

ASSIGNMENT BY R-BANDING OF X-INACTIVATION STATUS
FOR A PATIENT WITH A SEX CHROMOSOME ABNORMALITY

Submitted in partial Fulfillment of the Requirements for
Graduation with Honors to the Department of Biology and Chemistry at
Carroll College, Helena, Montana

Echo Velina Sanders
April 11, 1997

CORETTE LIBRARY CARROLL COLLEGE



3 5962 00108 387

This thesis for honors recognition has been approved for the Department of

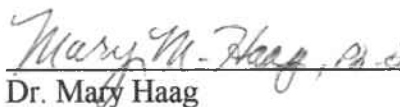
Biology and Chemistry by:



Dr. Marilyn Schendel, Advisor



Dr. Grant Hokit



Dr. Mary Haag

ACKNOWLEDGEMENTS

The design and execution of this project was a collaborative effort of several people to whom I am most grateful. I would especially like to thank Dr. Mary Haag for her dedication to student learning and commitment to her work. In addition, I would like to thank Linda Beischel and Sandy Phillips for their support and insights. Finally, I would like to extend my gratitude to Dr. Schendel for her time, energy, and encouragement from beginning to end.

ABSTRACT

An R-banding protocol was developed in order to study the X-inactivation pattern of a patient with a duplicated X chromosome. R-banding was used because it results in the differential banding of the active and inactive X chromosome due to differing replication patterns with the active X being early replicating and the inactive X being late replicating. The results demonstrate that the patient's normal X chromosome was early replicating as well as the duplicated region of the abnormal X chromosome. This suggests that the observed phenotype is a result of incomplete inactivation of the duplicated X chromosome.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	i
ABSTRACT	ii
LIST OF ILLUSTRATIONS	iv
INTRODUCTION AND LITERATURE REVIEW	1
MATERIALS AND METHODS	15
RESULTS	20
DISCUSSION	23
BIBLIOGRAPHY	26

LIST OF ILLUSTRATIONS

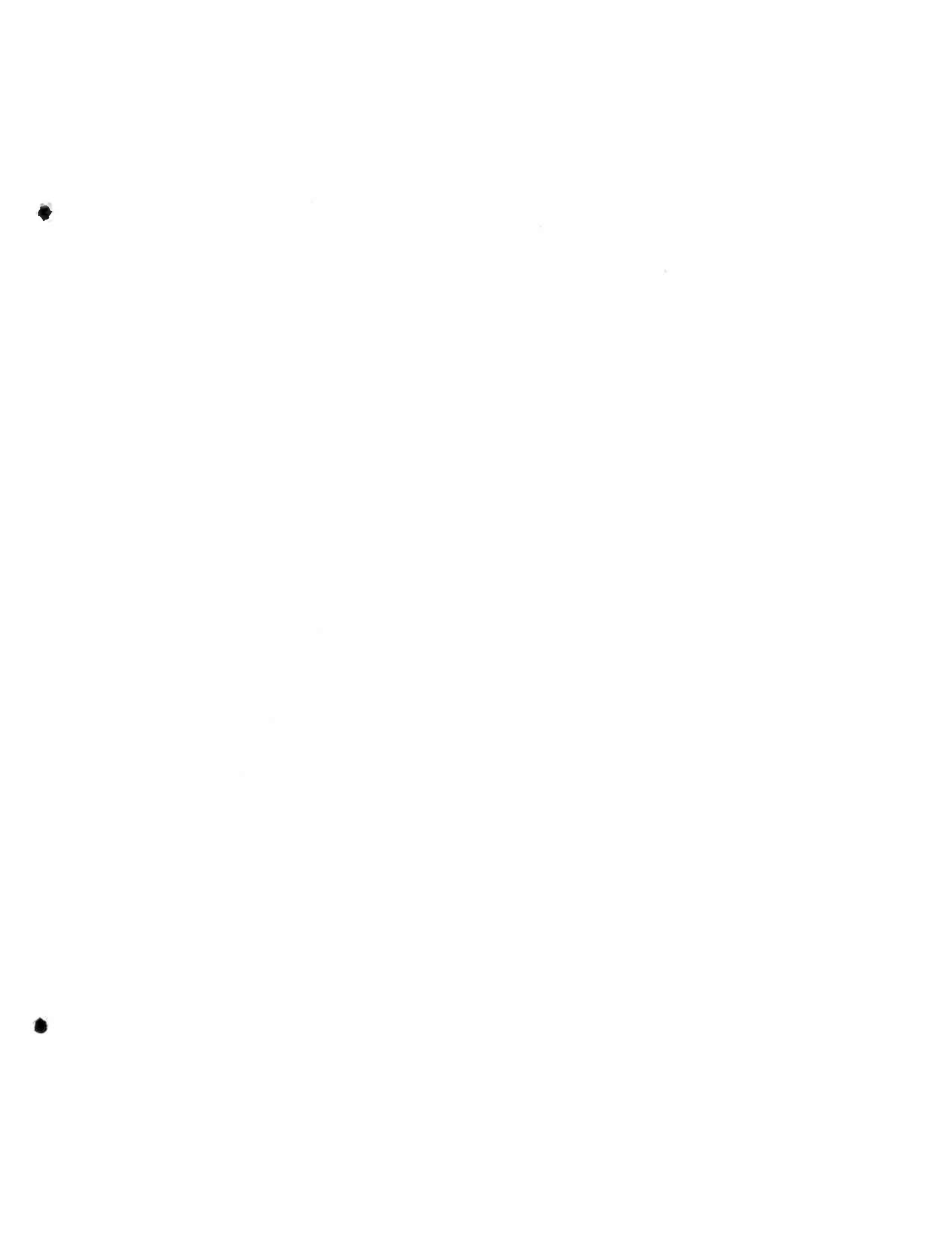
Figure 1	X-inactivation status of several genes	2
Figure 2	X:autosome translocation can result in balanced and unbalanced cells	7
Figure 3	Ideogram of the X chromosome	8
Figure 4	Pedigree of a family showing carriers of a duplicated X and affected individuals	10
Figure 5	Androgen receptor PCR shows X-inactivation skewing	13
Figure 6	R-banding results show the normal X and duplicated region of the abnormal X to be early replicating	22
Figure 7	FISH probes and DNA markers used to identify the duplicated region	24
Table 1	Summary of differences between initial and final protocols	21

INTRODUCTION

Humans normally have 46 chromosomes consisting of 22 pairs of autosomes and two sex chromosomes. The X chromosome is a sex chromosome which is present in both males and females. Females generally have two X chromosomes present in each cell (XX) and in males there is usually only one X chromosome and one Y chromosome (XY). Having two X chromosomes does not necessarily make a person female nor does having only one X chromosome necessarily make a person male (Mange and Mange 1990). In humans the presence or absence of the Y chromosome is more important to male development than is the number of X chromosomes (Mange and Mange 1990).

This difference in the number of X chromosomes between females and males results in an inequality of gene number. To equalize the phenotypes in males and females and rectify the inequality of genes, various dosage compensation mechanisms have evolved in different species. For example, in *Drosophila* dosage compensation mechanism is achieved by hypertranscription of the single male X chromosome (Kelley and Kuroda 1995). In mice, like all mammals, dosage compensation is achieved by inactivating one of the X chromosomes in females (Migeon 1994; Belmont 1996). This inactivation of the X chromosome is complete (Ashworth 1991).

In humans, however, some genes escape inactivation, making the X inactivation mechanism distinct from that of other mammals. Both X chromosomes function to some extent during early cleavage divisions of the zygote, but beginning in the late blastocyst stage, one X chromosome differentiates so that it no longer responds to signals that regulate the transcription of genes on its homologue (Migeon 1994). This results in expression from only one allele at the vast majority of X loci (Belmont 1996). However, some loci on the



inactivated human X chromosome are actually transcribed such as the blood group gene (XG), the steroid sulfatase (STS) gene and the X-inactive specific transcript (XIST) gene (Mänge and Mange 1990). The location of these genes and several others can be seen in Figure 1 (Davies 1994).

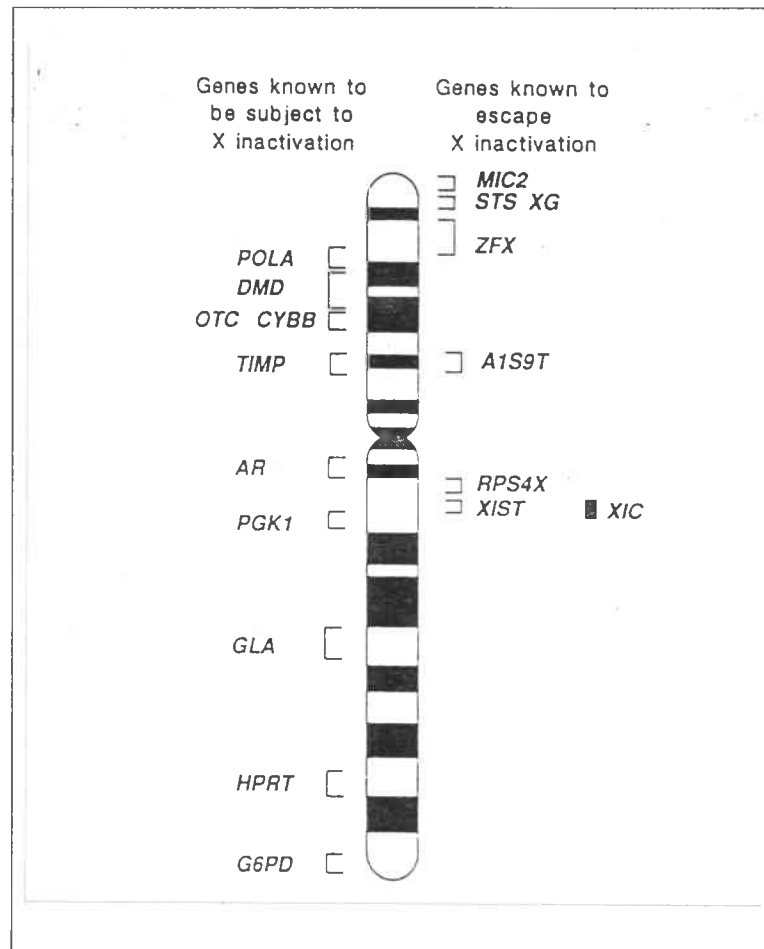


Figure 1. The X-inactivation status of several genes shows that some genes are known to be subject to X-inactivation while others escape inactivation.

X-inactivation involves multiple mechanisms including the initiation, spreading and maintenance of the inactivation. Although inhibition is maintained locus by locus, the initial events probably affect the chromosome as a whole since they lead to general inactivation of the one entire X chromosome rather than smaller portions of each X chromosome (Migeon 1994). It is likely that spreading of X inactivation results from changes in chromatin structure induced

by condensation of the X chromosome and DNA methylation (Migeon 1994). Furthermore, DNA methylation serves to lock in the silence of the locus once it has been inactivated and is probably the major mechanism for transmitting the inactive state through subsequent mitotic divisions (Migeon 1994).

X-inactivation also involves a counting mechanism of the number of Xs and the X:autosome ratio. The counting mechanism counts active rather than inactive X chromosomes, so as a result only one X may remain active (Migeon 1994; Belmont 1996).

The basic mechanism underlying X inactivation is inhibition of transcription. This inhibition is the result of methylation of certain nucleotides in the DNA (Allen et al. 1992). Methylation occurs on deoxycytosines located in CpG dinucleotides, the bulk of which occur in CpG-rich regions called CpG islands (Norris et al 1994). CpG islands are characterized by 60% GC content. There is experimental evidence that methylation of CpG islands result in total repression of the associated gene (Bird et al. 1992). As one would expect, the deoxycytosines in CpG islands are methylated in the inactive X chromosome but not in the active X chromosome (Jeppesen and Turner 1993).

The X inactivation center (XIC) is a cis-acting locus on the X chromosome which serves as the master regulatory switch controlling this process (Penny et al. 1996). XIC has been implicated in both the decision as to how many X chromosomes are inactivated (counting) and also which X chromosome is inactivated (choice) (Penny et al. 1996). Researchers have now provided evidence that XIC corresponds to the XIST gene which is transcribed solely from the inactive X chromosome (Penny et al. 1996; Belmont 1996). XIST does not code for a protein (Brown et al. 1992) but encodes a 15-17 kb ribosomal RNA that is localized predominantly within the nucleus (Brockdorff et al. 1992). Norris et al (1994)

demonstrated that the expressed *XIST* allele on the inactive X chromosome is unmethylated and that the *XIST* locus on the active X chromosome is completely methylated (Norris et al. 1994). This demonstrates the importance of DNA methylation in the inactivation of different loci on the X-chromosome.

The precise mechanisms of X inactivation are not yet known; however, the importance of X inactivation can be shown in cases involving aneuploidy. Aneuploidy refers to an unbalanced set of chromosomes in an organism due to an excess or deficiency of individual chromosomes (Mange and Mange 1990). In cases of aneuploidy of the X, such as XXX or XXY, all but one X chromosome will be inactivated (Belmont 1996), but it is unclear exactly how the cell senses how many X chromosomes are present. The number of sets of autosomes is somehow counted because in triploid individuals with a 69, XXY karyotype neither X chromosome is inactivated (Migeon 1994).

One of most noted examples of aneuploidy is Turner syndrome which is a term used to refer to a female with a 45 chromosomes with one X instead of two. The presence of only one X chromosome is highly lethal in embryos with at least 98% of all 45, X zygotes being lost in the first three months of pregnancy (Epstein 1986). The surviving Turner females are characterized by short stature, webbed necks, widely spaced nipples, and sterility (Mange and Mange 1990). This brings up an important question of why 45, X individuals would have any associated abnormal phenotype when X-inactivation occurs in normal 46, XX individuals leaving only one X active in each cell. This suggests that the inactivation of the X chromosome is not complete.

On the other hand, females with a 47, XXX karyotype have no distinctive phenotype and many appear to be completely normal (Mange and Mange 1990). This clearly

demonstrates the importance of a counting mechanism during X inactivation and the need for two copies of certain genes. Females with more than three X chromosomes are extremely rare, but at least 30 cases of 48, XXXX are known (Nielsen et al. 1977). Although these females are normal in sexual development, they are severely retarded and exhibit a wide range of physical abnormalities (Mange and Mange 1990).

In addition, males with Klinefelter's syndrome (47, XXY) inactivate one of the X chromosomes, further supporting the idea that a counting mechanism exists in the cell. Some Klinefelter males have no symptoms except infertility due to abnormal structure of the seminiferous tubules in the testes and androgen (male sex hormone) deficiency (Mange and Mange 1990). Other symptoms can include very small testes, poorly developed male secondary sexual characteristics and the presence of some feminine traits such as breast development (Mange and Mange 1990). The presence of the extra X clearly has some effect, probably due to the incomplete inactivation of the second X chromosome.

The importance of X inactivation can also be shown in cases in which nonrandom X inactivation occurs. Generally, X-chromosome inactivation occurs randomly with respect to parental origin of the chromosome resulting in cellular mosaicism, two cell lines with a genetic difference, in females (Migeon 1994). There are some exceptions to this general rule. For example, there is evidence that in the extraembryonic tissues, such as the placenta, the paternal X is selectively inactivated (Belmont 1996). This process is referred to as genomic imprinting in which a chromosome "remembers" its parental origin (Migeon 1994). X inactivation skewing, a term for nonrandom X inactivation, may result from a bias in the original decision as to which X to inactivate or from subsequent selective pressures which would cause cells with a certain inactive X to die off (Rupert et. al. 1995).

Structural abnormalities of the X chromosome may provide the selective pressure to skew X inactivation and result in abnormal phenotypes. Structural abnormalities can include X:autosome translocations, a deleted X, a duplicated X, or a ringed X chromosome. The subsequent X inactivation patterns can vary widely and often must be explained on a case by case basis because rarely are any two cases exactly the same unless inherited from one generation to the next.

Balanced X:autosome translocations occur when a portion of the X chromosome is attached to any other autosomal chromosome and a portion of that autosome is attached to the remaining portion of the X. Two copies of each chromosome are present but the information has been translocated from one chromosome to another. Balanced X:autosome translocations have been associated with skewing of X inactivation (Zabel et al. 1978). In general the preference is for inactivation of the normal X chromosome because spreading of inactivation into the autosomal region would give rise to a monosomy (only one copy) for that region of the autosome (Belmont 1996). This promotes selective pressure for the cells in which inactivation of the normal X has occurred to survive and for the other cells to die off.

Hypomelanosis of Ito (HI) is a sporadic multisystem disorder associated in several cases with X:autosome translocations (Hatchwell 1996). X inactivation analysis of peripheral blood in affected patients showed random X inactivation rather than the expected skewing. This means that in some cells the normal X was active and so there would be two active copies for a portion of the X instead of just one. In other cells the normal X was inactive and so there is a normal balance of X genes (see figure 2). The mosaic disomy, a term used to indicate that in some cells there are two active copies while in others only one, of X specific sequences is the most likely cause for HI (Hatchwell 1996; Hatchwell et al. 1996), and demonstrates how the X



inactivation pattern can contribute to certain phenotypes. In addition, a female with a balanced X:17 translocation and HI showed random X inactivation in affected areas of the skin while X inactivation in normal skin and blood was skewed as expected with the normal X being inactivated (Hodgson 1985).

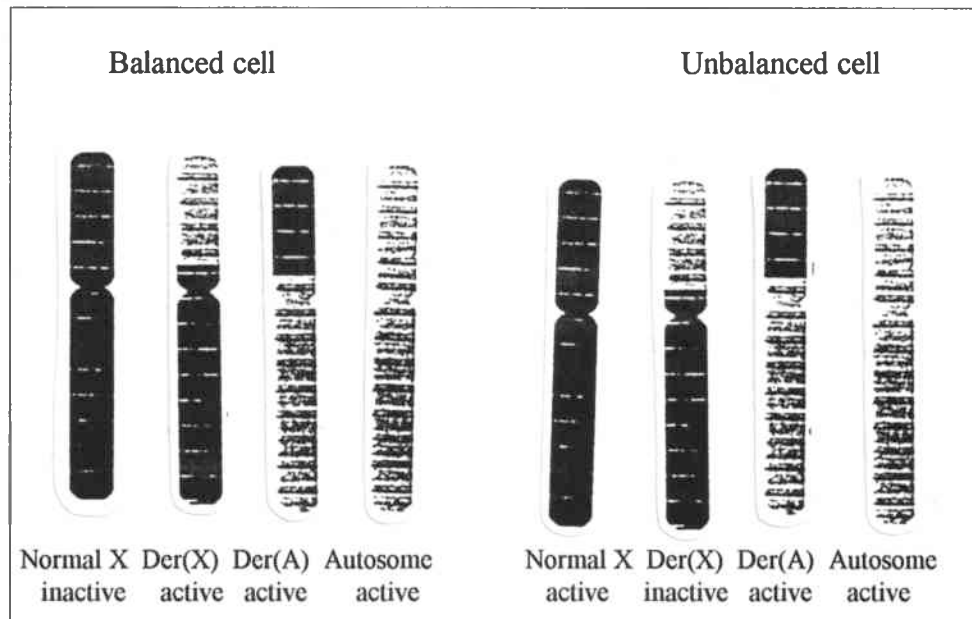


Figure 2. X:autosome translocations can result in a balanced or unbalanced cell. In the balanced cell all portions of the autosome are active and only one copy of genes on the X chromosome are active. In the unbalanced cell, a disomy exists for a portion of the X and a monosomy exists for a portion of the autosome which has become inactivated.

The next type of structural abnormality is a deletion in which a portion of the chromosome is absent. As a general rule, deleted X chromosomes are preferentially inactivated since cells in which the normal X is inactive would be preferentially lost since they would be missing vital genes (Goldman et al. 1982). However, Schmidt et al. (1989) report a case in which the deleted X chromosome is preferentially active, suggesting that the missing region plays a role in X inactivation or that rejoining of breakpoints in this particular region interferes with the inactivation process (Schmidt et al. 1989). The deleted region occurs between the region 27.1 to 27.3 on the q (long) arm of the X chromosome (see figure 3). The replication



chromosomes with only one X and the other cell line having 46 chromosomes with one normal X and one ring X (Kusnick et al. 1987; Van Dyke et al. 1992; Dennis et al. 1993). In contrast, females with a large ring X containing the XIST locus usually resemble individuals with Turner syndrome because the abnormal X chromosome is inactive in all cells and the normal X is the functional one (Migeon et al. 1994).

Duplications represent a fourth category of structural abnormalities. Duplications occur when a portion of a chromosome is copied twice instead of once and so that the region is duplicated. In males, duplication of a portion of the long arm of the X results in phenotypic abnormalities; whereas, most females having a duplication of the q-arm are phenotypically normal (Aughton et al. 1992). This can be attributed to the selective inactivation of the duplicated X chromosome in females as shown by replication studies which identify which X chromosome is early replicating (active) and which one is late replicating (inactive). However, at least six cases have been reported in which females with a duplicated q-arm of the X show phenotypic abnormalities including developmental delay, congenital anomalies, short stature and ovarian dysfunction, and in all but one case the duplicated X was found to be late replicating (inactive) (Varella-Garcia et al. 1981; Van Dyke et al. 1983; Morichon-Delvallez et al. 1988; Aughton et al. 1992; Vasquez et al. 1995).

One case study reports a three generation pedigree involving a rearranged X in which a region of the q-arm was duplicated and then attached to the p-arm (Vasquez et al. 1995). Two boys and one girl show significant clinical abnormalities while the boys' mothers and the girl's mother (see figure 4) are phenotypically normal despite having the same rearranged X (Vasquez et al. 1995). The boys' phenotype can be explained by a disomy for the duplicated region of the X; whereas, the girl's phenotype is due to a different inactivation pattern than that

of the other females in the family. The rearranged chromosome was late replicating in 97-100% of the metaphases in the unaffected sisters and mother, but the rearranged chromosome was early replicating in 43% of the lymphocytes in the affected female (Vasquez et al. 1995).

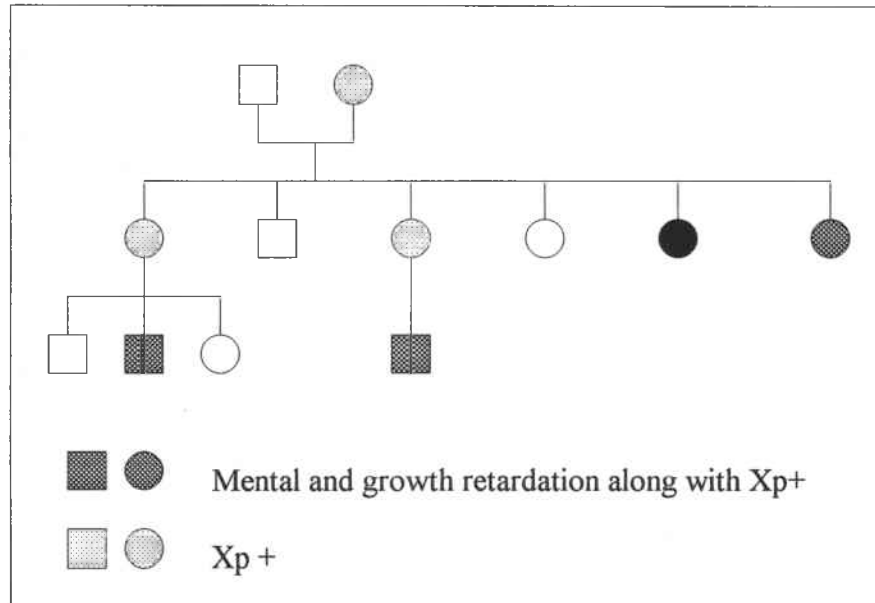


Figure 4. Pedigree of a family showing carriers of a duplicated X chromosome and affected individuals.

Aughton et al. (1993) report a female with a duplicated X from the region between band q13 to the end of the q-arm (qter) which is a very large duplication resulting in growth retardation, microcephaly, developmental delay, seizures, and minor anomalies. Replication studies found that in fifty of fifty cells (100%) the duplicated X was late-replicating (Aughton et al. 1993). The mechanism by which the abnormal phenotype of this patient arose is not clear, but several possible suggestions have been given. The role of imprinting in certain tissues may lead to phenotypic consequences if one X, whether duplicated or not, is preferentially inactivated in all cells. The expression of recessive genes from the active normal X chromosome may have some deleterious effect (Aughton et al. 1993). It is also possible that an increased dosage of genes within the duplicated region may contribute to the phenotype

since late replication of an X chromosome does not necessarily imply complete inactivation (Aughton et al. 1993). However, any such active regions should be early replicating and show up as darker bands in R-banding replication studies.

In another case a woman with a duplication of the region between bands q21 and q27 showed a skewed inactivation pattern in which the duplicated X chromosome is late replicating in 100% of cells (Varella-Garcia et al. 1981). This same pattern of inactivating the duplicated X chromosome held true for a patient with a duplication of bands q13 to q22 (Steinbach et al. 1980), one with duplication of the entire region from band q13.3 to q27 (Van Dyke et al. 1983), and a three generation transmission of a duplicated X chromosome from band q23 to q27 with only one phenotypically abnormal female (Morichon-Delvallez et al. 1988).

Explanations for the phenotypic manifestations of these cases involving duplications were reviewed by Van Dyke et al. (1983) and include the following possibilities: 1) random inactivation of the paternally and maternally derived X chromosomes may be essential in some tissues (role of imprinting); 2) two structurally normal X chromosomes may be essential for normal gonadal function; 3) equal doses of genes on Xp and Xq are essential; 4) the normal X might be inactivated in a few cells, producing a functional excess of genes in the duplicated region; and/or 5) a position effect created by breakpoints or new configurations of genetic material could alter interrelationships among genes (Van Dyke et al. 1983). Morichon-Delvallez et al. (1988) also bring up the possibility that the phenotype may be a coincidence such as in the case of the three generation transmission of the duplicated X. This is quite reasonable because the mother who has the same X duplication and pattern of inactivation is phenotypically normal.

A patient with a duplicated X chromosome was studied by the Medical Genetics department of Shodair Children's Hospital. She was described as having mild to moderate mental retardation, short stature, minimal facial anomalies, and excessive fat distribution on the extremities as compared with the trunk. The presence of extra material on the long arm of the X chromosome was first noted in 1976. A new karyotype completed at Shodair Hospital could not further identify the extra material, but several molecular and cytogenetic techniques were used to further characterize the extra material and to account for the patient's phenotype.

Fluorescence in situ hybridization (FISH) is a cytogenetic technique used to visualize the binding of DNA probes to metaphases chromosomes. The probes are sequences of DNA with a fluorescent label or antibody attached to them and can be specific for a single sequence on a chromosome or they can bind to a region such as the centromere which is common to more than one chromosome. The probe will bind to its complementary region on the chromosome(s) and then the metaphases can be analyzed under a fluorescence microscope to determine the number of fluorescent signals per metaphase. There would normally be two signals (two per chromosome pair) for a normal metaphase, one signal if there was a deletion of the region complementary to the probe, and three signals for a duplication of the region.

In the study of the Shodair patient, an X paint, which is a mixture of many specific probes for the X chromosome was used to determine if the extra material was derived from the X chromosome. The FISH results showed the entire length of both Xs hybridized with the FISH X paint probe suggesting that the extra material on the X chromosome was due to a duplication. Later several specific probes were used to try to identify which region of the X chromosome was duplicated.



Because skewed inactivation patterns are common in similar cases, an X inactivation assay was used. This molecular technique measures the amplification of a gene with two alleles from the X chromosome. In this case, an analysis of alleles for the androgen receptor gene was used. Methylation sensitive restriction enzymes are used to determine if the same allele, and thus the same chromosome, are always active (greater amplification of that allele) or if inactivation is random (equal amplification of the alleles). The result of the X inactivation assay showed almost complete inactivation of the maternal X chromosome (see figure 5). This left the question of which X, the normal or duplicated, was inherited from the mother and was thus being almost exclusively inactivated.

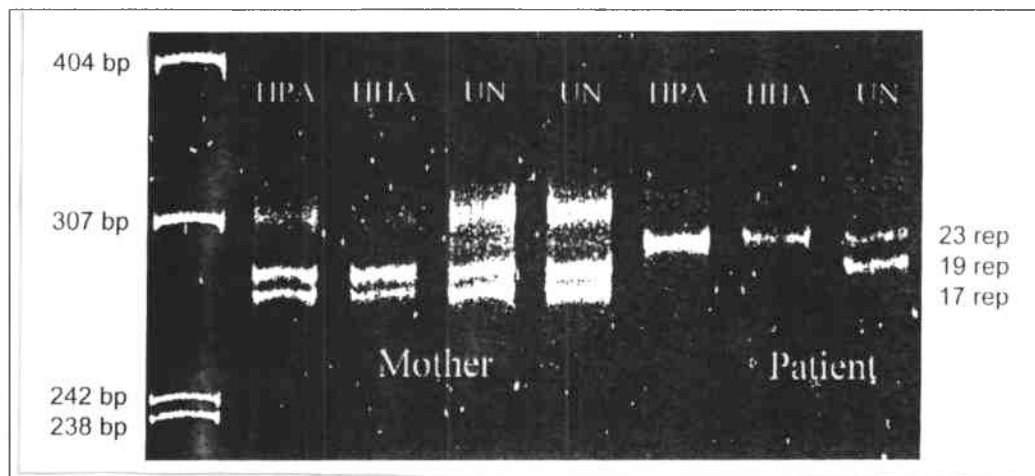


Figure 5. Androgen receptor PCR shows X-inactivation skewing. The paternally-derived X allele of 23 repeats is selectively inactivated as shown by the methylation sensitive restriction enzymes HPA and HHA.

One technique that held promise for answering this question was R-banding, a type of replication banding, which distinguishes between early and late replication within a single cell cycle. The early replicating areas correspond to chromosome bands containing actively coding gene sequences while the later replicating areas correspond to the largely highly repetitive or non-coding gene sequences (Denver protocol). The scope of my project was to first develop

an R-banding protocol that would give consistent, quality results. Then I applied this procedure to ascertain whether the duplicated X was early or late replicating which may help explain the patient's phenotype.

MATERIALS AND METHODS

Final Protocol

The peripheral blood was collected in a volume of 7-10 ml in a sodium heparinized vacutainer at a physician's office before it was sent to the lab. Once the sample had been received, it was allowed to sit at room temperature until the blood began to settle out, leaving a buffy coat layer between the particulate matter and the serum. This buffy coat contains the majority of nucleated white blood cells (Rooney and Czepulkowski 1992). Sometimes the blood was not allowed to settle out and instead well mixed whole blood was used instead of the buffy coat.

A sterilized hood and universal precautions were used in the handling of all blood products. First a 0.7 ml aliquot of buffy coat was taken for each culture tube using an electric pipette. The total volume needed for all culture tubes was drawn up and mixed inside a 5ml glass pipette tube before dispensing approximately 0.7 ml into each culture tube. Each culture tube contained 10 ml of growth medium which must be made fresh no more than a month before.

The lymphocyte growth medium was made by starting with a 100 ml bottle of RPMI-1640 medium purchased from Gibco Life Sciences Technologies, Inc. Then 10 ml of fetal calf serum was added plus 2 ml of phytohaemagglutinin (PHA) plus 2ml of reconstituted Antibiotic/Antimicotic plus 2 ml of L-glutamine. The PHA is a lymphocyte mitogen and stimulates cells to divide and grow while the antibiotics and antimicrobics protect against foreign particles. Next 0.5 ml of 5.0 ug/ml stock fluorodeoxyuridine (FudR) purchased from Sigma was added. The FudR promotes the uptake of bromodeoxyuridine (BrdU) which is a thymidine

analog (Rooney and Czepulkowski 1992). The bottle of medium was then mixed thoroughly and approximately 10 ml was dispensed into each culture tube.

Six cultures were set up from two sodium heparin vacutainers. The lymphocyte cultures were incubated at 37°C for 72 hours and were mixed well once each day by inverting the tubes.

Lymphocyte cultures were labeled with BrdU purchased from Sigma to produce the desired R(reverse)-Bands. BrdU is a thymidine analogue which is readily incorporated into chromosomes during the pulse period in which it is added (Rooney and Czepulkowski). A late pulse of BrdU during the last five to seven hours of culture will result in BrdU labeling of late replicating DNA which subsequently gives a Reverse or R-banding pattern on the chromosome (Denver protocol). R-bands are the reverse of Giemsa-banding (G-banding) which is more commonly used for chromosome staining and analysis. In this procedure 0.1 mls of 8 mg/ml stock (final concentration of 200 ug/ml) was added for the final six to seven hours of cell culture before harvesting. The BrdU also acts as a gate to hold cells at the interphase between G1 and S phases of the cell cycle. This allows many cells to reach the same stage before being released to synchronously complete cell division.

To harvest cells, 0.1 ml of 10 ug/ml colcemid was added for 15 minutes and the cultures were returned to the 37°C incubator. Colcemid is a drug that promotes microtubule breakdown (Kleinsmith and Kish, 1995) which causes the mitotic spindle to disappear; therefore, halting the nucleus in metaphase. Chromosomes halted in metaphase are condensed and can be visualized by a light microscope after proper preparation and staining.

After the colcemid incubation, the culture tubes were centrifuged at 800 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in approximately 13

mls of 0.057M KCl that was prewarmed to 37°C in a water bath. The culture tube was then left at room temperature for 15 minutes. During this time the pellet was broken up gently using a pipette. Care must be taken not to draw up any liquid into the pellet because cells may remain inside the pipette. The 0.057M KCl is hypotonic in comparison to red blood cells and so the red blood cells swell and burst, making them easier to remove.

Following the 15 minute incubation, a series of fixation steps were used to preserve the nucleated white blood cells in metaphase. A 3:1 absolute methanol:glacial acetic acid fixative was prepared for this purpose. In the first fixation step, 2 mls of fix was added to the hypotonic solution. The culture tube was recapped and inverted gently several times. Then the tubes were centrifuged for 10 minutes at 800 x g. The supernatant was aspirated off and 13 mls of fix was gradually added to resuspend the pellet. The tube was again centrifuged at 800 x g for 10 minutes. The supernatant was aspirated off and 10 mls of fixative were added. The pellet was vigorously resuspended before centrifuging for 10 minutes at 800 x g. The supernatant was aspirated off and a final 5 ml fix was added to the culture tube. The pellet was resuspended and centrifuged for 10 minutes at 800 x g.

After the final fixation, slides were prepared by removing the supernatant and resuspending in a few milliliters of fixative. The actual volume of fix depends on the size of the pellet and the mitotic index of the culture. The first slide serves as a gauge to measure the concentration of cells across the slide and more fix may be added to dilute the cells or the tube may have to be recentrifuged if the cells are spread too thinly.

Once the correct concentration of cells to fix was found, approximately 0.5 mls was drawn up into a bulb operated glass pipette. A slide rinsed and placed in distilled water was quickly drained on a paper towel to leave a thin film of water across the surface before several

drops of cell culture were dropped onto the slide. The water helps the cells spread evenly across the slide, and cells are dropped from about arms length above the slide, so the nucleus will burst and allow the chromosomes to spread apart to some extent.

Slides were then allowed to air dry or dried on a hot plate before being scanned under a phase contrast microscope. Then all of the usable slides were either baked overnight in a 60°C oven or aged for at least a week before staining.

Slides were stained by first covering them with a 1 mg/ml solution of Hoechst 33258 (stock purchased from Sigma, Inc.) for 10 minutes. Then each slide was covered with saline sodium citrate (2xSSC) in a petri dish and exposed to short wave ultraviolet light that was approximately three inches away for 90 minutes. Finally the slides were counterstained with a 5% Giemsa solution for 90 seconds.

The banding pattern is the result of staining fixed metaphases with Hoechst 33258 (bisbenzimidazole) which is quenched in those areas where BrdU was incorporated (Denver protocol). After UV degradation of the Hoechst-BrdU areas, Giemsa staining will reveal the same pattern as the Hoechst fluorescence with light Giemsa bands corresponding to the quenched BrdU-substituted areas and dark Giemsa bands relating to non-BrdU substituted areas (Denver protocol). The light areas are late-replicating and the dark bands are early replicating and as a result the late replicating, inactive X will appear washed out compared to the early replicating, active X chromosome.

When the R-banding procedure produced consistent, quality results it was tested on two other blood samples from a male and female to assure that the results were reproducible on other blood samples. Finally the R-banding technique was ready to be used on a blood sample from the patient of interest.

Once the slides were made and stained the microscopic analysis was done. In the analysis of the patient's chromosomes the following factors were recorded: (1) the total number of metaphases observed under high power, (2) the number of metaphases with discernible banding, (3) the number of cells in which both X chromosomes could be identified, and (4) the number of cells in which the abnormal X was active.

Initial Protocol

An initial protocol from Human Cytogenetics Volume I was used. Cultures were set up in the same manner as described in the final protocol except that the growth medium contained 20% fetal calf serum and lacked FudR. BrdU was added six hours before harvest for a final concentration of 1×10^{-4} . In addition deoxycytidine hydrochloride was added for a final concentration of 1×10^{-4} in an attempt to decrease the toxicity of BrdU. Colcemid was added one hour before harvest instead of 15 minutes, but the amount was the same at 0.1 ml of 10 ug/ml. The harvest technique was exactly the same, as was slide making. All slides were baked overnight in a 60°C oven.

The staining procedure had some significant differences. Slides were first soaked in phosphate buffered saline (PBS) for five minutes and then stained in 0.5 ug/ml Hoechst 33258 for ten minutes. Then slides were rinsed briefly in PBS and distilled water. Slides were mounted with MacIlvaines buffer of pH 7.5. Slides were irradiated for 15 minutes on a hot plate at 50°C. Next the coverslips were removed so slides could be rinsed in distilled water. Then they were incubated for 15 minutes in 2xSSC at 65°C before being stained in 5% Giemsa.

RESULTS

A major component of my summer research was the development of a workable R-banding protocol. The initial protocol was adapted from Human Cytogenetics Volume I, while the final protocol primarily followed a protocol developed by Art Daniel, a former director of the Shodair Cytogenetics Lab. Several blood samples from myself were used to experiment with variables such as the concentration of Hoechst, length of UV irradiation, concentration, and time in Giemsa counterstain.

The results of the initial protocol (as described in Materials and Methods) were very poor. No metaphases examined had a discernible banding pattern, but metaphases were present which indicated that the culture and harvest technique had worked as expected. Several variations of the staining time and procedure were repeated, but with little or no improvement in the quality of metaphases.

After reviewing several more procedures, I found that many of them called for a higher concentration of BrdU and a longer irradiation time. A protocol written by Art Daniels, a former director of the Cytogenetics Lab at Shodair, served as the main source of information and was experimented with in order to obtain consistent, high quality results.

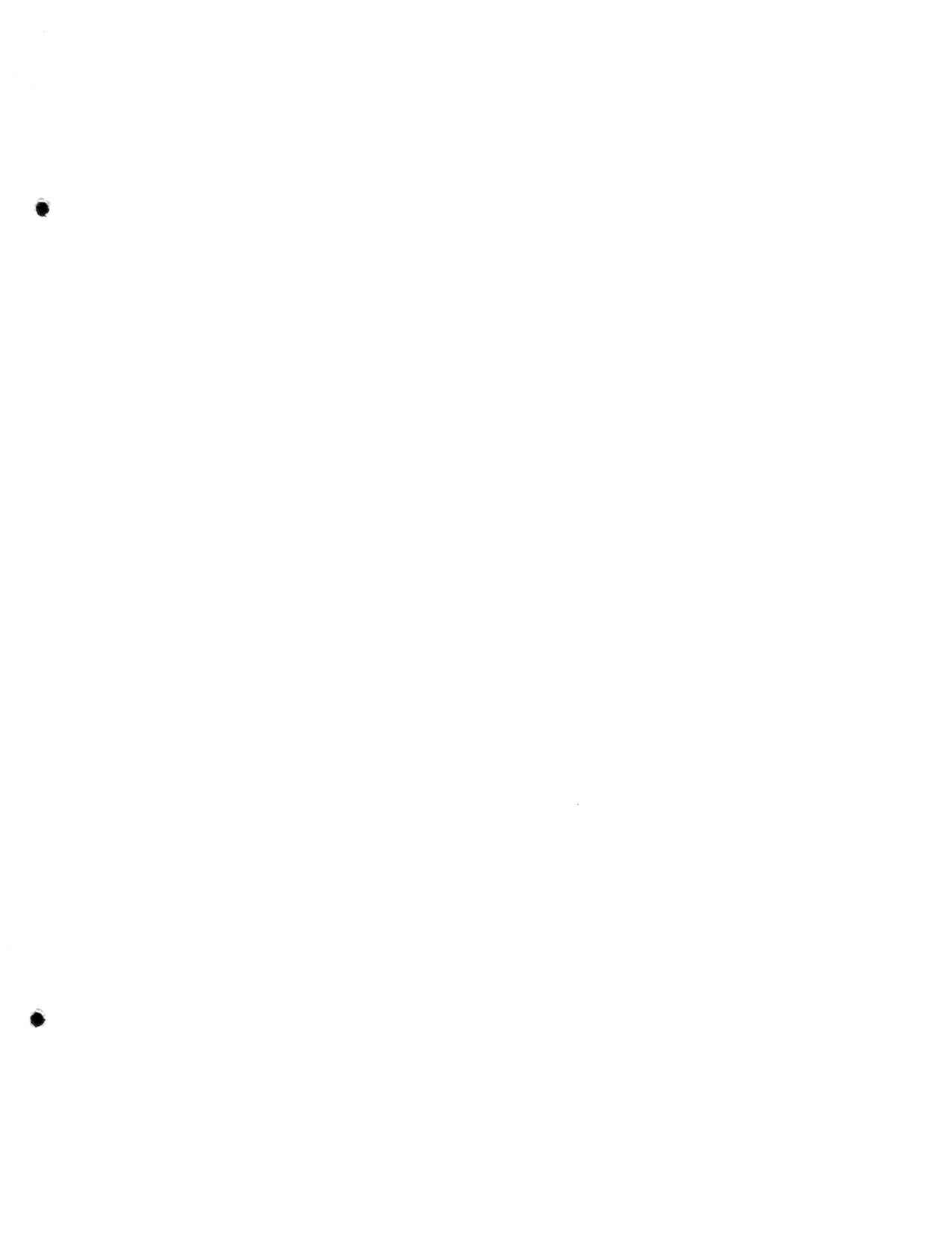
The initial and final protocols differed in several ways. Variables in the growth medium, the concentration of BrdU, irradiation time, slide treatment, and several other changes were made. A summary of the differences between the initial and final protocols can be seen in the following table, but these represent final values and do not include the numerous trial values and their combinations.

	Initial Protocol	Final Protocol
Fetal Calf Serum	20 %	10 %
FudR concentration	0	1x10 ⁻⁵ M
Deoxycytidine hydrochloride	1x10 ⁻⁴ M	0
BrdU concentration	1x10 ⁻⁴ M	6.5x10 ⁻⁴ M
Colcemid time	60 minutes	15 minutes
Irradiation time	15 minutes	90 minutes
Slides	MacIlvaine's buffer	2xSSC

Table 1. Summary of the differences between the initial and final R-banding protocols.

In refining the final protocol, the irradiation time seemed to be the most significant factor and several trials were done with times of 60, 80, 90, 100, 120, and 150 minutes. The best results were obtained at a time of 90 minutes. The time and concentration of the Giemsa counterstain did not have as much significance, but it was critical not to overstain due to poor quality of destained slides. A 5% Giemsa stain for 90 seconds consistently gave good quality stained slides.

In the analysis, the number of cells observed at high power (400X) was recorded and those with good morphology in which both X chromosome were distinguishable were analyzed as to whether the abnormal X was active or inactive. The total number of cells looked at under high power was 112. Of these 72 exhibited some degree of banding and both X's were discernible in 52 cells. In all 52 cells the late replicating X corresponded to the abnormal X chromosome.



The abnormal X often appeared slightly longer and always exhibited a dark band on the distal q arm which is not present on a normal female inactive X. In addition the dark region on the late replicating, abnormal X is larger than that on the normal, active X. The conclusion of R-banding studies show that the normal X is active as well as the duplicated portion of the abnormal X corresponding to the dark band located at the bottom of the chromosome (see figure 6).

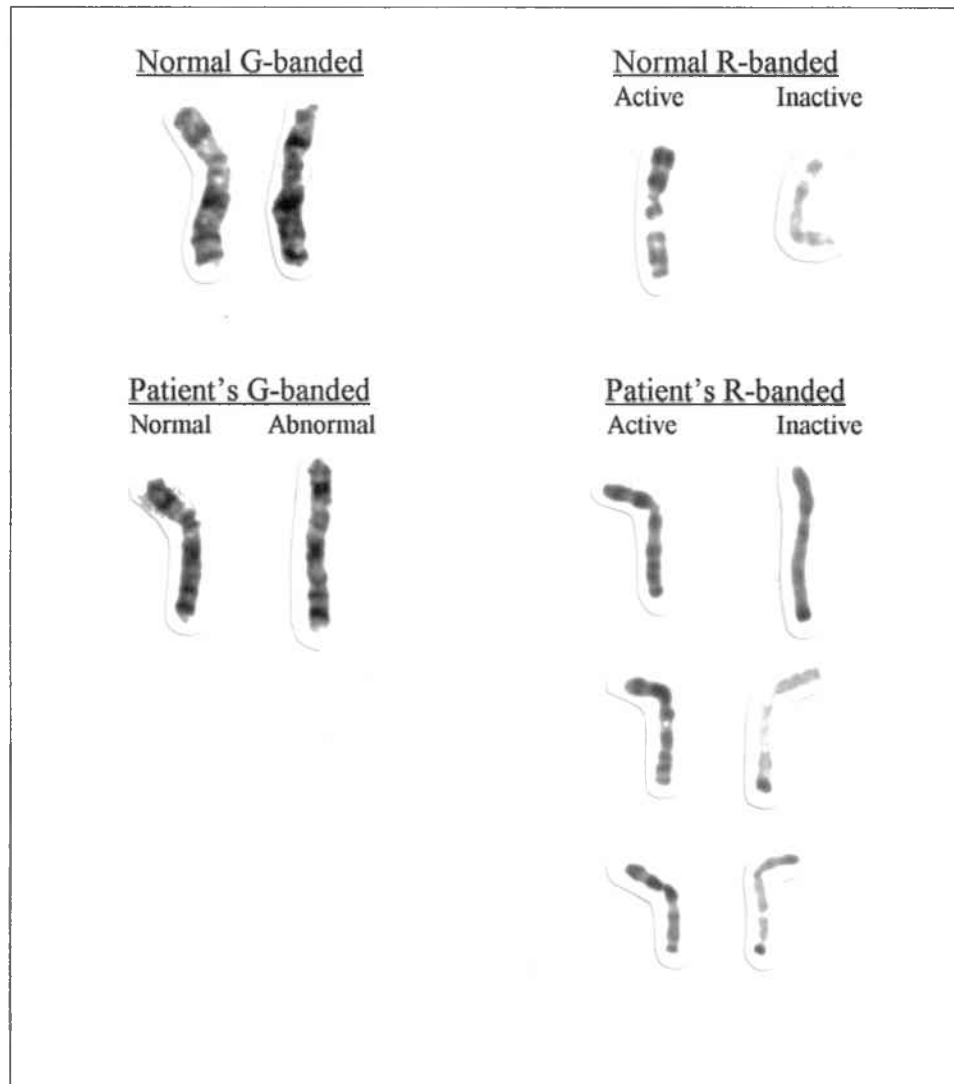


Figure 6. R-banding results show the normal X and duplicated region of the abnormal X to be early replicating while the abnormal X is overall, late replicating.

DISCUSSION

In general, females who carry a duplication of the q arm of the X chromosome are protected from genetic imbalance through preferential inactivation of the duplicated X chromosome (Thode et al. 1988). However there have been five cases in which an abnormal phenotype was associated with a duplicated Xq even though the duplicated X chromosome was late replicating (Aughton et al. 1993; Knuvtila et al. 1984; Morichon-Delvallez et al. 1988; Van Dyke et al. 1983; Varella-Garcia et al. 1990).

In these cases, several possible explanations were given for the observed abnormal phenotype. The explanation that appears to be most applicable to the Shodair case is that late replication does not necessarily imply complete inactivation (Aughton et al. 1993).

The results from my replication studies found that the normal X and the duplicated region on the abnormal X were early replicating. The dark band on the bottom of the inactive X is not present in a normal female and this implies that this region is early replicating. As a result, the patient has a functional disomy for that portion of the X chromosome which may account for her phenotype.

The results of the replication banding study enabled us to identify which X chromosome was being differentially inactivated. The nonrandom inactivation had been determined earlier by a PCR X inactivation assay of the androgen receptor gene. However, the extra material had not been further identified other than by whole chromosome painting using an X specific library which showed the extra material to be of X chromosome origin.

To follow up on this case, two approaches have been used to more specifically identify the duplicated region. Fluorescence In Situ Hybridization (FISH) of seven probes was tested for hybridization in the duplicated region. A positive result would show three signals per



metaphase, while a negative result would show the normal two signals per metaphase. Probes for STS (Xp22.3), KAL, DXS1140, Androgen receptor (Xq12), XIST (Xq13), DXZ4 (Xq24), and telomere Xq (Xq28) were all negative for hybridization within the duplicated region (see figure 7).

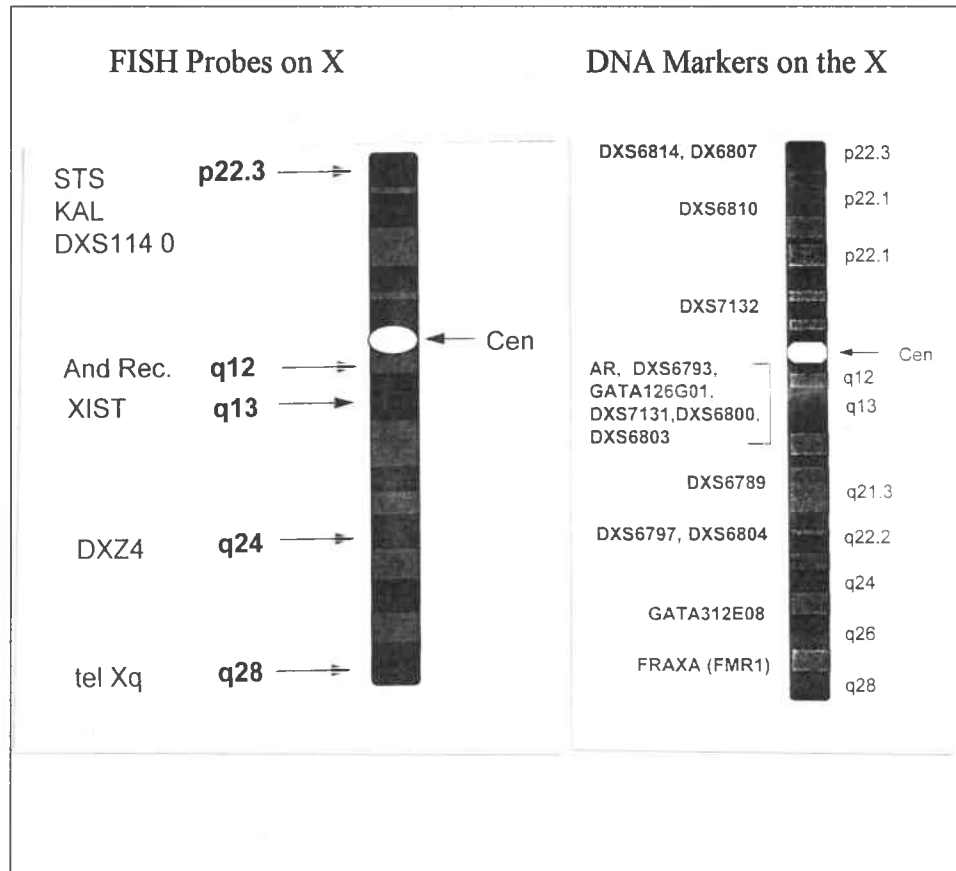


Figure 7. FISH probes and DNA markers were used to identify the duplicated region.

In addition, several DNA markers (see figure 7) on the X were examined by molecular studies using the polymerase chain reaction (PCR) to detect if there is amplification for any of these loci. This would help pinpoint which region of the X is duplicated. Just recently one marker located at the bottom of the q-arm gave a positive result, indicating that the duplicated region arose from the adjacent region of the X chromosome. However subsequent studies are still under way.

As technologies such as FISH and PCR have become available, they have been used to more specifically identify the duplicated portion of the X chromosome in the Shodair patient. Replication banding was also key to understanding this case in that R-banding studies showed which X chromosome was active and that the duplicated region of the abnormal X chromosome was early replicating and thus active. These molecular and cytological techniques are complementary to one another and have provided valuable information in the study of this patient and many others.

BIBLIOGRAPHY

- Ashworth A, Rastan S, Lovell-Badge R, Kay G. 1991. X-chromosome inactivation may explain the difference in viability of XO humans and mice. *Nature* Vol.351: 406-408.
- Aughton DJ, Abdual AA, Johnson JA, Transue DJ, Trock GL. 1993. Dir dup(X) (q13—qter) in a girl with growth retardation, microcephaly, developmental delay, seizures, and minor anomalies. *Am J Med Genet* 46:159-164.
- Belmont JW. 1996. Genetic control of X inactivation and processes leading to X-inactivation skewing. *Am J Hum Genet* 58:1101-1108.
- Bird AP. 1992. The essentials of DNA methylation. *Cell* 70:5-8.
- Daniels, Art: Personal communications. 840 Helena Avenue, Helena, MT 59604.
- Davies K. 1994. The essence of inactivity. *Nature* 349: 15-16.
- Dennis NR, Collins AL, Crolla JA, Cockwell AE, Fisher AM, Jacobs PA. 1993. Three patients with ring(X) chromosomes and a severe phenotype. *J Med Genet* 30:482-486.
- Denver protocol: Personal communications.
- Epstein CJ. 1986. The consequences of chromosome imbalance: principles, mechanisms and models. Cambridge University Press, Cambridge, MA.
- Goldman B, Polani PE, Daker MG, Angell RR. 1982. Clinical and cytogenetic aspects of X chromosome deletions. *Clin Genet* 21:36-52.
- Hatchwell E. 1996. Hypomelanosis of Ito and X;autosome translocations: a unifying hypothesis. *J Med Genet* 33:177-183.
- Hatchwell E, Robinson D, Crolla JA, Cockwell AE. 1996. X inactivation analysis in a female with hypomelanosis of Ito associated with a balanced X;17 translocation: evidence for functional disomy of Xp. *J Med Genet* 33:216-220.
- Hodgson SV, Neville B, Jones RW, Fear C, Bobrow M. 1985. Two cases of X;autosome translocation in females with incontinentia pigmenti. *Hum Genet* 71:231-234.
- Kelley RL, Kuroda MI. 1995. Equality for X chromosomes. *Science* Vol. 270:1607-1610
- Knuutila S, Heinonen K, Hongell K, Varonen S, Simell O. 1984. A duplication within the critical fertility region of X chromosome in a mentally retarded woman with normal menarche. *Hereditas* 101:253-255.

- Kusnick T, Irons TG, Wiley JE, Gettig EA, Rao KW, Bowyer S. 1987. 45X/46Xr(X) with syndactyly and severe mental retardation. *Am J Med Genet* 28:567-574.
- Mange, Arthur P. and Mange, Elaine J. 1990. *GENETICS: Human Aspects*. Sinauer Associates, Inc., Sunderland, Massachusetts.
- Migeon BR, Luo S, Jani M, Jeppesen P. 1994. The severe phenotype of females with tiny ring X chromosomes is associated with inability of these chromosomes to undergo X inactivation. *Am J Hum Genet* 55:497-504.
- Migeon BR, Jeppesen P, Torchia BS, Fu S, Dunn MA, Axelman J, Schmeckpeper BJ, Fantes J, Zori RT, Driscoll DJ. 1996. Lack of X inactivation associated with maternal X isodisomy: evidence for a counting mechanism prior to X inactivation during human embryogenesis. *Am J Hum Genet* 58:161-170.
- Migeon BR. 1996. X-chromosome inactivation: molecular mechanisms and genetic consequences. *TIG* Vol.10 No.7:230-235.
- Morichon-Delvallez N, Couturier J, Bourdrel V. 1988. Inherited Xq duplication due to a zygotic translocation t(X;X) (q23;q27). *Ann Genet* 31:117-119.
- Naumova AK, Plenge RM, Bird LM, Leppert M, Morgan K, Willard HF, Sapienze C. 1996. Heritability of X chromosome-inactivation phenotype in a large family. *Am J Hum Genet* 58:1111-1119.
- Norris DP, Patel D, Kay GF, Penny GD, Brockdorff N, Sheardown SA, Rastan S. 1994. Evidence that random and imprinted *Xist* expression is controlled by preepithelial methylation. *Cell* 77:41-51.
- Rooney DE and Czepulkowski BH. 1992. *Human Cytogenetics: A Practical approach to Constitutional analysis*. Volume I. Oxford University Press. New York, NY.
- Rupert JL, Brown CJ, Willard HF. 1995. Direct detection of non-random X-chromosome inactivation by use of a transcribed polymorphism in the XIST gene. *Eur J Hum Genet* 3:333-343.
- Schmidt M, Stolzmann WM. 1984. Replication variants of the human inactive X chromosome. II. Frequency and replication rate relative to the other chromosomes of the complement. *Chromosome* 89:68-75.
- Schmidt M, Certoma A, Du Sart D, Kalitsis P, Leversha M, Fowler K, Sheffield L, Jack I, Danks DM. 1990. Unusual X chromosome inactivation in a mentally retarded girl with an interstitial deletion Xq27: implications for the fragile X syndrome. *Hum Genet* 84:347-352.

- Steinbach P, Horstmann W, Scholz W. 1980. Tandem duplication dup(X) (q12 q22) in a male proband inherited from the mother showing mosaicism of X inactivation. *Hum Genet* 54:309-313.
- Thode A, Partington MW, Yip MY, Chapman C, Richardson VF, Turner G. 1988. A new syndrome with mental retardation, short stature and an Xq duplication. *Am J Med Genet* 30:239-250.
- Van Dyke DL, Miller MJ, Weiss L. 1983. The origin of inverted tandem duplications, and phenotypic effects of tandem duplication of the X chromosome long arm. *Am J Med Genet* 15:441-450.
- Van Dyke DL, Wiktor A, Palmer CG, et al. 1992. Ulrich-Turner syndrome with a small ring X chromosome and presence of mental retardation. *Am J Med Genet* 43:996-1005.
- Varella-Garcia M, Rajara EH, Gagliardi ART. 1981. Structural aberation of the X chromosome in a patient with gonadal dysgenesis: An approach to karyotype-phenotype correlation *J Med Genet* 18:228-231.
- Vasquez AI, Rivera H, Bobadilla L, Crolla JA. 1995. A familial Xp+ chromosome, dup (Xq26.3—qter). *J Med Genet* 32:891-893.
- Zabel BU, Baumann WA, Pirntke W, Gerhardt-Ratschow K. 1978. X-inactivation pattern in three cases of X/autosome translocation. *Am J Med Genet* 1:309-317.