Patient H are slow growing, and good chromosome preparations have been difficult to achieve.

Culturing in M199 did not demonstrate the fragile X chromosome in fibroblasts from Control I.

3.13 Comparison of the frequency of expression of fibroblasts and lymphocytes. All patients demonstrated the fragile X chromosome in peripheral blood lymphocytes. The frequency of expression of a fragile X chromosome in PHA stimulated lymphocytes cultured in folate deficient media for the eight patients and three normal controls are presented in Table V.

In both families there was a difference in the frequencies of expression between different mentally retarded males. In Family No. 1, Patient C exhibited the fragile X chromosome in 6.5% of the lymphocyte metaphases analyzed, whereas values of 15.9 - 19.3% were obtained for Patient D. In Family No. 2, Patient F demonstrated a frequency of expression of 13% compared to 32.6% for Patient G. The frequency of expression of

TABLE IV

Expression of the fragile X chromosome in cultured skin fibroblasts from patients of Family No. 2 and a normal control.

Patient	Fibroblast Passage No.	Media	No. Cells Expressing Marker / No. Cells Scored‡	Frequency in %
F Affected Male, 33 yrs.	3	M199	5/29	17.2
G Affected Male, 29 yrs.	3	M199	8/55	14.5
H Potential Carrier, 26 yrs.	3	M199	—	—
	5	M199	—	—
Control I Normal Male 29 yrs.	3	M199	0/50	<1

‡ all metaphases scored fell within the condensation criterion described in the text.

TABLE V

Expression of the fragile X chromosome in peripheral blood lymphocytes from patients of both families and normal controls.

Patient	Media	No. Cells Expressing Marker / No. Cells Scored‡	Frequency in %
Family No. 1			
A Obligate Carrier, 74 yrs.	M199 MEM-FA	4/39 2-3/19	10.3 11-16
B Obligate Carrier, 55 yrs.	M199 MEM-FA	9/175 4/98	5.1 4.3
C Affected Male, 49 yrs.	M199 MEM-FA	9/139 —	6.5 —
D Affected Male, 31 yrs.	M199 M199 MEM-FA	21/133 25/130 9/39	15.9 19.3 23.0
E Potential Carrier, 25 yrs.	M199 MEM-FA	29/89 16/105*	32.2 15.2
Family No. 2			
F Affected Male, 33 yrs.	M199	6/46	13.0
G Affected Male, 29 yrs.	M199	14/43	32.6
H Potential Carrier, 26 yrs.	M199	5/43	11.6
Control I Normal Male, 29 yrs.	M199	0/93	≤ 1
Control J Normal Male, 31 yrs.	M199	0/102	<1
Control K	M199	0/100	<1

‡ all metaphases scored fell within the condensation criterion described in the text.

* significantly different, re:M199. $\chi^2=8.14$, $.001<p<.01$.

the fragile X chromosome ranged from 4.3% to 32.2% for carrier females from both families.

Lymphocytes from four of five patients from Family No. 1 were successfully cultured in MEM-FA and M199. For Patients A, B, and D there was no difference in frequency of expression of the fragile X chromosome between M199 and MEM-FA, while Patient E showed a significantly lower frequency of expression of the fragile X chromosome in MEM-FA than in M199 ($p < .01$). This is a pattern similar to that observed in this patient's fibroblasts.

In comparing the frequencies of expression in lymphocytes and fibroblasts for a patient, there was no difference in the frequency of expression in these two cell types for three of the four mentally retarded males studied. One affected male, Patient G, exhibited the fragile X chromosome at a lower frequency in fibroblasts than in lymphocytes. One of the three carrier females, Patient B, showed a higher frequency of expression in fibroblasts than in lymphocytes. Another carrier female, Patient E, showed a lower frequency of expression in fibroblasts than in lymphocytes. The third carrier female showed no difference in frequency of expression between fibroblasts and lymphocytes.

3.2 Factors affecting a reliable determination of the frequency of expression.

3.21 Condensation criterion. Preliminary observations suggested that the expression of the fragile X chromosome was

in part a function of the degree of condensation of the metaphase chromosomes; the fragile X chromosome being more frequently detectable in metaphases where the chromosomes were not severely contracted. An actual study of this was therefore undertaken and the results are presented in Table VI. The frequency of expression of the fragile X chromosome in fibroblast metaphases where the No. 2 chromosome measured 4 - 7μm in length was 5.0%, whereas at 8 - 11 μm the frequency of expression increased to 20.5%, and at 12 - 15μm the frequency of expression was even higher, 37.1%. These differences in frequency of expression with longer chromosomes were statistically significant (Table VI). The decline in the total number of cells scored over the three length ranges of the No. 2 chromosome reflects difficulties encountered in scoring more extended chromosome preparations: scoring difficulties due to overlapping chromosomes and poor metaphase spreading.

On the basis of these results, it was decided to restrict analysis to metaphases in which the No. 2 chromosome used as an index measured 9 - 15μm in length. All metaphases scored in this study fell within this condensation criterion with the exception of the data shown in Table VI.

3.22 Variable appearance of the fragile X chromosome. There was also considerable variation in the appearance of the satellite on the terminal end of the long arm of the X chromosome independent of the culturing media conditions imposed on cells. In most cases the satellite appeared as a discrete

TABLE VI

Expression of the fragile X chromosome as a function of the degree of chromosome condensation. Fibroblasts from Patient D (MEM-FA).

Chromosome No. 2 Length	4-7 μm	8-11 μm	12-15 μm	Total
No. Cells Expressing Fragile X / No. Cells Scored	9/180	24/117+	26/70*	59/367
Frequency in %	5.0	20.5	37.1	16.1

\+ significantly different when compared to 4-7 μm condensation class: $\chi^2_1=17.28$, $p<.001$.

* significantly different when compared to 8-11 μm condensation class: $\chi^2_1=6.18$, $p<.05$.

bipartite piece of chromatin detached from the distal end of the long arm, frequently showing thread-like connections to the terminal end of the long arm (Fig. 3, a-e). Occasionally, the satellites were tightly juxtaposed, perhaps overlapping, and they appeared as a single chromatin mass but still separate from the long arms (3d, 3e). A single satellited chromatid is a variation of expression that was occasionally observed (Fig. 3f). Example g in Figure 3 shows an apparently bi-satellited X chromosome.

Another variation in the expression of the fragile X chromosome was a complete detachment or dissociation of the satellite fragment from the rest of the X chromosome (Fig. 4). These detached satellites were not included in any determination of a frequency of expression of the fragile X chromosome because of ambiguity with respect to the origin of the chromatin material.

3.23 Definitive association of the fragile site with the X chromosome by G-banding. Examples of the variable expression of the fragile X chromosome in Figure 3 were from metaphase chromosomes stained with aceto-orcein. Aceto-orcein rather than G-banding was used for most of the studies described (results in Tables III - VII) because it gave a higher incidence of metaphases in which the satellites could be clearly distinguished as distinct from the terminal end of the long arm of the X chromosome.

Examples in Figure 5, a-e, are typical of the appearance of the fragile X chromosome after G-banding. From these

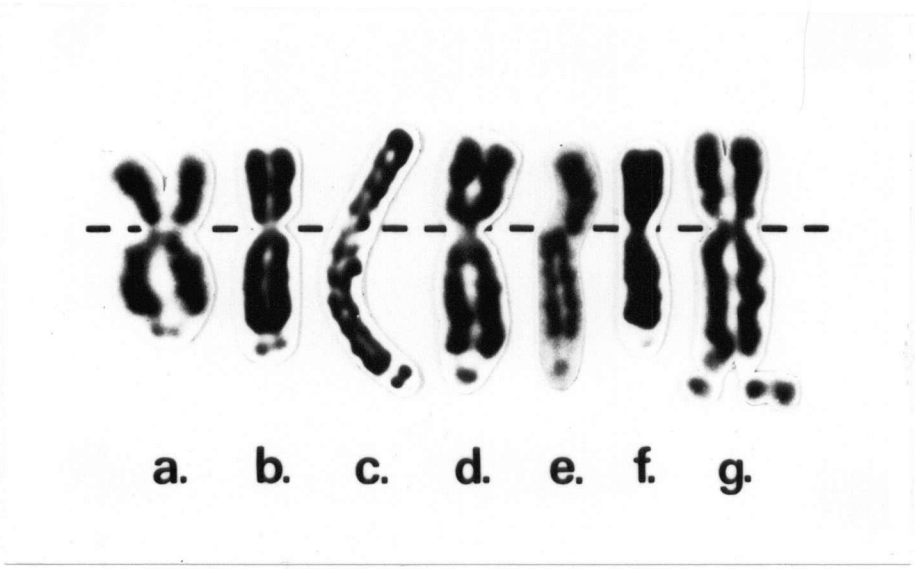

FIGURE 3

Variable expression of the fragile X chromosome: aceto-orcein staining.

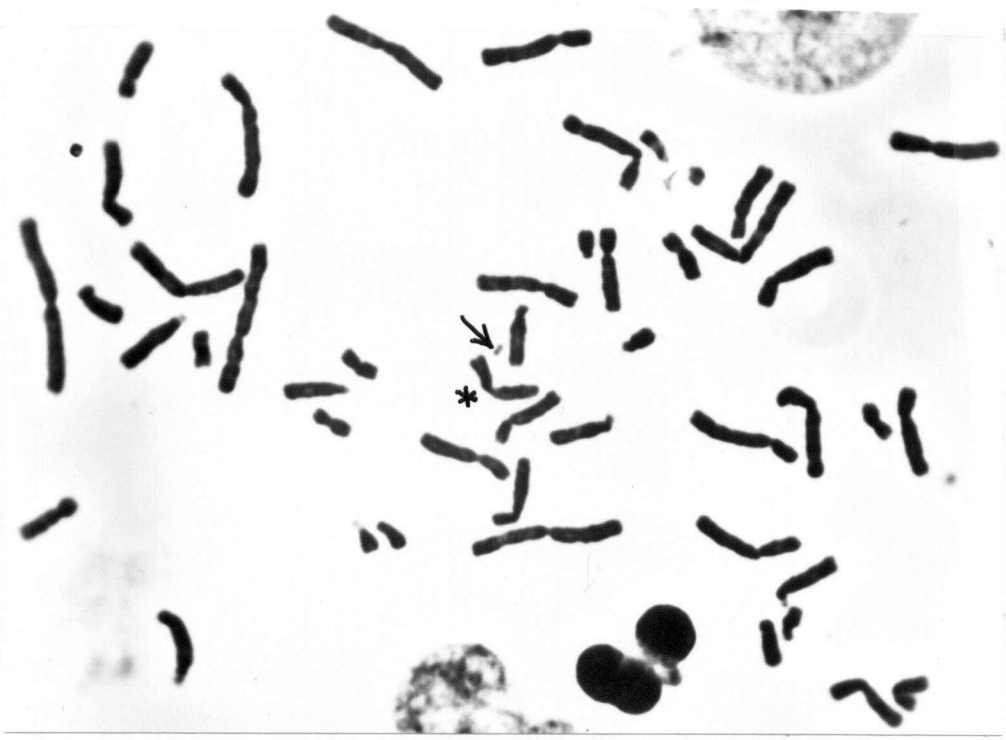

FIGURE 4

Detached satellite-like piece of chromatin (arrow) unassociated with the X chromosome (asterisk).

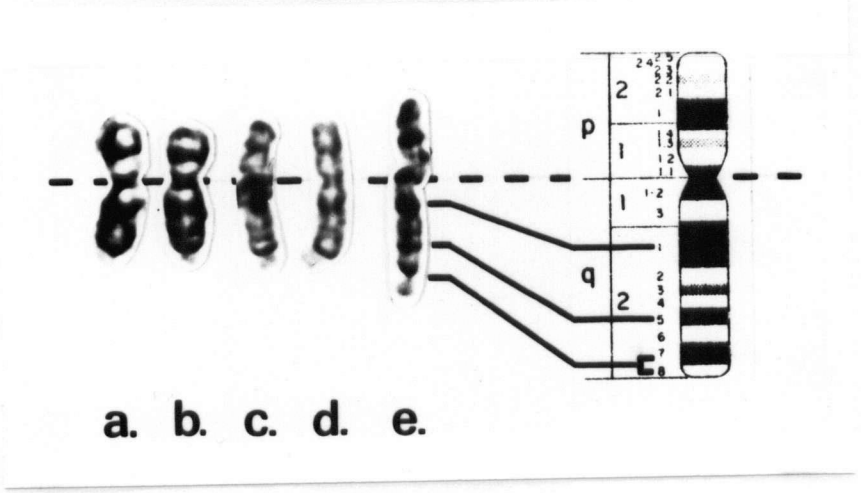

FIGURE 5

Variable expression of the fragile X chromosome: trypsin G-banding. Comparison to the standard banding pattern of the X chromosome (Paris Conference, 1971).

studies, the identification of the X chromosome as distinct from the other human C group cromosomes was definitively established. In addition, the few well G-banded preparations did permit more precise assignment of the position of the fragile site on the terminal end of the long arm. If the banding pattern of the fragile X chromosome is compared with the normal X chromosome (Fig. 5, d, e, and insert), it appears that the fragile site begins in the distal portion of band Xq27 or at the junction between bands q27 and q28.

3.24 Autosomal chromosome markers expressed under folate deprivation. Terminal markers were expressed on some of the autosomal chromosomes in both lymphocytes and fibroblasts cultured under folate deprivation from affected males and carrier females. These telomeric markers usually appeared as an isochromatid gap or break most often near the terminal end of the long arm. They frequently appeared to involve substantially more chromatin material than the satellites on the X chromosome (Fig. 6). These telomeric markers were expressed in all of the major chromosome groups with the exception of the G-group, and occurred most frequently on chromosomes No.'s 1, 3, two of the C-group chromosomes (probably 6 and 12), one of the D-group chromosomes (probably 13), and either 19 or 20 of the F group chromosomes. Autosomal telomeric markers were also expressed in normal control subjects in cells cultured under folate deprivation.

The frequency of expression of telomerically marked autosomal chromosomes in affected males and carrier females

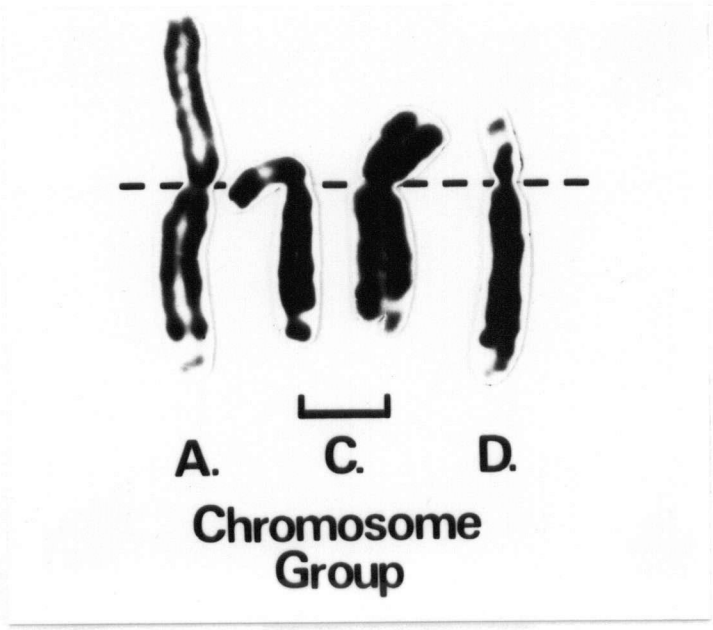

FIGURE 6

Autosomal telomeric markers expressed under folate deprivation in cell culture.

was extremely low when compared to the expression of the fragile X chromosome in these patients (Table VII). While control subjects did not exhibit the fragile X chromosome, there was no significant difference between patients and controls in the frequency of expression of telomerically marked autosomal chromosomes (Table VII).

3.3 Factors that influence the frequency of expression of the fragile X chromosome.

3.31 Culturing factors: MEM-Ad, BrdU, and actinomycin-D. Results presented in the first two sections demonstrated that folate deprivation and the degree of condensation of the metaphase chromosomes appeared to be important in the expression of the fragile X chromosome in cultured fibroblasts. Further experimentation was undertaken to test the mode of action of folate deprivation and to examine the effects of particular agents, known to affect chromosome condensation, on the frequency of expression of the fragile X chromosome. In an attempt to test the notion that both the availability of thymidylate and DNA replication were at least in part responsible for expression of the Xq27-28 fragile site, cells from two patients were cultured in a medium deficient in adenine (MEM-Ad). Unfortunately cultures could not be sustained in this medium, and no metaphases were observed at chromosome harvest. Therefore this medium was not examined further.

TABLE VII

Expression of autosomal telomeric markers in cultured skin fibroblasts from patients and normal controls (MEM-FA).

Patients	No. of Telomerically Marked Chromosomes (Frequency in %)								
	Group A	Group B	Group C	Group D	Group E	Group F	Total	Xq27-28	Total Cells Scored
C and D Affected Males	13 (2.2)	7 (1.2)	13 (2.2)	5 (0.8)	3 (0.5)	1 (0.2)	42* (7.1)	112 (19.0)	589
I and J Control Males	8 (3.4)	— —	9 (3.8)	3 (1.3)	6 (2.6)	— —	26 (11.1)	1? (0.4)	235
B and E Carrier Females	23 (2.4)	12 (1.3)	11 (1.2)	5 (0.5)	3 (0.3)	4 (0.4)	58* (6.1)	124 (13.0)	955
K Control Female	1 (0.5)	2 (1.0)	4 (2.1)	4 (2.1)	1 (0.5)	3 (1.6)	15 (7.8)		193

* not significantly different when compared to matched control. Males: $\chi^2_1=3.43$, $p>.05$. Females: $\chi^2_1=0.77$, $p>.05$.

The addition of 5-BrdU and actinomycin-D to folate deprived culture media was also investigated since these agents are both known to interfere with the chromosome condensation process. To examine the effects of 5-BrdU on the frequency of expression of the fragile X chromosome, $BrdU^{(10^{-5}M)}$ was added to folate deprived media 3 - 5 hrs. prior to chromosome harvest in parallel cultures of lymphocytes from Patients B and E and a parallel culture of fibroblasts from Patient B. While the addition of BrdU did result in more extended chromosomes, with particular chromosome regions showing pronounced decondensation effects, namely the paracentromeric heterochromatic regions on chromosomes 1, 9, and 16, and the short arm satellites on the D and G group chromosomes, the addition of BrdU did not produce a significant change in the frequency of expression of the fragile X chromosome in either of the two patients studied (Table VIII).

The incorporation of actinomycin-D (5mcg/ml) into the culture medium was examined using peripheral blood lymphocytes from two patients and skin fibroblasts from one patient from Family No. 1 (Table VIII). Actinomycin-D did produce more extended chromosome preparations, and, while it gave the impression of significantly improving the frequency of marker expression for Patient B, the frequency of chromosome breaks and gaps in cells grown in folate deprived media supplemented with actinomycin-D was so extensive that a reliable determination of the frequency of expression was not possible. An example of this breakage phenomenon is given in Figure 7.

TABLE VIII

Effects of 5-BrdU and actinomycin-D on the frequency of expression of the fragile X chromosome in lymphocytes and fibroblasts.

Patient	Tissue	Media	No. Cells Expressing Marker / No. Cells Scored	Frequency in %
B Obligate Carrier, 55 yrs.	Lymphocytes	M199 std.	9/175*	5.1
		M199 + actin-D	?9/65	13.9?
		MEM-FA	4/93*	4.3
		MEM-FA + BrdU	9/105	8.6
	Fibroblasts	MEM-FA	9/49*	18.3
		MEM-FA + BrdU	26/104	25.0
D Affected Male, 31 yrs.	Lymphocytes	M199 std.	21/133*	15.9
		M199 + actin-D	?2/13	13.3?
E Potential Carrier, 25 yrs.	Lymphocytes	MEM-FA	16/105*	15.2
		MEM-FA + BrdU	22/99	22.2
	Fibroblasts	MEM-FA	11/119*	9.2
		MEM-FA + actin-D	?3/15	20.0?

* previously reported frequencies (Tables III and V).

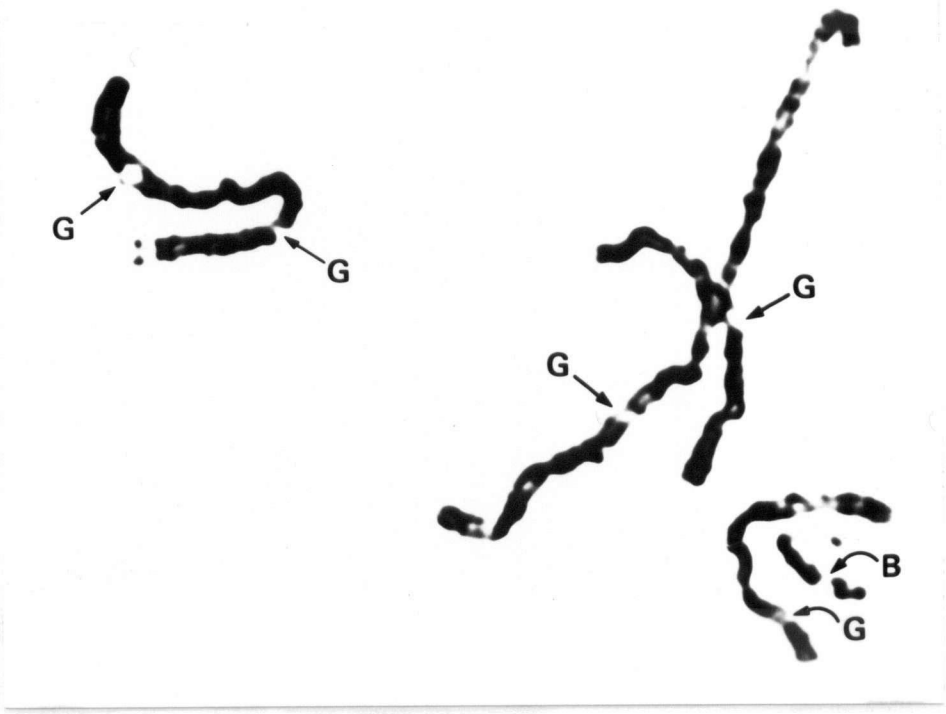

FIGURE 7

Extended chromosomes and gaps(G) and breaks(B) produced by actinomycin-D in combination with folate deprivation in cell culture. The chromosome at the upper left may be the fragile X.

3.32 Hypotonic effects at chromosome harvest. Results presented in the first section demonstrated the fragile X chromosome in skin fibroblasts from three patients (B, C, and D) cultured in medium containing normal levels of folic acid (MEM). Because this result suggested that aspects of the fibroblast method other than folate deprivation were influencing fragile site expression, experiments were undertaken to determine the effects of different hypotonic treatments at chromosome harvest on the frequency of expression of the fragile X chromosome in fibroblast culture. Parallel cultures of cells from an affected male (Patient D) were established and maintained in accordance with the protocol described above in media deficient in folic acid (M199 and MEM-FA) and medium containing normal levels of folic acid (MEM). At chromosome harvest, fibroblasts cultured in each of the different media were further subdivided and treated separately with one of three hypotonics, 1% NaCitrate, 0.075 M KCl, or distilled H_2O.

Under culturing conditions of folate deprivation, the expression of the fragile X chromosome was significantly higher with a hypotonic treatment in 1% NaCitrate than when the hypotonic treatment employed was 0.075 M KCl (Table IX). The use of distilled water as hypotonic with this culturing method proved unsatisfactory under all media conditions because it resulted in a very low, virtually unscorable, mitotic index. As previously noted, the frequency of expression of a fragile X chromosome in media containing normal levels of

TABLE IX

Effects of different hypotonic treatments at chromosome harvest on the frequency of expression of the fragile X chromosome in fibroblasts.

Patient	Media	Hypotonic	No. Cells Expressing Marker / No. Cells Scored	Frequency in %
D Affected Male	MEM	1% NaCitrate	4/93+	4.3
	MEM	0.075M KCl	0/89	<1
	M199	1% NaCitrate	15/79*	19.0
	M199	0.075 M KCl	2/99	2.0
	MEM-FA	1% NaCitrate	46/202*	22.9
	MEM-FA	0.075 M KCl	7/239	2.9

\+ not significantly different when compared to a hypotonic treatment in 0.075 M KCl for the same medium. Fisher's Exact Test: $p=0.12$.

* significantly different when compared to a hypotonic treatment in 0.075 M KCl for the same medium. M199: $\chi^2_1=7.32$, $p<.01$. MEM-FA: $\chi^2_1=20.39$, $p<.001$.

folic acid (MEM) with a hypotonic treatment in NaCitrate was about 5% for this patient. Using a KCl hypotonic, the frequency of expression was even lower, less than 1%. This difference in frequency was not statistically significant ($p=0.12$).

It was apparent that chromosome preparations treated with NaCitrate as a hypotonic were different in appearance from those treated with KCl. NaCitrate appeared to substantially interact with, or disrupt, the structural integrity of the chromatin. This was particularly evident along the periphery of the chromosomes where the chromatin appeared loose and poorly organized. With a hypotonic treatment in KCl the integrity of the chromatin along the periphery of the chromosome appeared more compact and undisrupted (Fig. 8).

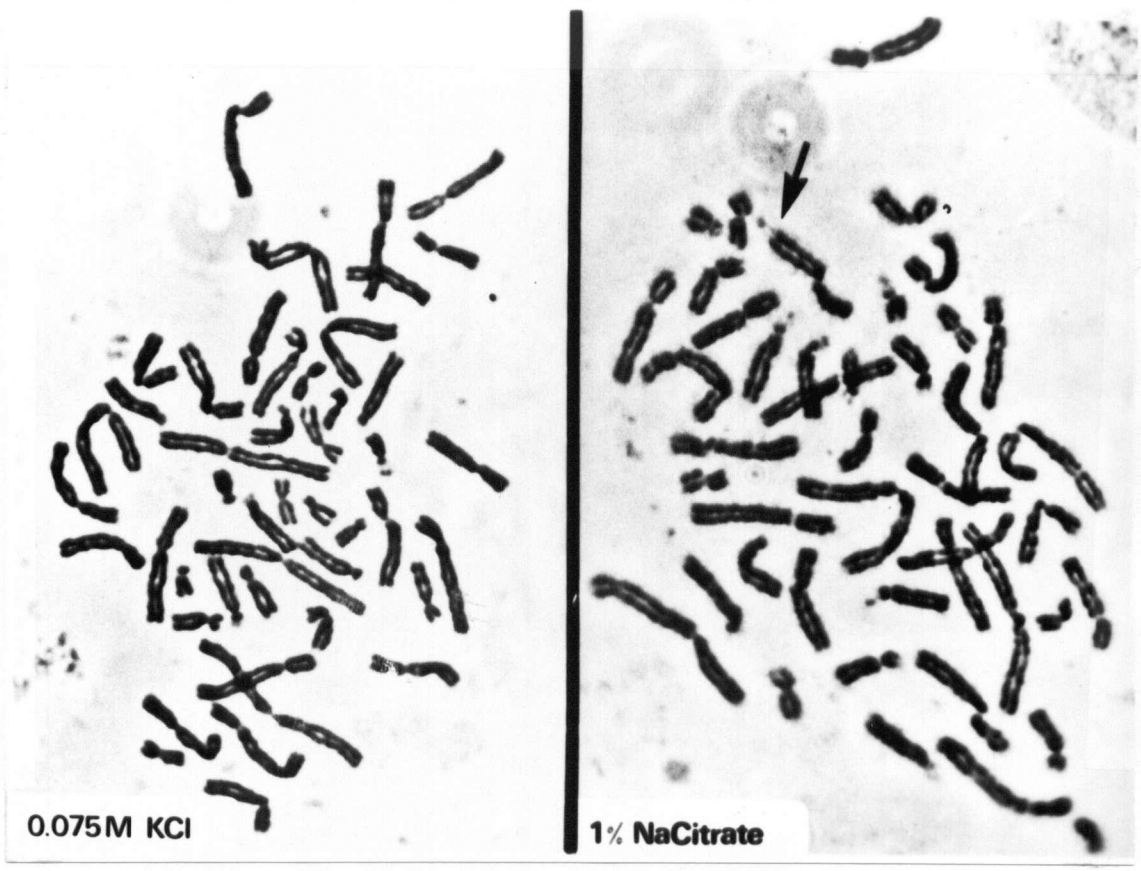

FIGURE 8

Effects of different hypotonic treatments at chromosome harvest on chromosome morphology.

CHAPTER 4

DISCUSSION

4.1 Fragile X expression in fibroblasts from mentally retarded males and their female relatives.

Using the fibroblast method developed for this study, it was possible to elicit the expression of the fragile X chromosome in this tissue type. The fragile X chromosome was demonstrated in skin fibroblast cell lines established from all five patients from Family No. 1. The highest frequencies of expression obtained were for the two mentally retarded males from this family, at frequencies in excess of 25% in fibroblasts cultured in medium without folic acid. A higher frequency of expression in mentally retarded males in fibroblasts is consistent with the generally higher frequencies of expression reported in lymphocytes for mentally retarded males (see Table I, section 1.13).

The data on frequencies of marker expression in carrier females for fibroblasts (as well as lymphocytes) show neither a correlation with female age nor a lack of expression in older obligate carriers. For example, in Family No. 1, a 25 yr. old potential carrier demonstrated the fragile X in 17.8% of fibroblast cells cultured in M199, a 55 yr. old obligate carrier showed a frequency of 19.3% in fibroblasts in M199, and a 74 yr. old obligate carrier demonstrated it in 9.7% of fibroblast cells cultured in M199. These results are not consistent with results in lymphocytes for fragile X

expression in carrier females recently reported by Sutherland (1979c), indicating that the frequency of expression in carrier females decreases with age and that fragile X chromosome expression is frequently difficult if not impossible to elicit in females over the age of 30 yrs.

The study of later passage fibroblasts from two of the patients from Family No. 1 (Patients C and D) did not show a significant difference in the frequency of expression from that obtained in earlier passage cells. This suggests that the method is reproducible. Furthermore, the successful application of the fibroblast method to established cell lines from two patients from a second unrelated family, in an attempt to assess the reliability of the fibroblast method, suggests that the method should be applicable to other families in which the disorder is segregating.

In comparing the frequencies of expression in fibroblasts and lymphocytes for all patients studied, who demonstrated the fragile X in both tissues, no general statement can be made about the greater effectiveness of either fibroblast culture or lymphocyte culture in eliciting the expression of the fragile X chromosome. All eight patients studied showed the fragile X in lymphocytes cultured in folate deficient media over a wide range of frequencies(4.3 to 32.6%). Seven of the eight patients showed the fragile X chromosome in fibroblasts cultured in folate deficient media at comparable frequencies (5.9 to 35.0%). While four of the eight patients studied showed no difference in the frequency of expression between fibroblasts and lymphocytes, two patients showed a signifi-

cantly lower frequency of expression in fibroblasts, and one patient showed a higher frequency of expression in fibroblasts. Thus, there appeared to be no consistent pattern of either tissue being more effective in demonstrating the fragile X chromosome when the comparison was possible.

The demonstration of the fragile X chromosome in cultured skin fibroblasts has not solved the diagnostic dilemma of demonstrating the marker chromosome reliably in carrier females. None of the females studied in this project failed to show the marker in lymphocytes, so it remains to be seen whether fibroblasts will be successful in demonstrating the fragile X chromosome in female patients who do not express it in lymphocytes. However, those females who do exhibit the marker in even a small percentage of cells in either fibroblasts or lymphocytes can be considered carriers. On the basis of data collected in this study, two potential carrier females, Patients E and H, can now be considered heterozygous carriers of the disorder.

4.2 Expression of the fragile X chromosome in cultured skin fibroblasts: culturing and chromosome harvesting factors.

While it is difficult to say precisely why previous investigators have not been successful in demonstrating the fragile X chromosome in fibroblasts, the fact is that few attempts have been made to study fibroblast chromosomes from patients from families in which the disorder is known to be segregating (Lubs, 1969; Sutherland, 1979b; Turner et al., 1980).

Probably the most important features, which differentiate the method developed in this study from earlier attempts to demonstrate the fragile X chromosome in fibroblasts, are the transfer of cells to folate deficient media after routine maintenance in medium containing normal levels of folic acid and a hypotonic treatment at chromosome harvest in NaCitrate rather than KCl.

4.21 Folate deprivation in cell culture. The feature of the fibroblast method which appeared to have the greatest influence on the frequency of expression of the fragile X chromosome was folate deprivation in cell culture. While it was possible to elicit marker expression in cells cultured in MEM, there was a significantly higher frequency of expression in cells transferred to either M199 or MEM-FA. The fragile X chromosome was expressed at comparable frequencies in fibroblasts cultured in M199, which is low in folic acid, and MEM-FA, a complete medium from which folic acid was deleted. The lack of any consistent difference between M199 and MEM-FA in demonstrating the fragile X chromosome in either lymphocyte or fibroblast culture parallels results reported by Sutherland (1979a) on the effectiveness of folate deficient media in eliciting the expression of the fragile X chromosome in lymphocyte culture. A very recent report of the expression of the fragile X chromosome in fibroblasts, from a mentally retarded male patient, cultured in medium containing normal levels of folate plus the addition of the folic acid antagonist, methotrexate during the last 48 hrs. of culture is

consistent with results reported in this study on the effectiveness of folate deprivation for eliciting fragile X expression in cultured skin fibroblasts (Jennings et al., 1979).

While results from this study do support the hypothesis that folate deprivation is indeed of significance in fragile site expression, they do not clarify Sutherland's (1979a) proposal for the mode of its action, which suggested that folate deprivation leads to reduced pyrimidine biosynthesis, specifically, reducing the level of thymidylate available for de novo DNA synthesis. Experiments using MEM-Ad in cell culture were not useful in testing this hypothesis since the medium would not support cell growth. However, experiments which were undertaken to examine the chromosome decondensation effects of BrdU did offer indirect evidence in support of Sutherland's proposal. In its capacity to act as a thymidine analog, BrdU, when added to the culture medium late in the replicative cycle, had no effect on the frequency of fragile X expression. This result was consistent with Sutherland's (1979a) findings that the addition of thymidine or BrdU resulted in a lower frequency of fragile X expression only if added prior to or during, the earlier stages of DNA replication. The late incorporation of BrdU had little or no effect on the frequency of expression of the fragile X chromosome.

4.22 Hypotonic effects at chromosome harvest. The expression of the fragile X chromosome in MEM, containing normal levels of folate, suggested that features of the fibroblast method, other than folate deprivation in cell culture, were

important for the expression of the fragile site. The comparison of different hypotonic treatments of fibroblasts cultured in media deficient in folic acid revealed that NaCitrate provided a significantly higher frequency of expression than KCl. That the data did not show a significant difference between these two hypotonic treatments for fibroblasts cultured in medium containing normal levels of folate is more likely due to the very low frequencies of marker expression using this medium than to a lack of effect of the NaCitrate in the absence of folate deficiency. However, the data do show the highest frequencies of fragile X expression when these two features are combined: folate deprived culturing conditions and hypotonic treatment at chromosome harvest with NaCitrate.

These results do not explain the discrepancy between the frequencies of marker expression in fibroblasts and in lymphocytes using KCl as a hypotonic. For the same culturing conditions, lymphocytes that were treated with KCl at harvest showed frequencies of expression comparable to those obtained in fibroblasts using NaCitrate as a hypotonic.

The effects of different hypotonic treatments at chromosome harvest have not been extensively studied by other investigators of heritable fragile sites. The analysis of harvest methods, when reported in studies of the fragile X chromosome by previous investigators, has indicated that all prior studies used KCl. The only indication in the literature that heritable fragile sites are sensitive to different hypo-

tonic treatments is in a report by Bühler et al. (1970) which showed that the 2q12 fragile site is expressed at a higher frequency when the lymphocyte chromosome preparations are treated with a hypotonic of NaCitrate rather than KCl. Although the effects of a NaCitrate hypotonic treatment on lymphocytes was not examined here, its application might result in a higher frequency of expression of the Xq27-28 fragile site in that tissue and may be particularly useful in determining the carrier status of potential female heterozygotes.

One could speculate that the mechanism underlying the improved frequency of expression with NaCitrate involves the chelation of divalent cations by the citrate moiety of the hypotonic. In discussing the nature and origin of achromatic lesions in chromosomes, Chaudhuri (1972) suggested that, in addition to disturbances caused in the DNA itself, altered concentrations of divalent cations can produce decondensation irregularities. Earlier work by Kabat (1967) and Steffensen (1961) also stressed the importance of divalent metal ions in the organization and structural maintenance of the nucleoprotein complex of the chromosome. Golomb and Bahr (1974) showed that the chelation of divalent cations, particularly Ca^{+2}, can interfere with the normal condensation process, and, further, that excess Ca^{+2} can lead to severe chromosome contraction under some circumstances.

The marked difference between the appearance of chromosomes following treatment in KCl and NaCitrate suggests that

NaCitrate interacts more vigorously with the chromatin. In general the chromosomes appeared less contracted, and the integrity of the chromatin appeared more relaxed. With KCl, the peripheries of the chromosomes appeared more distinct, which seems to indicate that KCl interacts less directly with the chromatin material than NaCitrate. Studies to test the effects of other chelating agents, such as EDTA or EGTA, during cell culture or chromosome harvest, might be useful in accentuating the frequency of expression of the Xq27-28 fragile site. These studies may also be useful for gaining a better understanding of the role of divalent cations in fragile site expression.

Both folate deprivation and NaCitrate as a hypotonic have been shown to influence the frequency of expression of the fragile X chromosome in fibroblast culture. The finding of a higher frequency of expression when both of these conditions were imposed on cells suggests that the two features of the method may interact with one another. On the basis of proposed modes of action for folate deprivation and NaCitrate hypotonic, it could be hypothesized that by causing abnormal replicative behavior or other disturbances in the DNA, folate deprivation may make the Xq27-28 fragile site particularly sensitive to agents that interrupt the condensation process or effectively decondense the chromatin at the fragile site and so elicit fragile site expression.

4.3 Reliable determination of the frequency of expression of the fragile X chromosome in fibroblasts.

4.31 Variable appearance of the fragile X chromosome. Reliable determination of a frequency of marker expression required recognition of the variation in appearance of the expressed fragile site. For the purposes of this study, slide preparations were stained with aceto-orcein on a routine basis. Although, unlike G-banding, orcein does not permit an absolute distinction between the X chromosome and the human C group autosomal chromosomes of comparable size, it was visually more effective than trypsin G-banding in revealing expression of the fragile site.

The better resolution of the fragile site, seen with aceto-orcein, might be attributed to its higher affinity as a stain for the nucleic acid component of chromatin rather than the associated protein components of the chromosome (Pearse, 1961). Giemsa is thought to stain protein components associated with particular DNA regions of the chromosome (Chuprevich et al., 1973; Brown et al., 1975; Sumner, 1976) which may make it less useful for distinguishing discontinuities in chromosome structure.

The variation in expression of the fragile X chromosome reported in the results (Fig. 3) with aceto-orcein staining is consistent with the observations of other investigators (Lubs, 1969; Giraud et al., 1976; Sutherland, 1979b; Turleau et al., 1979). Most often, a terminal satellite was expressed on both chromatids, giving a bipartite appearance to the

satellite chromatin as a whole. Occasionally the terminal satellite appeared as single chromatin mass with the individual chromatid satellites possibly fused or overlapping (Fig. 3 d, e), and, more rarely, only one of the chromatids expressed a (single) satellite (Fig. 3 f).

A double satellited X chromosome (Fig. 3 g) was a morphological variation observed only three times in the course of this study. This phenomenon has been reported by other investigators at similarly low frequencies and has been attributed to selective endoreduplication of the satellite chromatin (Lubs, 1969; Giraud et al., 1976; Sutherland, 1979b; Turleau, 1979). Analogous duplications of the long arm segment of the chromosome arm distal to the fragile site have been reported for the 2q12 and 10q23 fragile sites (Lejeune, 1966, 1968; Fraccaro et al., 1972; Ferguson-Smith, 1973; Noël et al., 1977). For the 2q12 and 10q23 fragile sites, duplication of the long arm segment distal to the fragile site has generally been thought to be the result of mitotic non-dysjunction and subsequent replication of the chromatids distal to the fragile site rather than selective endoreduplication of the long arm fragment. Selective endoreduplication has, however, been the preferred interpretation of multiple copies of the chromosome fragment distal to the fragile site when the piece of chromatin is small. Magenis et al. (1970) reported the presence of multiple copies (2 - 12) of the small long arm fragment distal to the fragile site at 16q22 in lymphocyte preparations from several patients.

Well extended chromosome preparations permitted better definition of the fragile site. However, on the basis of G-banding, it was not possible to precisely assign the fragile site to either the G-positive band at Xq27 or the G-negative band at Xq28. Most often, the fragile site appeared at the junction of the two bands. As previously stated (section 1.22), precise assignment of the fragile site is to some extent a function of the banding method employed, and, because of reported discrepancies in the location of the fragile site on the X chromosome, it is reported as Xq27-28 (Sutherland, 1979b).

Well extended chromosome preparations stained with aceto-orcein frequently showed fine threads traversing the fragile site, connecting the displaced satellite with the rest of the long arm of the chromosome (Fig. 3 d, e). This suggests that the chromatin or DNA is continuous across the fragile site, and that the fragile site is a region of localized decondensation rather than an actual interruption or break of the chromosome. That the fragile site has a tendency to shear or break off, however, is suggested from the observation of detached satellites in some chromosome preparations. Since detached satellites were not included in a determination of the frequency of expression of the fragile X, they may be responsible for the unexpectedly low frequencies of expression of the fragile X occasionally observed in some patients (e.g., detached satellites occurred 1½ times more frequently than did intact fragile X chromosomes for Patient

E in fibroblasts cultured in MEM-FA, Table III).

4.32 Condensation. The results showed that the frequency of expression of a fragile X chromosome in cultured skin fibroblasts was significantly higher in more extended chromosome preparations. In order to obtain a more reliable determination of the frequency of expression, a criterion was established which metaphases had to meet before being scored for the fragile X chromosome.

The imposition of this scoring criterion may have contributed to higher frequencies of expression of the fragile X chromosome reported here. Lymphocyte studies using similar culturing conditions conducted by Jacobs et al. (1980) of all five patients from Family No. 1 demonstrated the marker chromosome only in Patients C (5%), D (13.2%), and E (13.5%). Other lymphocyte studies by Dunn et al. (1980), of three patients from Family No. 1 (A, B, and E), demonstrated the presence of the marker chromosome only in Patient E (19%). [Patients A and B, reported here, expressed the marker in 10.3% and 5.1% of cells scored respectively. For Patient C, the frequency of expression was 6.5%, for Patient D, 15.9% and 19.3%, and for Patient E, 32.2% of the cells scored (Table V).]

A fibroblast slide preparation of Patient D, the frequency of marker expression for which was determined here to be 19.3%, was sent to two other laboratories for study. While these examinations found the marker to be present, it was expressed at a lower frequency (Sutherland, personal communication; Jacobs and Glover, personal communication). These inter-

laboratory discrepancies in determining the frequency of marker expression are difficult to assess, but may in part be due to variation in what is considered an acceptable degree of chromosome condensation in scoring for the marker chromosome.[2] Higher frequencies of expression of the fragile X chromosome in less condensed chromosome preparations has also been noted by other investigators (Fox and Gerrard, personal communication). Looking at more extended chromosomes permits better definition of the separation between the satellite and the rest of the long arm of the X chromosome. This reduces the scoring ambiguity encountered with more condensed chromosome preparations, where the distinction between the terminal end of the long arm and the satellite is obscured.

Whether expression of the Xq27-28 fragile site is actually the result of an abnormality in chromosome condensation, perhaps due to premitotic alterations in the chromatin of the fragile site, or whether the higher frequency of expression in less condensed chromosome preparations is simply the result of better resolution of the fragile site, is not known. The fact that the fragile site was only expressed in

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[2] All observations here of the fragile X were done using phase contrast microscopy. While it has not been determined to what extent this may be important in the observed frequency of marker expression, other studies have employed, on a regular basis, bright field illumination for observing the fragile X. This technical difference may be in part responsible for discrepancies between the frequencies of expression reported by different laboratories. Slides examined here from a laboratory which uses bright field illumination were stained to a greater intensity than the slides examined for this report. It is possible that overstaining may mask the expression of the fragile site in some metaphases and thereby contribute to a lower frequency of expression of the fragile X chromosome.

a fraction of well extended chromosome preparations (Table VI) suggests that condensation effects may be secondary to irregularities in the chromatin established earlier in the cell cycle, i.e., during DNA replication. As mentioned above, decondensation at the 2q12 fragile site has been shown to parallel late or faulty replicative behavior of the chromatin at the fragile site (Bühler et al., 1970). In autoradiographic studies of the 2q12 fragile site, Fraccaro et al. (1972) showed that while the abnormal chromosome No. 2 was synchronous in replication with its homologue in both arms, the incorporation of ^{3}H thymidine label into the fragile site was not observed. They interpreted this finding as indicating that either the DNA in the fragile site did not replicate, or there was not enough DNA in the discontinuity itself to be revealed by autoradiography.

Methods employed to improve the frequency of expression by inhibiting chromosome condensation just prior to the time of harvest, that is, the use of 5-BrdU and actinomycin-D, failed to significantly alter the frequency. While both actinomycin-D and BrdU produced more extended chromosomes, neither had any effect on the frequency of expression. Actinomycin-D, in combination with folate deprivation, produced such extensive chromosome breakage that it was felt that any significant improvement in the frequency of expression would have to be interpreted cautiously.

BrdU was effective in eliciting specific decondensation effects in some regions of some chromosome. In its capacity

to act as a thymidine analog, BrdU produced significant decondensation effects in the AT-rich paracentromeric regions of heterochromatin on chromosomes 1, 9, and 16. The 2^{o} constriction regions on the D and G group chromosomes also showed specific decondensation effects. These observations were consistent with earlier reports of chromosomal segments particularly sensitive to BrdU incorporation and decondensation (Palmer, 1970; Zakharov et al., 1974).

The effects of BrdU on the 2^{o} constriction of the D and G group chromosomes, and the unresponsiveness of the Xq27-28 fragile site to BrdU, may be taken as indirect evidence in support of the belief that the Xq27-28 fragile site is not the result of a translocation of chromatin from one of these acrocentric chromosomes.

4.33 Autosomal telomeric markers. The expression of terminal markers on some of the autosomal chromosomes under folate deprived culturing conditions has led some investigators to caution against assuming absolute diagnostic specifity for the fragile X chromosome (Jennings et al., 1980). In this study, autosomal markers were expressed in both patients and controls at low frequencies, whereas the fragile X chromosome was expressed only in patients at relatively much higher frequencies, suggesting that the expression of autosomal markers does not represent a threat to the diagnostic usefulness of the fragile X chromosome.

Expression of terminally marked autosomes in chromosome preparations from lymphocytes cultured in M199 is well docu-

mented by other investigators studying the fragile X chromosome (Howard-Peebles, personal communication; Dunn et al., 1980; Jacobs et al., 1980; Jennings et al., 1980; Martin et al., 1980; Soudek et al., 1980). With some consistency, terminal markers have been noted on chromosomes No. 1, 3, 6, a small C group chromosome (probably 12), 13, and 19. The median interstitial fragile site at 16q22 has also been reported by several workers. Because these autosomal markers are expressed in both normal controls and patients, they have generally been accepted as a peculiarity of cells cultured in M199.

The frequency of expression of these autosomal markers in patients expressing the fragile X chromosome has also been reported to be significantly lower than the frequency of expression of the fragile X chromosome (Jacobs, et al., 1980; Jennings et al., 1980).[3] While some workers have suggested that components or deficiencies in M199 other than its low folate content may be responsible for the general chromosome instability resulting from culture in M199 (Emerit et al., 1974; Keck and Emerit, 1979), the demonstration here of ana-

[3]In fibroblasts cultured in MEM-FA from Patient D (Table VII), the frequency of expression of telomerically marked autosomes in total was 42 in 289 cells scored or about 7%. In the same patient, the frequency of expression of the fragile X was 112 in 589 cells or about 19%. A more accurate appreciation of the significance of telomerically marked autosomes can be achieved if the number of these markers in any one autosomal group is considered separately. For example, all of the autosomal markers in the A group chromosomes for Patient D (Table VII) were actually confined to chromosomes 1 and 3. As there are two representatives of each of these chromosomes for every X in this male patient, the frequency of A group marked autosomes for this patient would be 13/589 x 4 or about 0.5%.

logous telomeric changes on chromosomes cultured in MEM-FA suggests that the relatively low level of folic acid in M199 is responsible for the destabilizing effects of this medium.

Analogous effects on chromosomes *in vivo* have been reported in patients suffering from vitamin B_{12} deficiency, folate deficiency, or both (Heath, 1966). Recently, Côté and Papadakou-Lagoyanni (1979) reported that an *in vivo* folate deficiency associated with β-thalassaemia may be involved in the increased incidence of spontaneous chromosome breakage seen in some patients. Autosomal telomeric markers appear to represent a nonspecific chromosomal response to either *in vivo* or *in vitro* folate deficiency.

4.4 Conclusions and future prospects.

This study has demonstrated that the fragile X chromosome is expressed in cultured skin fibroblasts. Culturing conditions of folate deprivation, which had previously been reported to influence the expression of the fragile X chromosome in peripheral blood lymphocytes, have a similar effect on the expression of the marker chromosome in cultured fibroblasts. A NaCitrate hypotonic treatment at chromosome harvest was shown to influence the frequency of expression of the fragile site in fibroblast tissue, and the degree of chromosome condensation was shown to be important in the determination of a frequency of expression of the fragile X chromosome.

While the use of fibroblasts does not at this time repre-

sent an improvement over lymphocytes for determining whether an affected male possesses the fragile X chromosome or whether or not a female is a carrier, a technique for demonstrating the marker in fibroblasts does make the prospect of demonstrating it in amniocytes more likely. Antenatal diagnosis of familial sex-linked mental retardation associated with the fragile X chromosome will depend on whether or not the fragile X chromosome can be reliably detected in cultured amniotic fluid cells. Since it has been shown that amniocytes behave similarly to populations of fibroblasts in terms of chromosome analysis (Bryant et al, 1978), the development of a method to elicit the expression of the fragile X chromosome in fibroblasts is an important step toward that goal.

In order for the fibroblast technique to be clinically valuable, further attempts should be made to increase the frequency of expression of the marker. Also, in order that it be considered reliable for diagnostic purposes, this technique should be applied to further subjects in other families in which the disorder is segregating.

Fibroblasts do provide a useful laboratory cell population to study cell culturing and chromosome harvesting conditions that influence the expression of the Xq27-28 fragile site. Further application of histochemical and immunofluorescent chromosome banding techniques may permit better definition of the DNA base composition of the fragile region and add to our general understanding of the chromosome structural irregularities of a fragile site. These techniques may also

contribute to a clearer understanding of the relationship that exists between the fragile X chromosome, its culturing peculiarities, and the mental retardation associated with this chromosome anomaly.

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APPENDIX A

Consent forms and certificate of approval for clinical research and other studies involving human subjects.

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DEPARTMENT OF MEDICAL GENETICS
VANCOUVER, B.C., CANADA

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Consent Form

It has been explained to me that the mental retardation that has occurred in my family is a sex-linked hereditary disorder affecting mostly males, while females may act as carriers of the disorder when they are outwardly quite healthy. I understand that cultures of blood cells have, in some recent studies, demonstrated a chromosome irregularity that could provide information for determining whether male infants are liable to develop such retardation, and perhaps whether females are carriers or not.

Accordingly, I give my permission to obtain a blood sample by venepuncture and a tissue sample obtained by skin biopsy. I understand that the skin biopsy procedure involves lifting a very small piece of skin (about this "0" size) with a hypodermic needle and snipping the skin away with a pair of sharp eye lid scissors. I understand that cells from the blood and tissue samples will be grown in tissue culture and the chromosomes(material which carries information about inheritance) will be examined.

I understand that I am free to withdraw my consent and to discontinue my participation in this study at any time without prejudice to future care. I understand that my participation will be kept confidential at all times.

Date: _____, 19__

Patient's Signature: _____

Parent's Signature (if applicable): _____

Witness' Signature: _____

continued...

APPENDIX B

Formulation of tissue culture media.

Component	MEM mg/L	M199 mg/L	MEM-FA mg/L	MEM-Ad mg/L
Inorganic Salts				
$CaCl_2$	200.00	140.00	200.00	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	—	0.72	—	—
KCl	400.00	400.00	400.00	400.00
KH_2PO_4	—	60.00	—	—
$MgSO_4 \cdot H_2O$	200.00	200.00	200.00	200.00
NaCl	6800.00	8000.00	6800.00	6800.00
$NaHCO_3$	2200.00	350.00	2200.00	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00	—	140.00	140.00
$Na_2HPO_4 \cdot 7H_2O$	—	90.00	—	—
Other Components				
Adenine sulfate	—	10.00	—	—
Adenosinetriphosphate (Disodium salt)	—	1.00	—	—
Adenylic acid	—	0.20	—	—
Azaserine	—	—	—	0.20
Cholesterol	—	0.20	—	—
Deoxyribose	—	0.50	—	—
Glucose	1000.00	1000.00	1000.00	1000.00
Glutathione	—	0.50	—	—
Guanine HCl (Free base)	—	0.30	—	0.30
Hypoxanthine	—	0.30	—	—
Phenol red	10.00	20.00	10.00	10.00
Ribose	—	0.50	—	—
Sodium acetate	—	50.00	—	—
Thymine	—	0.30	—	—
Tween 80	—	20.00	—	—
Uracil	—	0.30	—	—
Xanthine	—	0.30	—	—

continued...

Component	MEM mg/L	M199 mg/L	MEM-FA mg/L	MEM-Ad mg/L
Amino Acids				
L-Alanine	8.90	50.00	8.90	8.90
L-Arginine·HCl	126.00	70.00	126.00	126.00
L-Asparagine·H_2O	15.00	—	15.00	15.00
L-Aspartic acid	13.30	60.00	13.30	13.30
L-Cysteine HCl·H_2O	—	0.11	—	—
L-Cystine	24.00	20.00	24.00	24.00
L-Glutamic acid·H_2O	14.70	150.00	14.70	14.70
L-Glutamine	292.00	100.00	292.00	292.00
Glycine	7.50	50.00	7.50	7.50
L-Histidine HCl·H_2O	42.00	21.88	42.00	42.00
L-Hydroxyproline	—	10.00	—	—
L-Isoleucine	52.00	40.00	52.00	52.00
L-Leucine	52.00	120.00	52.00	52.00
L-Lysine HCl	72.50	70.00	72.50	72.50
L-Methionine	15.00	30.00	15.00	15.00
L-Phenylaline	32.00	50.00	32.00	32.00
L-Proline	11.50	40.00	11.50	11.50
L-Serine	10.50	50.00	10.50	10.50
L-Threnine	48.00	60.00	48.00	48.00
L-Tryptophane	10.00	20.00	10.00	10.00
L-Tyrosine	36.00	40.00	36.00	36.00
L-Valine	46.00	50.00	46.00	46.00
Vitamins				
Ascorbic acid	—	0.05	—	—
Alpha tocopherol phosphate (disodium salt)	—	0.01	—	—
d-Biotin	1.00	0.01	1.00	1.00
Calciferol	—	0.10	—	—
Ca-pantothenate	1.00	0.01	1.00	1.00
Choline chloride	1.00	0.50	1.00	1.00
Folic acid	1.00	0.01	—	1.00
i-Inositol	2.00	0.05	2.00	2.00
Menadione	—	0.01	—	—
Niacin	—	0.025	—	—
Niacinamide	1.00	0.025	1.00	1.00
Para-aminobenzoic acid	—	0.05	—	—
Pyridoxal HCl	1.00	0.025	1.00	1.00
Pyridoxine HCl	—	0.025	—	—
Riboflavin	0.10	0.01	0.10	0.10
Thiamine HCl	1.00	0.01	1.00	1.00
Vitamin A (acetate)	—	0.14	—	—

APPENDIX C

Folate determination on various laboratory lots of fetal serum and Medium 199 with and without a serum supplement. (Assayed by Dr. George Gray, Department of Hematology, Vancouver General Hospital, Vancouver).

No.	Sample	Manufacturer	Lot No.	Folate $x10^{-3}$ mg/L
A	Human cord serum	—	—	7.0+
B	Calf serum	Gibco	R191223	9.2
C	Fetal calf serum (FCS)	Gibco	R695618	10.5
D	Fetal calf serum (FCS)	Wild Life Serums	802072	14.0
E	Fetal calf serum (FCS)	Flow Laboratories	29101111	2.2
F	Calf serum	Gibco	R595419	9.1
G	Fetal calf serum (FCS)	Gibco	C184511	5.9
H	Fetal Calf serum (FCS)	Gibco	C184511*	5.4
I	Medium 199 w/o FCS	Gibco	R090814	16.5
J	Medium 199 w/ FCS_E	Gibco	A891012	12.0

\+ normal human serum value range 3-16 x 10^{-3}mg/L
* heat inactivated

All FCS media supplements in the study were taken from Flow Laboratories Lot No. 29101111 (Sample E). While it is of interest that the folate level in this serum is particularly low relative to other samples tested, it is difficult to imagine that a 1:20 dilution (5%) of serum into a culture medium would contribute significantly to the folate level. Furthermore, in comparing Samples I and J of M199 with and without a FCS supplement, there appeared to be more variation in folic acid content between different lots of M199 than would be contributed by different lots of FCS supplemented at 5%.