379
N81
No. 6143

A COMPARISON OF STRAIGHT-STAINED, Q-STAINED, AND REVERSE FLUORESCENT-STAINED CELL LINES FOR DETECTION OF FRAGILE SITES ON THE HUMAN X CHROMOSOME

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Susan L. Coultas, B.S.

Denton, Texas

May, 1985

Coultas, Susan L., *A Comparison of Straight-Stained, Q-Stained, and Reverse Fluorescent-Stained Cell Lines for Detection of Fragile Sites on the Human X Chromosome*. Master of Science (Biology), May, 1985, 35 pp., 2 tables, 5 illustrations, bibliography, 21 titles.

Cell cultures were examined for percentage of fragile sites seen in straight-stained, Q-stained, and reverse fluorescent-stained preparations. In all cases, percentage of fragile site expression was decreased when compared to straight-stained preparations. However, fragile sites seen in Q- and RF-stain could be identified as on X chromosomes. This was not the case in straight-stain due to lack of banding. It is proposed that screening labs routinely employ fluorescent banding methods when identifying chromosomal fragile sites. If at all possible, straight-stain should be used to identify chromosomes exhibiting fragile sites. These slides should then be destained and restained to identify on which chromosome the fragile site is located.

TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF ILLUSTRATIONS	v
Chapter	
I. INTRODUCTION	1
II. CLINICAL AND CYTOGENETIC ASPECTS OF FRAGILE-X SYNDROME	2
III. BIOCHEMICAL ASPECTS OF FRAGILE-X SYNDROME	8
IV. PROPOSED RESEARCH	12
V. MATERIALS AND METHODS	14
VI. RESULTS AND DISCUSSION	19
REFERENCES	34

LIST OF TABLES

Table Page

I. Percentage of Fragile Sites Present in Straight-Stain, Q-Stain, and RF-Stain for the Six Cultures Examined. 20

II. Chi-square Values and Probabilities Associated with the Differences Found in Various Staining Methods. 24

LIST OF ILLUSTRATIONS

Figure Page

1. Fragile-X in a Large Kindred: Transmission Through Normal Males 6

2. Diagrammatic Representation of G-Banded X Chromosome - The Fragile Site Occurs at Distal End of Long Arm Between Regions q27 and q28. 21

3. Photographs of Straight-Stained Fragile-X Positive Cell Lines. 26

4. Photographs of Q-Stained Fragile-X Positive Cell Lines. 28

5. Photographs of Reverse Fluorescent-Stained Fragile-X Positive Cell Lines. 30

CHAPTER I

INTRODUCTION

A high incidence of a broken or fragile site on the X chromosome in humans at the end of the long arm (between Xq27 and Xq28) in cases of a form of X-linked mental retardation has been found. This condition has been termed Fragile-X Syndrome due to the marker on the X that has been associated with the mental deficiency found in some males. Turner et al. [1] reported that in about 30% of families with males affected with non-specific X-linked mental retardation, those males showed fragile sites on the X chromosome in some proportion of the cultured lymphocytes. Among the many clinical manifestations of Fragile-X Syndrome are mental retardation, high forehead, prominent ears, prominent supraorbital ridge, and especially macro-orchidism. One should note, however, that none of these findings are constant throughout the syndrome.

CHAPTER II

CLINICAL AND CYTOGENETIC ASPECTS OF FRAGILE-X SYNDROME

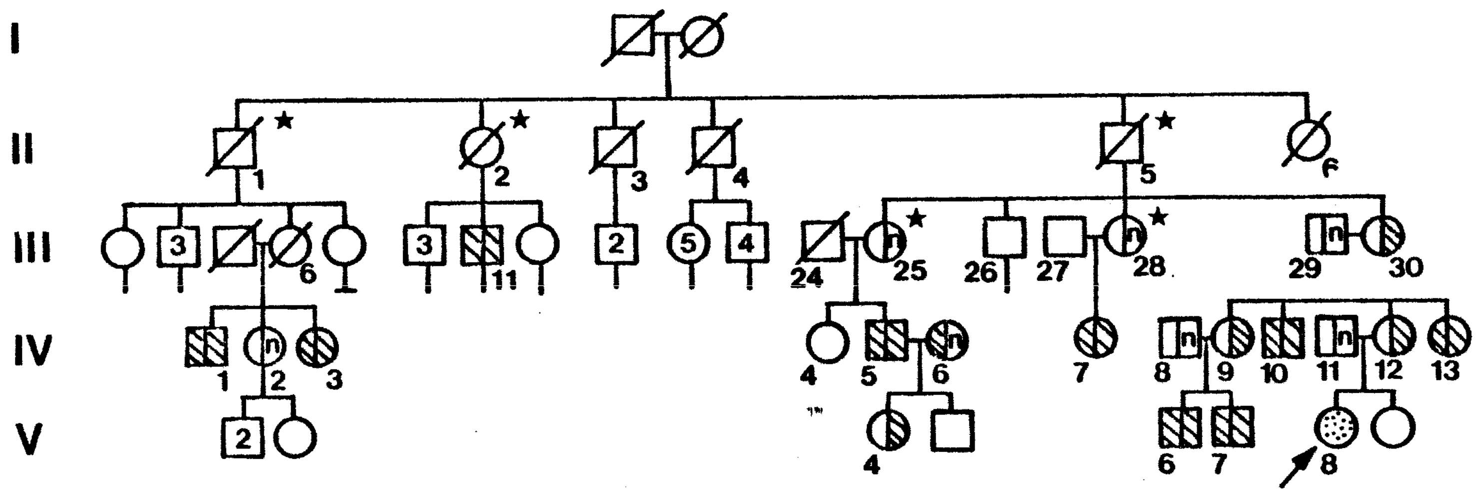
Since the fragile-X site was discovered by Lubs in 1969 [2], many similar sites have been found on other chromosomes (i.e., 2, 6, 7, 9-12, 16, and 20). Steinbach et al. [3] suggest that telomeric lesions similar to the fragile-X formation can probably occur on every chromosome arm. Laboratory interest in fragile sites increased in 1977 when Sutherland [4] discovered that the fragile-X phenomenon was folic acid sensitive. With depletion of folic acid in culture medium, breaks may occur at Xq27/28. The medium normally used is Medium 199 (more recently MEM-FA) due to low folate content. There has been some uncertainty as to whether the break occurs at q27, q28, or at the interface between the two. An elongation of the chromosome does occur, and it displays a negatively heterochromatic gap between the two bands mentioned. Enhancement of fragile sites in lymphocyte cell lines can be accomplished by prolonging the culture time to 96 hours, elevating the pH, diminishing the colcemid, and air drying of slides [5]. Other advances with fragile-X include the addition of methionine, trifluorothymidine, or FUdR to culture medium. The addition of FUdR was found to be of

help in demonstrating the fragile-X in lymphoblastoid cell lines and fibroblasts [5]. One major aspect of fragile-X is that patients who suffer from this type of X-linked mental retardation never show the fragile site in all of the observed metaphases [6]. Indeed, there seems to be a characteristic level of fragile-X in each individual which does not change even after several years. Eberle et al. [7] have shown this to be the case by examination of three brothers with fragile-X. They repeated examination of the blood cultures of the brothers under the exact same conditions three times at intervals of several months and found that the percentage fragile-X seen remained fairly constant. More recently, a variant of Fragile-X Syndrome has been investigated. Buhler et al. [8] were presented with a case of a male child with growth and development retardation as well as dysmorphic syndrome. The proband's blood was cultured under normal rather than folic acid deprived conditions. The findings included a decondensed site at Xq26 rather than between q27 and q28. This decondensation could be clearly seen in G, Q, and R-banded preparations, although not as clearly in Q-banded preparations. The pattern of the family history correlated that seen in X-linked mental retardation, however, the clinical findings were not similar to those seen in Fragile-X Syndrome. This proband exhibited retarded growth, hearing impairment, epicanthus, flat bridge of the

nose, high forehead, high arched palate, head circumference below the third percentile, cryptorchidism, rudimentary scrotum, and small penis. Most importantly, the area of decondensation was found in all mitoses, as opposed to only a certain percentage found in normal fragile-X positive cases. Brown et al. [9] correlated a significant concordance for autism in identical twins and a male to female ratio of about 4:1 to possible genetic inheritance through fragile-X. Indeed, they found five fragile-X males who also displayed autism suggesting that the association may show a significant frequency [9]. Many investigators have found families in which the only way the fragile-X could have been inherited was through a male carrier who was normal. The case reported by Van Roy et al. [10] arose when the parents of a child with Down Syndrome were referred for genetic counseling. Due to this referral, several other mentally retarded persons of both sexes were investigated. Upon examination of all but one of the mentally retarded family members for such parameters as testicular volume, stature, head circumference, and length of right ear the following data were recorded. [10]. The fragile-X was found in 16-37% of cells in the six mentally retarded male patients, 18-42% in the three mentally retarded female patients, and in 0.5-26% in some of their normal or nearly normal female relatives. The major point of this study was that the fragile-X had to be transmitted

through two normal males (II.1 and II.5) to affected grandchildren (Fig. 1). The arguments are strong in favor of this hypothesis, but there can be no proof in this case. Fryns and Van Den Berghe [11] and Daker et al. [12] have also found normal males that carry the fragile-X. In the case of Daker et al. [12], a 42-year-old man showed fragile-X in 15% of metaphase spreads. The man, in interview, showed a level of comprehension and conversation that suggested an IQ well within the normal range. The man's brother also had the fragile site which indicates its heritable nature in this instance. In another study by Turner et al. [13] on 128 mildly retarded schoolgirls in New South Wales, approximately four percent of the girls carried the fragile-X. When the families of the girls were examined, affected males with macro-orchidism and fragile-X were also found. Physical examination of the girls with fragile-X yielded essentially normal results. This suggested that the presence of the fragile-X marker should be suspected in mildly retarded but physically normal girls. Since prenatal diagnosis has recently become feasible for detection of heterozygous females as well as hemizygotes, the decision faced by families could become extremely complicated in light of the current research findings.

Fig. 1--Fragile-X in a large kindred: Transmission through normal males.



Normal phenotype, not examined cytogenetically

Normal phenotype, no fra (x) chromosome found

Probable heterozygate

Normal offspring only

Fra (x) chromosome found

Mental retardation

Obligate hemizygate

Down's syndrome

CHAPTER III

BIOCHEMICAL ASPECTS OF FRAGILE-X SYNDROME

Many researchers have investigated the biochemical mechanism behind fragile sites. As previously mentioned, Sutherland [4] discovered that Medium 199, which has a low folic acid content, was necessary to bring about the expression of a fragile site at Xq27/28. Folic acid acts as a pre-cofactor for thymidylate synthetase, and it is seen that fragile-X expression is suppressed by adding thymidine to the medium. It has also been shown that fluorodeoxyuridine (FUdR) will induce fragile-X even in the presence of folic acid [14]. FUdR blocks _de novo_ thymidylate synthesis by inhibiting thymidylate synthetase. Since exogenous thymidine can enter DNA through conversion to thymidylic acid by the "scavenger" thymidine kinase pathway, these results suggest that folic acid deficiency in absence of exogenous thymidine induces fragile-X expression by depletion of thymidylic acid available for DNA synthesis. The experiments set up by Gardiner et al. [15] suggested the possibility of using exogenous thymidine to suppress fragile-X even after the fragile site has been induced by FUdR. They removed 99.4% of the exogenous thymidine, resulting in the only thymidylic acid available

to DNA of lymphocytes cultured in MEM supplemented with 20% deoxy fetal bovine serum being through the de novo cell pathway. Their data supported findings that fragile site, once induced by addition of FUdR or decreased folic acid, can be suppressed by addition of exogenous thymidine, thus confirming that the mechanism of action of fragile site induction by FUdR is thymidylic acid starvation. This information may point out a possible therapeutic mechanism if the actual mental retardation can be definitely linked to the fragile site as its cause. Perhaps the fragile site could be repaired early enough in development to negate the destructive influence. Eberle et al. [16,17] reported diminished proportions of fragile-X chromosomes in co-cultures of blood cells taken from fragile-X patients with normal blood cells. However, Soudek and Emanuel [18] did not observe suppression of fragile-X in media where fragile-X negative cells had previously been cultivated for two days. They used equally active mitotic cell lines in equal proportions demonstrating that none of the cell line were suppressed. They also observed no "infection" of a fragility causing factor from fragile-X cells that would have caused normal cell lines to show fragile sites. Vekemans et al. [19] addressed the possibility of a general chromosomal instability in cells of patients with the Fragile-X Syndrome. Chromosome preparations were screened and scored for both chromatid and chromosomal breaks.

Their data showed that the Fragile-X Syndrome is not associated with a general chromosomal instability expressed in folate deficient medium and assessed in terms of chromosome breakage [19]. The number of chromosome and chromatid breaks observed in control males, control females, and Fragile-X males was not significantly different from the number expected. Mixon and Dev [20] performed several experiments designed to illucidate the importance of methionine in culture medium for fragile site expression. They found that when methionine was supplemented into medium with FUdR, the percent fragile sites climbed to 50% and more. When S-adenosylmethionine (SAM), which is a direct methyl group donor, and FUdR supplement were used, increased fragile sites were observed for two of the three concentrations. The trend was not statistically significant, however. 5-Azacytidine (5-aza), which inhibits methylation at the DNA level, methionine, and FUdR supplement causes a significant decrease in the frequency of fragile-X. This would be seen as a reversal of the apparent effect of FUdR and methionine. Finally, the addition of S-adenosylhomocysteine (SAH), which inhibits the production of SAM from methionine, methionine, and FUdR also causes a nearly linear decrease in the appearance of fragile sites with an increase in concentration. The methylation of DNA when blocked at either the methylation step itself or at the formation of

the methyl donor, produced the same effect. As the completeness of the block increased the expression of the fragile site decreased. The expression of the fragile sites was enhanced by those molecules that actually participate in DNA methylation but was depressed by those molecules that inhibit DNA methylation.

CHAPTER IV

PROPOSED RESEARCH

All of the screening of fragile sites (on the X especially) has been by using straight-stain as the stain of choice. This may not be a logical choice since there are no bands present with this staining method. Since there are other chromosomes that can show fragile sites, it would seem likely that in counting spreads for fragility, some of those counted could contain other chromosomal fragile sites. The percentage fragile site expression would be artificially higher than it should be under such conditions. To use a staining method that will show bands on the individual chromosomes would seem to be a more logical choice. By doing this, those chromosomes that have fragile sites and are not X's would be eliminated. It might be possible to use only one staining method that shows banding, yet not lose the expressivity. However, it is possible that percent expressivity would decrease in an alternate staining method. The best possible technique for identification would probably be to stain and locate possible fragile X's in straight-stain. These slides could then be destained and restained by means of a banding method. Those cells with fragile sites should be relocated

and karyotyped for positive identification of the X. This should all be done in the interest of accuracy. As legislation of the field of genetics becomes more concentrated, those labs that show concise and accurate results will be better equipped to handle any questions or problems that could possibly arise.

CHAPTER V

MATERIALS AND METHODS

Blood of patients was collected in heparinized tubes, and 0.25-0.30 ml of lymphocytes are placed in Falcon (#2037) tubes of Media 199 (Gibco) supplemented with 2.0% fetal calf serum (Gibco), 0.8% antibiotics (Gibco), 2.0% phytohemagglutinin (Gibco), and 0.3% L-glutamine for 72 or 96 hours at 37 degrees centigrade in a carbon dioxide incubator. Colcemid (0.2 ml) was added to each culture for 2.5 hours. The culture was mixed by flipping the tube, and the suspension was poured into a clean 12 ml graduated centrifuge tube. The tube was spun at 800-1000 rpm for 8-10 minutes using a bench top centrifuge. The supernatant was aspirated off being careful not to disturb the cell button. The button was resuspended by flipping the bottom of the tube. Room temperature 0.075 M KCl (4 ml) was added to each tube, mixing completely. The tubes were incubated for 15-25 minutes, depending on spreading produced. Approximately 0.25 ml of fresh fixative (3 parts methyl alcohol:1 part glacial acetic acid) was added to each tube, and air was gently bubbled through cell suspension with a Pasteur Pipette. The cell suspension must sit at room temperature for 8 minutes. The cell suspension was

centrifuged at 800-1000 rpm for 8 minutes, supernatant aspirated, and cell button resuspended. Approximately 2 ml of room temperature fixative was gently added to cell button by layering it on the cells, trying not to mix completely. The tube was then placed in the refrigerator for at least two minutes. The tube was removed from refrigerator, and the culture was bubbled by injecting air with a Pasteur Pipette in order to break clumps and centrifuged for 6 minutes at 1000 rpm. The supernatant was aspirated off, and the button was resuspended. Approximately 2 ml fixative was added to tube, and air was bubbled thoroughly and repeatedly through cell suspension. The tube was centrifuged for 6 minutes at 1000 rpm. The supernatant was aspirated off, and the button was resuspended. Another 2 ml of fixative was added to tube, and the tube was centrifuged for 6 minutes at 1000 rpm. The supernatant was aspirated, and the button was resuspended. Fixative (0.3-1.0 ml) was added to tube, and air was bubbled through the cell suspension. Drops of the cell suspension were placed on a cleaned slide and allowed to dry overnight at room temperature. The dried slides were either straight-stained, Q-stained, or RF-stained. Slides were straight-stained in 3 ml of Improved R66 Giemsa Stain (Gurr's) mixed with 45 ml pH=6.8 Gurr's buffer for 10 minutes or until stained darkly enough. The straight-staining solution was freshly mixed. These slides were

photographed on Leitz Dialux microscope using a normal light source and the Planapochromat (63X). DIN was set at 19-20 for about a 2 second exposure. McIlvaine's pH=6.8 buffer solution (Buffer A) was prepared by adding 9.1 ml 0.1 M Citric Acid (Solution A) and 40.9 ml 0.2 M Sodium Phosphate dibasic (Solution B) to 50 ml double distilled water or reverse osmosis water. The solution was mixed on a magnetic stir plate, and pH was adjusted to 6.8 with Solution A. McIlvaine's pH=7.0 buffer solution (Buffer B) was prepared by adding 13 ml Solution A and 87.0 ml Solution B to 1.00 ml distilled water or reverse osmosis water, adjusted pH to 7.0 with Solution A. The specifications for Quinacrine Mustard (QM) were: Quinacrine Mustard dihydrochloride; Sigma Brand, Grade II, approximately 90%, Anhydrous Molecular Weight = 541.8. Stock solution was made by dissolving 100 mg QM Powder in 100 ml double distilled water or reverse osmosis water in a light-protected container. The solution was stirred on a magnetic plate for eight hours. Four ml stock per tube were aliquoted and stored at zero degrees centigrade. The working solution was prepared by adding 1 aliquot QM stock to 75.0 ml McIlvaine's pH=6.8 buffer for a final concentration of 50 g/ml. The slides were rinsed in deionized water and placed in 100 ml pH=6.8 buffer for 2 minutes. They were then placed in 70 ml QM working solution for 30 minutes and quickly rinsed with deionized

water. Approximately 70 ml of pH=6.8 McIlvaine's was poured into a Coplin jar with the rinsed slides and allowed to sit for 2 minutes. The Coplin jar was agitated and the solution poured off. The previous rinsing procedure was repeated twice. The Glycerol/McIlvaine's Mounting Media was prepared by adding in 100 ml Solution A (0.1 M Citric Acid) to 10 ml Solution B (0.2 M Sodium Phosphate dibasic), adjusting the pH to 5.5 with Solution A. An equal volume of glycerol was added to the above solution and stirred on a magnetic stir plate until the glycerol was completely dissolved. Coverslips were mounted on Q-stained slides by placing two drops of mounting media on slide and dropping the coverslip on top. Photography of slides was under Leitz Dialux Q-stained microscope variomat with Pleomopak 2-4 fluorescence illuminator. Reverse fluorescent-bands were accomplished on aged slides (not less than one day old). McIlvaine's pH=6.8 (Buffer A) and pH=7.0 (Buffer B) buffer solutions were prepared as described for Q-staining Chromomycin A3 solution was prepared by adding 30 mg Chromomycin A3 to 60 ml buffer B. It was then placed in a plastic Coplin jar and frozen. Distamycin A was prepared by adding 10 mg distamycin A-HCl (Boehringer, Serva, or Sigma) to 50 ml Buffer A. The solution was put in a plastic Coplin jar and frozen immediately. Slides were placed in thawed chromomycin A3 for 45 minutes and dipped once into pH=6.8 buffer. Drops of the distamycin were

placed on the slides and covered with a coverslip for 8-15 minutes. Coverslips were removed, and the slides were dipped once in ph=6.8 buffer solution. The slides were mounted in Glycerol/McIlvaine's mounting media before drying completely occurs. They were also stored in a black box to await photography. Photography of RF-stained preparations was done under Leitz Dialux microscope variomat with Pleomopak 2-4 fluorescence illuminator.

CHAPTER VI

RESULTS AND DISCUSSION

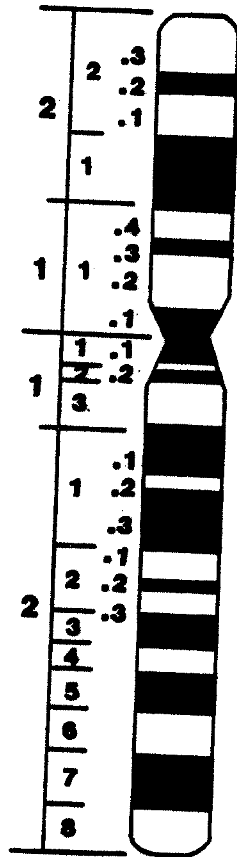
Six blood cultures of positive fragile-X males were examined in this fragile-X survey. Each of the cultures had been straight-stained and scored for percentage fragile-X (Table I). In scoring the cases, 50 cell spreads were examined for fragile sites per case. Approximately eight new slides were prepared according to standard procedures using the stored pellet for each culture. Two of the new slides per case were stained in straight-stain. Exact locations were found and photographed for spreads that exhibited fragile sites. These slides were then destained in 95% ethyl alcohol and restained in either quinacrine mustard stain or reverse fluorescent stain. The previously located spreads were relocated and photographed under ultraviolet microscope. Approximately three more of the slides were stained for Q-bands, and three more were stained for RF-bands. These were then scored for percentage fragile-X present. Examples spreads were photographed. G-banded cell lines do not show fragile sites well due to the last band (q28) being a light band (Fig. 2).

TABLE I

PERCENTAGE FRAGILE SITES PRESENT IN STRAIGHT-STAIN, Q-STAIN, AND RF-STAIN FOR THE SIX CULTURES EXAMINED

	Straight-stain	Q-stain	RF-stain
CI11	18%	4%	12%
CO37	18%	12%	10%
CR37	24%	6%	12%
CS09	22%	2%	2%
CS40	24%	18%	8%
CV47	32%	20%	24%
Mean	23.2%	10.4%	11.4%
S.D.	± 5.3	± 7.6	± 7.3

Fig. 2.--Diagrammatic representation of G-banded X chromosome - The fragile site occurs at distal end of long arm between regions q27 and q28.


2
2
.3
.2
.1
1
1
1
.4
.3
.2
.1
1
1
.1
2
.2
3
1
.1
.2
.3
2
2
.1
.2
.3
3
4
5
6
7
8

In all of the cases examined, a reduction of expressivity resulted in either Q-stained slides or RF-stained slides. However, the reduction was not uniform throughout all of the cases. Logically, one might expect that the greater the percentage fragile-X expressed in straight-stained preparations, the higher the percentage in other staining methods. This was not the case. All of the cases that were examined were at least 18% fragile-X in straight-stain. However, two cases (CI11 and CO37, 18% in straight-stain) showed a greater percentage of fragile sites when quinacrine-stained and reverse fluorescent-stained cells (CI11--4% in Q and 12% in RF; CO37--12% in Q and 10% in RF) than did a case that exhibited 22% fragile-X in straight-stain (CS09--2% in both Q and RF). One cannot predict what the percent expressivity will be in another staining method by what it was in straight-stain. In some instances, the differences were statistically significant, and in some instances they were not. Table II reports the findings of straight-stained, Q-stained, and RF-stained preparations and corresponding chi-square values. There was a significant difference between percentage fragile-X expressed in straight-stain and Q stain in CR37 (X^2 = 5.020, $0.05>p>0.025$) and CS09 (X^2 = 7.670, $0.025>p>0.005$). In RF-stain versus straight-stain, there was a significant difference in percentage fragile-X expression in CS09

TABLE II

CHI-SQUARE VALUES AND PROBABILITIES ASSOCIATED WITH THE DIFFERENCES FOUND IN THE VARIOUS STAINING METHODS

	Straight-stain vs. Q-stain		Straight-stain vs. RF-stain	
	X^2	probability	X^2	probability
CI11	3.677	$0.10>p>0.05$	0.314	$0.75>p>0.50$
CO37	0.314	$0.75>p>0.50$	0.747	$0.50>p>0.25$
CR37	5.020	$0.05>p>0.025$	1.694	$0.25>p>0.10$
CS09	7.670	$0.025>p>0.005$	7.670	$0.025>p>0.005$
CS40	0.241	$0.75>p>0.50$	3.646	$0.10>p>0.05$
CV47	1.299	$0.25>p>0.10$	0.446	$0.75>p>0.50$

(X^2 = 7.670, 0.025>p>0.005). All other cases showed a reduction in percentage fragile-X expression, but that reduction was not significant.

Although each case exhibited a reduction in fragile site expressivity, many positive conclusions may be drawn from this study. Some ambiguity was seen when actually locating fragile sites in Q- and RF-stained preparations. Photographic differences between straight-stained, Q-stained, and RF-stained preparations demonstrate the fragile sites can vary from quite distinct to almost indistinguishable (Figs. 3-5). Especially in Q-stained preparations, the fragile sites, when seen, were quite faded. When slides were straight-stained, and fragile sites were located, the sites were usually easily seen. Destained and restained slides prepared in Q-stain were dim and "fuzzy." This could have been part of the reason that the percentage of fragile site expressivity was reduced in the alternate staining method. Those spreads that had "fuzzy" edges where the fragile sites could have been engulfed in the "fuzziness" would probably have been counted as a negative fragile site spread. Those cells destained and restained in RF were somewhat more easily distinguishable. One difficulty with RF-stain is that it takes some time for the bands to show up well under ultraviolet light. This might cause the total time for preparation of the case to be extended. Therefore, it can

Fig. 3--Photographs of straight-stained fragile-X positive cell lines.

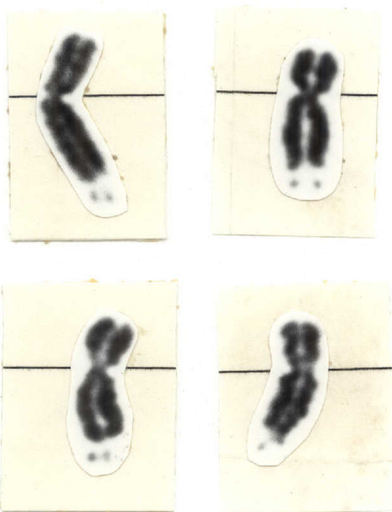


Fig. 4.--Photographs of Q-stained fragile-x positive cell lines.

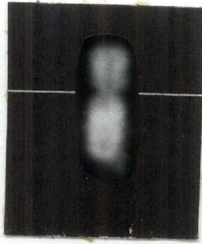
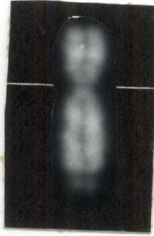
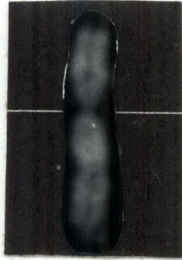
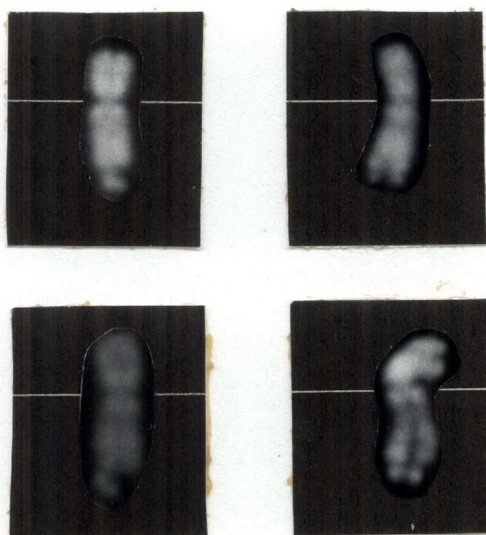




Fig. 5.--Photographs of reverse fluorescent-stained fragile-X positive cell lines.



be seen that Q- and RF-stained spreads cannot be used to indicate percentage fragile-X. However, one of the methods should be used to correctly determine that the fragile sites seen are actually located on the X.

An attempt to identify autosomes that might have had fragile sites was not made. The only chromosomes that were closely examined were those that could be identified as X's in Q- and RF-stain. Since there is evidence that other chromosomes can have fragile sites, the reduction of fragile sites in the cases examined could have been due to the fact that in straight-stain, the sites were not all on X's. If this were the case, the alternate staining methods might be considered better indicators of the true percentage of cells that have fragile-X's. One should remember that in most facilities, any case that exhibits 2% or more fragile site spreads will be considered fragile-X positive.

In conclusion, Fragile-X Syndrome is a disorder in which the symptoms are varied and indistinct. The one symptom other than the obvious, mental retardation, that is seen quite often is macro-orchidism. The actual fragile sites can be observed when an agent is added to culture medium that will participate in the methylation of DNA. Due to the high incidence of non-specific X-linked mental retardation, this syndrome has become highly reported in the literature. In the interest of accuracy, more precise

identification methods are needed to insure that the fragile sites occur on the X in routine screening. This accuracy can be obtained by utilizing the staining methods described in this paper.

REFERENCES

1. Turner, G., R. Till and A. Daniel. 1978. Marker X chromosomes, mental retardation, and macro-orchidism. The New England Journal of Medicine 299:1472.

2. Lubs, H.A. 1969. A marker X chromosome. American Journal of Human Genetics 21:231-244.

3. Steinbach, P., G. Barbi and T. Boller. 1982. On the frequency of telomeric chromosomal changes induced by culture conditions suitable for fragile-X expression. Human Genetics 61:160-162.

4. Sutherland, G.R. 1977. Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science 197:265-266.

5. Hecht, F., P.B. Jacky and G.R. Sutherland. 1982. The fragile-X chromosome: current methods. American Journal of Medical Genetics 11:489-495.

6. Jacobs, P.A., M. Mayer, J. Matsuura, F. Rhoads and S.C. Yee. 1983. A cytogenetic study of a population of mentally retarded males with special reference to the marker(X) syndrome. Human Genetics 63:139-148.

7. Eberle, G., M. Zankl and H. Zankl. 1982. The expression of fragile-X chromosomes in members of the same family at different times of examination. Human Genetics 61:254-255.

8. Buhler, E.M., F. Hadziselimovic and U. Pira. 1982. A variant of the fra(X) syndrome. Human Genetics 61:273-275.

9. Brown, W.T., E.C. Jenkins, E. Friedman, J. Brooks, K. Wisniewski, S. Raguthu and J. French. 1982. Autism is associated with the fragile-X syndrome. Journal of Autism and Development Disorders 12:303-308.

10. Van Roy, B.C., M.C. DeSmedt, R.A. Raes, J.E. Dumon, and J.G. Leroy. 1983. Fragile-X trait in a large kindred: transmission through normal males. Journal of Medical Genetics 20:286-289.

11. Fryns, J.P. and H. Van Den Berghe. 1982. Transmission of fragile(X)(q27) from normal males(s). Human Genetics 61:262-263.

12. Daker, M.G., P. Chidiac, C.N. Fear and A.C. Berry. 1981. Fragile-X in a normal male: A cautionary tale. The Lancet 1:780.

13. Turner, G., R. Brookwell, A. Daniel, M. Selikowitz and M. Zilibowitz. 1980. Heterozygous expression of X-linked mental retardation and X-chromosome marker fra(X)(q27). The New England Journal of Medicine 303:662-664.

14. Tommerup, N., H. Pousel and K. Brondum-Nielsen. 1981. 5-fluoro-2-deoxyuridine induction of the fragiles site Xq28 associated with X-linked mental retardation. Journal of Medical Genetics 18:374-376.

15. Gardiner, G.B., S.L. Wenger and M.W. Steele. 1984. In vitro reversal of fragile-X expression by exogenous thymidine. Clinical Genetics 25:135-139.

16. Eberle, G., M. Zankl and H. Zankl. 1981. Sources of error in fragile-X determination. The Lancet 1:557.

17. Eberle, G., G. Zankl and H. Zankl. 1982. Co-cultivation studies with cells of patients bearing the fragile-X chromosomes. Human Genetics 61:163-164.

18. Soudek, D. and M. Emanuel. 1983. A fragile-X suppresser in the normal human blood? Human Genetics 65:88-89.

19. Vekemans, M., B. Popovich, D. Rosenblatt and P. Monroe. 1983. Chromosomal breakage in normal and fragile-X subjects using low folate culture conditions. Journal of Medical Genetics 20:404-407.

20. Mixon, J.C. and V.G. Dev. 1983. Fragile-X expression is decreased by 5-azacytidine and S-adenosylhomocysteine. American Journal of Human Genetics 35:1270-1275.

21. All materials and methods were taken directly from those formulated by the Genetic Screening and Counseling Service Cytogenetic Laboratory, Denton, Texas.