This is to certify that the

thesis entitled
Identification and characterization of human chromosome contents in somatic cell hybrids from a patient with Menkes syndrome by using G-11 stain technique

presented by

Shirong Wang

has been accepted towards fulfillment
of the requirements for

M.S. degree in Zoology

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Date April 8, 1991

IDENTIFICATION AND CHARACTERIZATION OF HUMAN CHROMOSOME CONTENTS IN SOMATIC CELL HYBRIDS FROM A PATIENT WITH MENKES SYNDROME BY USING G-11 STAIN TECHNIQUE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1991

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF HUMAN CHROMOSOME CONTENTS IN SOMATIC CELL HYBRIDS FROM A PATIENT WITH MENKES SYNDROME BY USING G-11 STAIN TECHNIQUE

By

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A female patient carrying a de novo balanced chromosome translocation t(X;2) (q13;q32.2) has recently been reported to fully express an X-linked recessive condition, Menkes disease. This case is utilized to map the Menkes gene on the human X chromosome. A modification of the sequential G-11 banding procedure of Wyandt was employed to identify human chromosome contents in four somatic cell hybrid clones obtained from this patient. Differential staining appeared at pH 11.97-12.05 and stain time 3-3.5 minutes. All cell lines were found to contain either the intact or part of the human derivative 2, including the breakpoint at Xq13. A detailed discussion of the human chromosome contents in each clone, the chromosomal analysis of the derivative 2 and inconsistency of the G-11 stain technique is presented.

To

My Parents

for their love and support

ACKNOWLEDGEMENTS

I would like especially thank my advisor Dr. James V. Higgins for his guidance, support and encouragement during the course of this experimental study.

I also thank Dr. Hackel and Dr. Champness for serving on my committee and providing pointed comments.

I am grateful to Beverly Sankey for her technical assistance and generously viewing the karyotypes.

Thanks are likewise due Dr. Glover, Vera Verga and Bryan Hall for providing the specimens and for constructive suggestion in performing G-11 staining.

Finally, I am indebted for the friendship of many people in the Genetics lab who have made these years enjoyable and memorable to me. They are: Julie Shreeve, Mickey Lopez, Mecky Howell, Sirinunt Hope, Gillian Bice, Charlotte Wilks, Lisa Stempek, Joseph Marrazza, Kim Brown, Blair Bullard, John Bice, Rachel Eggleton, Paul Love, Donna Lehman, Linda Kentfield and Alma Cantu. Thanks also to Mike Klusowski and David Loomis for their technical assistance in word-processing this thesis.

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INTRODUCTION

Menkes Syndrome is an X-linked recessive disease. Previous studies with comparative gene mapping on an animal model of Menkes disease suggested that the possible locus for this gene in humans is at band Xq13 (Horn et al, 1984). A female patient with Menkes Syndrome was reported carrying a de novo balanced translocation t(X;2) (q13;q32.2). Her normal X was found non-randomly inactivated and presumably the translocation disrupted the normal function of the Menkes gene on the rearranged X, resulting in expression of the disease. This case is being utilized at the University of Michigan to map precisely the locus for Menkes gene in humans. The lymphoblastoid from the patient C.G. was hybridized with the Chinese hamster fibroblast cell line and HAS medium was used to isolate the translocated human X;2 chromosome. The hybrid cells from different clones were harvested and sequentially banded with Giemsa and G-11 techniques to identify the human DNA materials in these clones.

The G-11 technique is a Giemsa staining procedure conducted under pH of 11. It was primarily used to stain selectively some heterochromatic regions in human chromosomes, especially the secondary constriction of chromosome 1, 9 and 16, but was later found to be more valuable in differentiating

species-specific chromosomes in somatic cell hybrids and in gene mapping (Bobrow, 1974; Burgerhout, 1975; Friend et al, 1976).

The purpose of this study is therefore utilization of G-11 banding technique to identify and characterize human chromosome contents in hybrid clones obtained from the patient with Menkes Syndrome and to select cell lines containing the rearranged human X;2 chromosome for further mapping of the Menkes gene.

LITERATURE REVIEW

1. Menkes Syndrome:

In 1962, Menkes[1] described an apparent X-linked recessive disease found in a family of English-Irish descent. Five boys were affected with peculiar hair (depigmented, sparse and short), growth failure, severe psychomotor retardation and focal or generalized seizures. Their failure to grow brought these affected infants to medical attention at the age of a few weeks and death occurred in the first or second year of life. Their hair was examined microscopically and showed twisting spirally, varying in diameter along the length of the shaft and fracturing at regular intervals. Focal cerebral degeneration and cerebellar atrophy were reported based on two autopsies. Although the disorder, named Menkes Syndrome, was thought to be metabolic, no obvious plausible cause could be discovered at that time. In 1972, Danks[2] and his collaborators found that a type of Australian sheep with copper deficiency produced abnormal wool similar to kinky hair in Menkes disease. This discovery lead them to investigate the copper metabolism in 7 patients with Menkes Syndrome. They noted that all of these patients had low serum copper and ceruloplasmin, as well as abnormal intestinal absorption of orally administered copper salt. In the paper published in

1972, they suggested that Menkes Syndrome was in fact an expression of defective copper metabolism. Besides the low serum copper and ceruloplasmin levels in Menkes disease, liver copper levels were found to be grossly reduced, and copper content in brain was also decreased. All other organs and tissues showed an excessive amount of copper, especially in the kidney which had the highest level of copper of all the tissues. Copper absorption from the intestine has been reported to be only 15% of normal (Dekaban et al[3], 1975). The renal copper loss was excessively great. Studies on cultured fibroblast cells from the skin of patients demonstrated the accumulation of excessive amounts of copper and the increased sensitivity to the toxic effects of additional copper added to the culture medium (Danks[4], 1977). Based on these observations, Danks (1977) and William[5] (1978) postulated that the basic defect in Menkes Syndrome resided on a mutation of an intracellular copper-binding molecule causing an increased binding affinity to copper. As a result, copper enzyme synthesis would be impaired despite a normal level of cellular copper. This binding molecule, named metallothionein, was found to be present in increased amounts in most tissues. However, this earlier hypothesis has been discarded when evidence showed the location of the gene for metallothionein to be on chromosome 8 in mice and chromosome 16 in humans, and not on the X chromosome (Danks[6], 1989). In recent years, metallothionein cDNAs has been cloned from several species and

the expression of this gene has been well-studied. Measurement of its mRNA indicates that the increased induction of the gene is due to increasing levels of copper rather than the pre-existing overexpression of the genes (Danks, 1989). The explanation for this finding is that a gene encoding for an intracellular copper transport protein has mutated and this abnormal protein loses its function of carrying copper to the intracellular sites where copper enzymes are assembled. As a result, excessive "free" copper induces metallothionein synthesis. It is apparent that, instead of being a disease of simple copper deficiency, Menkes Syndrome is a storage disease in which copper is trapped by metallothionein in several tissues, most notably in the kidney. The resultant deficiency of copper in other tissues and the impairment of the biosynthesis of copper enzymes accounts for the generalized defects. The incidence of the Menkes disease is estimated 1 in 50,000 to 100,000 (Danks et al, 1989).

In 1987, Kapur et al[7] reported a female patient with Menkes disease having a de novo balanced translocation between chromosomes X and 2. The patient C.G. was an 11 month old baby born to a 28-year-old G2, P1 mother and an 33-year-old father. She was delivered by Cesarean section at 35 weeks. Her birth weight was 2,610 gram, length 48.5 cm, and head circumference 35 cm. She was normal at birth and discharged from the hospital at the normal time. Starting from 6 months she developed seizures and had feeding difficulty as well as

growth retardation. Her hair was scanty with a visible fine kink. Microscopical examination of the hair showed typical pili torti. Serum copper was as low as 14 μg/dl compared to the normal level of 70-150 μg/dl and serum ceruloplasmin 8 mg/dl (normal 18-45 mg/dl). The peripheral blood chromosomes were studied and karyotype was reported as 46,X,t(X;2)(q13;q32.2) at 550 band level (Figure 1). Her blood showed late replication of the normal X chromosome in all 24 spreads. This result indicated that the normal X chromosome was inactivated in this patient. Both her parents had a normal karyotype. Since Menkes Syndrome is an X-linked recessive disease, females are usually not affected. It was therefore hypothesized that non-random inactivation of the normal X chromosome resulted in the expression of the defective Menkes gene on her active X translocated chromosome.

There are similar case reports where a known X-linked recessive condition is fully expressed in females bearing an X-autosomal translocations. In his review article, Mattei[8] analyzed 105 cases with translocations between X and different autosomes. He found that in nearly 95% of balanced t(X-Autosome) and 91% of unbalanced t(X-Autosome), X chromosome inactivation occurred in the most favorable model rather than as a random event (Lyon[9] 1961). In the case of a balanced translocation, inactivation of the translocated X will lead to spreading of the inactivation into the adjoining autosomal material, thus creating a partial autosomal monosomic

condition. Therefore, the normal X is seen to nonrandomly inactivate. The converse is true in an unbalanced translocation in which spreading of the inactivation into the partially trisomic or monosomic autosome confers a selective advantage, and the translocated X will be nonrandomly inactivated. Mattei concluded that most females with a balanced X/autosome translocation showed preferential inactivation of the normal X. This would allow for the full expression of a recessive allele on the translocated X, whether it was caused by an inherited gene mutation or by the chromosome rearrangement. The same conclusion was also made by Gartler and Sparkes[10] (1963).

2. Gene mapping:

There are many different ways to map a gene. From the cytogenetic point of view, a powerful method to map a gene to a specific band of the chromosome is the utilization of a balanced X/autosome translocation in female patients with full expression of X-linked recessive diseases. Duchenne muscular dystrophy (D.M.D) is an example. Boyd et al[11] (1986) reviewed 20 cases of female patients carrying X-autosome translocations. These translocations involved different autosomes but the breakpoints on X chromosome were all in the short arm at band 21, without exception. Presumably the rearrangement at this specific site had disrupted the normal function of the gene for D.M.D and this, coupled with the

preferential inactivation of the normal X chromosome, was responsible for the occurrence of the disease. It would be reasonable to hypothesize therefore that the chromosome breakage was at the gene locus for D.M.D., and had created a mutation and subsequent full expression of phenotypes due to non-random inactivation of the normal X chromosome. The assignment to Xp21 has been confirmed by family studies using the probes which detect restriction fragment length polymorphisms of the X chromosome short arm (Goodfellow et al[12], 1985). Theoretically, the gene for Menkes could also be mapped in the same way by using translocation as a tool, but practically it is not feasible since Menkes disease is a relatively rare disorder and no second case has been reported to carry a translocation between the chromosome X and an autosome.

Nevertheless, some advances have been made in recent years in mapping the human gene for Menkes Syndrome by using DNA hybridization technique. Wieacker[13] (1983) performed a linkage study in a large kindred with Menkes Syndrome by using RFLP probe 1.28, which mapped to the distal part of Xp between X centromere and Xp11.3. He found at least two crossovers. The distance was estimated at 18 cM. Other data using different RFLPs probes, such as MGU22 (close to the centromere) and a centromeric C-banding polymorphism also indicated the linkage of Menkes gene to the centromeric region (Ropers et al[14], 1983; Friedrich et al[15], 1983). Wienker et al[16] (1983) assigned

Menkes gene on the short arm of X and proposed the most likely gene order as Xpter-Menkes-L1.28-MGU22. However, a comparative mapping conducted by Horn et al[17] (1984) suggested that the Menkes locus was likely on the long arm close to band q13. This conclusion was reached based on an animal model of Menkes disease in the mouse.

The Mottled mice (an animal model of Menkes Syndrome) were initially noted by their variegated depigmentation of the coat color in females heterozygous for this X-linked gene. The deficiency of male offspring suggested lethality of this disorder in male. A number of allelic mutations have been recognized in mouse. The males with less severe mutations had severe depigmentation, abnormal vibrissae, skeletal defects, neurological degeneration and arterial abnormalities (Philips[18], 1961; Rowe[19], 1974). The mutation in the mottled mouse is considered to be homologous to Menkes Syndrome in the human being with the same pattern of inheritance and similarly neuropathologic, skeletal, vascular, and clinical abnormalities, as well as similar copper abnormalities in tissue and serum and abnormalities in copper metabolism (Danks, 1977). X-linkage of the Mo locus ('mottled') in the mouse has been proven and the locus has been mapped on the X chromosome of the mouse near the loci for phosphoglycerate kinase (Pgk-1) and alphagalactosidase (Ags) and closely linked to Pgk-1 (McKusick[20], 1978). Since the order of Pgk-1/Pgk and Ags/Gal in mice and humans is the same with respect to the

centromere (Horn et al, 1984) and the human PGK has previously been mapped to band Xq13.2-13.3, the most likely position of the gene for Menkes in humans is on the long arm of X chromosome, within the band Xq13.

3. Somatic cell hybridization:

Somatic cell hybridization was begun in 1960's as a product of the development in somatic cell genetics. As early as 1961, Barski et al[21] first noted proliferating cells arising from fusion of the two lines which contain the chromosomes of both cell lines. These results were confirmed by others, and Littlefield[22] (1964) added a selective system, hypoxanthine-aminopterin-thymidine (HAT), to isolate hybrids between the two drug-resistant cell lines. Ephrussi et al[23] (1965) were able to fuse human-rodent hybrids and isolate them in a HAT selective medium. The medium contains hypoxanthine and thymidine (H and T), the normal precursors of DNA, and aminopterin (A), a drug that specifically blocks one pathway leading to DNA synthesis. Normal cells can synthesize DNA by way of two metabolic pathways: the major pathway, which is blocked by aminopterin, and a salvage pathway which uses nucleoside as a precursor to synthesize DNA. Two enzymes are required for completion of the salvage pathway: thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT). Cells that are unable to produce either TK or HPRT cannot convert precursors into DNA along the salvage

pathway and must depend on the major pathway for DNA synthesis. Thus, if the major pathway is blocked by the presence of aminopterin in the medium, such cells would be unable to grow. Grzeschik et al[24] (1972) made hybridization between a human cell strain derived from a female heterozygous for an X-autosome translocation in which the structurally abnormal X was genetically active and a HPRT deficient mouse cell line which could not survive in HAT medium and therefore required the human HPRT enzyme to proliferate. In these hybrids, cells underwent progressive reduction in the number of human chromosomes, but selective retention of the human X chromosome on which the HPRT gene is located. By this way, the reduced hybrids containing only one of the two segments of the structurally abnormal X chromosome can be obtained. These kinds of cell clones are very useful to map a specific gene on the X chromosome. The DNA samples from these clones and from the patient's family members can be digested with the appropriate restriction endonucleases and analyzed for RFLPs by Southern blot hybridization with selected radiolabeled DNA probes.

Human lymphocytes are often used for somatic cell hybridization, but its limited lifetime prevented its use in long-term culture. Usually at about 40-45 days it will lose viability and die. Gerrer et al[25] (1969) in vitro infected buffy-coat cells from the peripheral blood of a healthy adult with a herpes-like virus designated Epstein-Barr virus (EBV)

and successfully established a long-term proliferated lymphoblast culture. In this culture, seventy-five percent of the cells which had been transformed by a virus showed striking changes in morphology: lymphocytes grew in size from small cells to medium and large ones with sparse cytoplasms and the enlarged rounded nuclei contained prominent nucleoli. These transformed cells obtained much extended life span, therefore could be used for the establishment of long-term cell clones for DNA analysis.

4. Human cytogenetics:

The study of human chromosomes can be said to date from 1879 when Arnold observed chromosomes in dividing human cells (Makino[26], 1975). In the following years, many scientists attempted to determine chromosome numbers in humans. However, the correct number was not established until 1956 when Tjio and Levan[27] reported consistent counts of 46 chromosomes in fibroblast cultures from the lungs of human abortuses. This discovery was attributed to technical improvements in cytogenetics in the 1950s which allowed for the accurate and precise examination of chromosomes.

One of these achievements was obtaining satisfactory preparations of dividing cells at metaphase. For the majority of the cell cycle time, the chromosomes are in an interphase in which the DNA is diffuse and no details of the chromosome can be seen. Whereas during metaphase, the DNA is maximally

condensed and has distinctive morphology. However, only a small proportion of cells are in metaphase at any one time. Even though metaphase cells can be observed, the clumping and overlapping of chromosomes always make results uncertain. In the 1950s, several improvements in technical methods were obtained in the following fields:

The first of these was the development of techniques for culturing tissues and cells in vitro. Classical studies of human chromosomes were limited to testicular specimens which had the problem of the relative scanty number of cells undergoing division. By systematic addition of the various nutrients required for mammalian cells, people were able to culture in vitro a variety of tissues. Newly developed techniques involved the use of short-term cell cultures. These cultures furnish actively dividing cells without a loss or gain of chromosomes. A higher proportion of mitoses could be obtained through cell harvest from these short-term cultures.

A second technical development was the application of colchicine or its derivative Colcemid on tissue culture. This compound interferes with the formation of the mitotic spindle so that when a cell enters mitosis under the influence of colchicine, the spindle fails to form and the cell is therefore "arrested" at metaphase (Levan[28], 1938). Thus, colchicine treatment increases the opportunity of finding cells suitable for examination. The use of Colcemid can eliminate some toxic side effects of colchicine such as a

chromosomal contraction.

A third development was the treatment of cells with hypotonic solutions of various kinds for the purpose of swelling cells in order to improve the chromosome dispersion. Makino and Nishimura[29] (1952) had used water pretreatment of cells for the study of the chromosomes in animals. Hsu[30] (1952) developed a method of using hypotonic culture medium on human fibroblasts. The chromosomes examined by this method appeared as sharply defined bodies with considerable spreading in the cells, making them more amenable for study. There was minimum overlapping in well-prepared metaphase and the chromosomes could be counted readily by means of photomicrography. Other various solutions used included hypotonic sodium citrate and sodium chloride, 0.65M potassium glycerophosphate, 0.075M potassium chloride, and diluted balanced salt solutions of varying complexity (Hanks, Ringer, Tyrode, etc).

Improvements were also made on slide preparations. The previous method introduced by Hietz in 1936 was called "the squash technique" (reviewed by Hamerton[31], 1971). It involved putting the materials between a coverslip and a slide, and with gentle but firm pressure, pressing the material until the chromosomes spread. One problem with squash preparations, however, was that they were temporary and slowly deteriorated. Also, mammalian cells were very soft after hypotonic solution pretreatment and pressing too hard would break cells. Therefore, when "air-drying technique" was developed by

Rothfels and Siminovitch[32] (1958), it became a welcome technical improvement and quickly replaced the previous "squash method". "Air-drying technique" basically consisted of placing a drop of cell suspension on a clean slide, allowing it to spread out, and then drying quickly. This method gave good reproducible results with a high yield of well-spread metaphase chromosomes. Since then variations were made on this technique, such as the method developed by Moorhead[33] (1960) who used a methanol-acetic acid mixture as fixative to force chromosomes spreading without any mechanical pressure. Methanol-acetic acid has a property of quick evaporation. It takes water out of cells quickly so that cells are able to flatten out and the chromosomes will spread.

Another major revolutionary breakthrough in cytogenetic technology was the discovery of various banding methods which allow the accurate identification of individual human chromosomes. Eighty years ago, a German scientist, Gustav Giemsa, found that a degradation product of methylene blue, called methylene azure, was able to stain chromatin and that another compound, eosin, contributed to it by binding the azure-incorporated chromatin. He then mixed methylene azure, eosin, glycerine and methanol together to form a stain solution, later named Giemsa stain (reviewed by Hsu[34], 1979). Prior to that time cytologists often used acetic orcein, acetic carmine and other stains, rather than Giemsa, for chromosome preparations. Those stains had certain limitations

in the identification of the human chromosomes on morphological criteria, and consequently structural rearrangements occurring in the chromosome segment will often escape detection. Thus, there was a need for more objective methods that would allow the identification of individual chromosomes with accuracy. In response to this demand, several banding techniques were developed in late 60's and early 70's: Caspersson et al[35] (1969) discovered Q-band, quinacrine fluorescent banding, using quinacrine mustard. He found characteristic fluorescent segments (or bands) of various degrees of brightness along each metaphase chromosome. Thus chromosome pairs with identical gross morphology could be recognized individually by their fluorescence banding patterns; Arrighi et al[36] (1971) established C-band (a term for constitutive heterochromatin) technique by which heterochromatic (including centromeric) regions showed darker Giemsa stain than the chromosome arms; By applying the C-band procedure to flame-dried slides, Patil et al[37] (1971) was able to find a new banding pattern, later named as G-band (Giemsa-band). A comparison was made between this new Giemsa banding and Q-banding in the Paris Conference that fall. The two systems matched almost band by band, with the brightly fluorescent Q-bands being equivalent to deeply stained G-bands, and the dull or non-fluorescent Q-bands corresponding to the lightly stained or unstained G-bands. In the same year, Seabright[38] modified the Giemsa technique by pretreatment of

the mitotic spreads with trypsin and obtained sharp chromosomal banding patterns. This trypsin Giemsa technique has replaced the older Giemsa-band methods and has become a routine chromosomal banding technique used in most cytogenetic laboratories since. Simultaneously with the discovery of G-band, Dutrillaux found R-band (Reverse Giemsa band) which is exactly the reverse of G-band in the sense of the darkly and lightly stained zones (reviewed by Hsu, 1979). As demonstrated with the Q-, G-, R-or C-staining methods, each individual chromosome appears as consisting of a continuous series of light and dark bands organized in a well defined differential patterns. All of these banding techniques gave a similar staining pattern of the chromosomes, therefore they provided a useful and reliable tool for identifying each chromosome by recognizable characteristic band patterns produced along the chromosomes.

5. Differential staining techniques:

To map human gene, a very useful technique is to produce interspecific hybrid via somatic cell hybridization. Such crosses often undergo an initial random loss of human chromosomes until karyotype stability is reached. By comparison of the cosegregation of human gene products in a number of hybrid clones, linkage of the gene loci determining those products can be inferred. This technique requires distinguishing and identifying human genetic material from a

rodent's background so that a gene locus could be assigned to a given human chromosome. A variety of techniques have so far been reported to obtain staining differences between different species. Two such methods are Hoechst 33258 staining and C-banding techniques, which offer a rapid means of distinguishing human and mouse chromosomes.

In 1972, Hilwig et al[39] conducted a test to treat chromosome preparations from mouse cells with the bibenzimidazlo compound "Hoechst 33258" (H 33258). He found intense staining of the centromeric regions on the mouse chromosome. Kucherlapati et al[40] (1975) applied the same procedure on mouse-human somatic cell hybrid and observed a differential staining between mouse and human chromosomes. With this stain, the mouse chromosomes in the hybrid cells showed bright fluorescence whereas the human chromosomes were non-fluorescent or weakly fluorescent.

The C-band method developed by Arrighi and Hsu (1971) uniquely stains heterochromatin regions and is also used to identify specific human and mouse homologous chromosomes. In the hybrids stained with C-band technique, the mouse chromosomes had a deeply staining block of heterochromatin which accounts for approximately 10% of the total length in the longer chromosomes and up to 50% of the total length in the shorter chromosomes. In contrast, the centromeres of human chromosomes were not stained as intensely as those of mouse chromosomes because constitutive centromeric heterochromatin

in man is quantitatively reduced compared with the mouse (Chen et al[41], 1971).

Although C-banding technique and staining with Hoechst 33258 could provide a rapid means of identifying mouse and human chromosomes, they usually fail to detect parts of and small translocations of the human chromosomes. In 1972, Bobrow et al[42] reported a new Giemsa stain technique, named G-11 staining. The basic mechanism is that under highly alkaline conditions (Ph=11), Giemsa mixtures can differentially stain certain heterochromatic regions into magenta and remaining portion of human chromosomes pale blue. It was originally adopted for use in cytogenetic analyses of patients with hematologic conditions, primarily leukemia. It was also used to demonstrate polymorphisms, translocations and pericentric inversions. Modifications of this procedure have been useful for differentiating species-specific origin of a chromosome in human-rodent hybrid cells (Bobrow et al[43], 1974). Under optimal conditions, mouse chromosomes in the hybrids stain a dark magenta with pale blue centromeres whereas human chromosomes show blue color with red differentiation at paracentromeric regions. By using Giemsa-11 technique, Burgerhout[44] (1975) and Friend[45] (1976) were able to successfully detect two-colored chromosomes which represented interspecific translocations in the mouse and human hybrids. Such an identification would be very helpful to aid in the regional localization of human genes.

MATERIALS AND METHODS

1. culture:

The somatic cell hybrids between lymphoblastoid cells from the patient C.G. and Chinese hamster fibroblast cell line RJK88 was constructed in the genetics laboratory of the University of Michigan. Fusions were performed using polyethylene glycol (PEG) and hybrids were selected in HAS medium (hypoxanthine/ azaserine) for the retention of the derivative chromosome 2 (2pter-2q32:Xq13-Xqter). Fourteen hybrids were isolated and four of them were obtained from the University of Michigan for further identification by G-banding and G-11 staining. These frozen cell cultures were thawed by removal of the freezing ampoules from the styrofoam box containing dried ice and immediate immersion in a 37°C waterbath, agitating until defrosted. Cells were then transferred into the 25 cm^2 Falcon flasks with the Pasteur pipettes, suspended in 5 ml HAS growth medium immediately, and finally incubated at 37°C in a 5% CO_2 incubator. HAS medium was made from 500 ml D-MEM supplemented by 10% fetal calf serum, 2% L-glutamine, 10 ml 10^{-2} M L-proline, 20 ml $2.5x10^{-3}$ M hypoxanthine, 5 ml 10^{-3} M azoserine and 10 ml Penicillin/streptomycin.

Cultures were inspected under the microscope for the

growing density every other day. If it reached 1/2 confluence, cells would be subcultured into another flask. To do this, cells were first rinsed with 1X PBS, then exposed to warmed (37°C) 1.0 ml 0.25% GIBCO trypsin-EDTA for 1 minute or until most cells were round up and detached from the flasks. 0.7 ml cell suspension was discarded from each flask, followed by addition of 5 ml complete HAS medium and mixed. When cultures showed healthy growth with 1/2 confluence or less, enzymatic disposal was not necessary; instead, a change of fresh medium would be sufficient. If individual cultures were growing poorly, only half of medium was discarded from the flask each time and replaced by an equal amount of fresh HAS medium. By microscopic monitoring, cultures were allowed to be in the active growth condition until they were ready for harvest.

2. harvest:

Harvest was usually accomplished when cells were 2/3 confluent. Twelve hours before harvest, cultures were fed with the fresh HAS medium in order to synchronize the cell cycle. Just before the harvest, they were washed with 1X PBS followed by 1 ml GIBCO Trypsin-EDTA digestion for 1-2 minutes. Free cells were transferred to centrifuge tubes and treated with colcemid at final concentration 0.1 μg/ml for 20 minutes to accumulate metaphase cells. Cultures were then removed from incubation and spun for 5 minutes at 1000 RPM in a centrifuge. After the supernatant was drawn down to 1 ml, pellets were

tapped gently to mix well. Cultures were then treated with 7 ml prewarmed (37°C) 0.075 M KCl and allowed to incubate for 12 minutes. Timing is the critical step in minimizing the amount of cytoplasmic background in order to obtain good color differentiation. Hypotonic treatment for 12 minutes in 0.075M KCl is sufficient to achieve a minimum background. At the end of 12 minutes, 1 ml freshly-made fix (3:1 100% methanol and acetic acid) was added directly into hypotonic solution and mixed well. Then the suspension was centrifuged at 1000 RPM for 10 minutes. After supernatant was removed, cultures were treated with 8 ml 3:1 fix and allowed to be refrigerated at 4°C for 10 more minutes. Two changes of fix were required before the completion of harvest. At the end, pellets were stored at 4°C for the future use.

3. <u>making slides</u>:

The pellet was taken out from the refrigerator and washed once or twice with fresh fixative. The supernatant was drawn down to the appropriate level and cell suspension was gently mixed by a Pasteur pipette. The slides were precleaned with 100% ethanol and then rinsed thoroughly in double distilled water. Spreading of the metaphase preparation was achieved by pipetting three drops of low density cell suspension to separate areas of each wet slide from 10-15" height. The slides were then passed through a steamer for about 2-3 seconds and placed on a 65°C slide warmer for 1-2

minutes or until the water was removed. Slides on each cell clone were scanned to determine their quality on a low power lens using a phase contrast scope. The methods utilized were adjusted to obtain appropriate cell density, high mitotic index, optimal chromosome spreading and minimal cytoplasmic background. Adjustments could be made by increasing or decreasing the angle of slide, dropping cell suspension from more or less height, passing slides through a steamer for longer or shorter time, blowing over slides or avoiding blowing, as well as changing the ratio of methanol:acetic acid in the fix, etc. Once the optimal method was determined, all other slides on the same clone would be made in the exactly same way to ensure the consistency of each slide. The slides were then aged in 90°C oven for 1 hour and 15 minutes and stored in an air tight container. The critical point in making slides was to keep cell density low. Excess cells would result in an overall blue appearance of chromosomes after staining. The density was thought to be appropriate if cells spread over 1/3 to 1/2 area of a slide.

4. Giemsa banding:

Trypsin stock solution was made by dissolving 2.5 g SIGMA type III Trypsin powder in 100 ml Tyrode solution which contained NaCl 8.0 g, KCl 0.2 g, $NaH_2PO_4 \cdot H_2O$ 0.05 g, Glucose 1.0 g, $NaHCO_3$ 1.0 g and 1000 ml distilled water. This Trypsin solution was then aliguoted into Falcon tubes, 1 ml in each,

and stored at 0°C. Before banding, one tube was removed from the freezer and thawed out under room temperature. 0.3 ml Trypsin stock solution was diluted with 100 ml 1X PBS, forming Trypsin working solution.

To make the Wright stain solution, 2.5 g of EM Wright blood stain powder was mixed with 1000 ml methanol, stirred for four hours and then filtered through two combined #1 filters.

Slides were trypsinized for 10-20 seconds, rinsed thoroughly with two changes of 0.9% NaCl and blotted. They were then stained for 1 minute 10 seconds to 1 minute 20 seconds with 4 ml Wright working solution (1 ml Wright stain solution + 3 ml Gurr's buffer-pH 6.8). When the staining was complete, the slides were rinsed with double distilled water, then air dried and examined for banding quality by using 100X bright field oil immersion lens: if chromosomes in most mitoses appeared bloated, empty or fuzzy, over-trypsinization was suggested and the next slide would be trypsinized for a shorter time; if chromosomes were under-trypsinized as indicated by decreased contrast and indistinct bands, exposure time to the trypsin would be increased. Staining time was also adjusted by monitoring color intensity of chromosomes.

Stained slides were scanned on low power bright-field objective to select well-banded, well spread mitoses, with as few overlapping chromosomes as possible. These metaphases were further examined on 100X bright field oil immersion lens and

photographed with 35 mm high contrast copy films. The co-ordinates of the scope was written for the record.

5. destaining slides:

After pictures were taken, slides were cleaned with xylene. The destain process involved dipping these slides sequentially in 70% ethanol, 80% ethanol, 95% ethanol + 2% hydrochloric acid, 95% ethanol and 100% methanol, and finally blotting dry.

6. Giemsa-11 staining:

The method used was modified from Wyandt et al (1976). To make G-11 working solution, CAPS buffer and G-11 stock solution were needed. The buffer was made by dissolving 2.213 gram SIGMA CAPS powder in 1000 ml distilled water to obtain the final concentration 0.01 M. CAPS was warmed in 37°C water bath for 1 hour. Before making G-11 working solution, the pH of the buffer was adjusted to the desired value with a pH meter.

Giemsa-11 stain solution was made by combining 0.5 grams powdered Giemsa (Fisher G-146) and 33 ml glycerol, stirring for 2 hours followed by incubation overnight at 37°C. On the second day, 33 ml methanol was added and thoroughly mixed.

The destained slides were placed in a Coplin jar and soaked in distilled water for 1-2 hours. Just before staining,

they were transferred directly from distilled water to 49 ml prewarmed (37°C) and pH pre-adjusted CAPS buffer in water bath. Immediately 0.8-1 ml Giemsa-11 stain solution was added into the buffer and mixed gently to minimize formation of surface film which would adhere to a slide during its removal from the solution. Staining of the slide was monitored by removing them at various time intervals and reviewing microscopically for color intensity. If the chromosomes appeared pale blue overall, the slides would be stained longer; if the chromosomes were very dark magenta, the staining time would be reduced. The average time was 3 - 3.5 minutes for all the slides. At the end of the staining time, slides were removed, rinsed briefly in distilled water, dried with compressed air and mounted with cover slips. The pre-photographed slides were viewed again under the same microscope and those metaphases formerly photographed were relocated with the previous co-ordinater record. If color differentiation occurred on these mitoses, a second photograph was obtained by using either colored or black/white film and two prints of the same spread were compared to determine the human chromosomal contents. The stain solution was always prepared fresh for each set of 2-3 slides and could not be reused.

RESULTS

With well stained slides, human chromosomes are light blue with red dots at paracentromeric region of chromosomes 1, 4, 5, 7, 9, 10, 13, 14, 15, 20, 21, and 22. Chinese hamster chromosomes are stained a dark magenta, with pale blue centromere region (Bobrow, 1974). In hybrids, human X chromosome can be identified by its lighter color and by the absence of the markedly paler centromere region. Occasionally a translocation can occur between human and Chinese hamster chromosomes. Under these conditions, translocated chromosomes will be stained blue and magenta respectively (Figure 2); in a black-white photograph, the respective areas are displayed as light and dark gray.

1. Optimal pH of the buffer and the staining time:

Four cell lines were tested, MHCG 1A, MHCG 9, MHCG 10 and MHCG 11. For each of them, a minimum 10 slides were made to ensure that at least three slides would be able to show differentiation after G-11 staining.

The starting pH of CAPS buffer was 11.50, at which all the chromosomes were stained pink without any color differentiation. This indicated the pH of CAPS solution was too low. The pH was then raised by 0.1 each experiment and

slides were monitored microscopically to direct the adjustment. The optimal differentiation was achieved at the staining time of 3 - 3.5 minutes and pH of the buffer in the range of 11.97-12.05. At this pH, all four cell lines showed distinct color differentiation with human chromosomes stained light blue and Chinese hamster chromosomes dark magenta. The same differential staining was also found on the translocated chromosomes. The blue areas of these chromosomes coincided with the parts which were identified as being human-derived on regular Giemsa-banded slides.

Table 1 Total Number of Cells Photographed After G-banding

cell lines	# of slides	# of cells
MHCG 1A	5	86
MHCG 9	9	81
MHCG 10	4	109
MHCG 11	8	135
Total	26	411

As shown in Table 1, a total 26 slides and 411 cells

Table 2 Numbers of Slides and Cells Analyzed and Usable after G-11 Staining

cell line	# of slides analyzed	# of cells analyzed	# of cells with differentiation	# of useful cells
MHCG 1A	3	55	24	22
MHCG 9	3	23	5	4
MHCG 10	3	79	22	19
MHCG 11	3	52	17	9
TOTAL	12	209	68	54

were photographed after Giemsa banding. Table 2 lists the number of cells with differentiation after G-11 staining. The total number of cells photographed was 411, however only 68 of them showed color differentiation. Among these 68 cells, 2/3 (46/68 cells) had distinctively blue and pink colors on the human and Chinese hamster chromosomes, respectively.

In Table 2, the column " # of slides analyzed" refers to the slides in which color differentiation was evident. It does not include those in which differential staining was absent in the previously photographed cells, even though present in some other metaphases in the same slide. "The # of cells analyzed" specifically indicates the chromosome spreads which had already been photographed after Giemsa banding. "The # of cells with differentiation" was counted from the analyzed cells only, as described above. Again, the color differentiation could also be found in other cells in the same slides, but they were unable to be utilized because of the lack of the sequential G-11 staining.

2. <u>Human chromosomal composition in four cell lines</u>:

Although the number and content of human chromosomes varied from cell line to cell line, the derivative 2 was found in all four clones in either intact or translocated form. Cell lines MHCG 1A, MHCG 9 and MHCG 11 did not bear the detectable intact nor translocated normal or derivative X, based on karyotyping analysis. The exception was MHCG 10, which will be

discussed later. MHCG 1A, 9 and 10 also carried human chromosomes other than the derivative 2, as seen in Table 3. Table 4 and 5 give details of the human chromosomal contents in each clone.

Table 3 Human Chromosomal Content in Four Clones

cell lines	# of cells analyzed	presence of der(2)	additional human chromosomes
MHCG 1A	22	yes	8,12,14,22
MHCG 9	4	yes	14
MHCG 10	19	yes	8,13,22
MHCG 11	9	yes	NONE
TOTAL	54		8,12,13,14,22

MHCG 1A: Selected by HAS medium, the derivative 2 was present in 18 out of 22 spreads, and found to be missing in the other four cells. Besides the derivative 2, each spread carried either one, two, three or all four of the following human chromosomes--8, 12, 14, and 22. These four chromosomes, together with the derivative 2, consisted of the basic

component of the human chromosome in MHCG 1A cell line (Figure 3). Among the 22 spreads analyzed, 1 had all 5 of these chromosomes, 7 had 4, 11 cells had 3 of them, 1 cell had 2 and the remaining 2 cells had only one chromosome of human origin. Thus the majority of cells (18/22) contained 3-4 human chromosomes, which probably represented the stable number of human chromosomes in this specific clone.

Three cells in MHCG 1A were marked with " ? " in Table 4. One of them had distinct color differentiation on all chromosomes except one, which was stained between blue and magenta on the short arm and dark magenta on long arm. When reviewing its Giemsa-banded karyotype, the short arm of this chromosome failed to match any part of human chromosomes. Since the color alteration was not distinguished on this specific chromosome, it is likely that this segment of chromosome is not human-derived. The other two questionable cells containing the same piece of chromosome in light blue color with two positive bands and without apparent centromere visible in Giemsa banded karyotype. From the color and morphology, it could represent a part of a broken human chromosome. But since these pieces of chromosomes were small, it was not possible to tell, by their morphology, from which chromosome they had derived.

MHCG 9: Four cells were analyzed. The long arm of the derivative 2 was again found in the majority of the cells (3/4), human 14 was also present in 3/4 cells. These were

Table 4 Human Chromosome Composition in Different Cells

cell lines	human chromosomal composition/cell	# of cells	total
MHCG 1A	22	1	22
	14	1	
	14, 22	1	
	22, t(der 2q), ?	1	
	14, 22, t(der 2q)	8	
	12, 14, 22	1	
	12, 14, 22, t(der 2q)	5	
	12, 14, t(der 2q)	1	
	14, 22, t(der 2q), ?	2	
	8, 12, 14, 22, t(der 2q)	1	
MHCG 9	14	1	4
	t(der 2q)	1	
	14, t(der 2q)	2	
MHCG 10	8	4	19
	t(der 2q)	1	
	der 2	1	
	13, der 2	1	
	8, t(Xq)	4	
	22, der 2	5	
	8, t(der 2q)	3	
MHCG 11	t(der 2q)	9	9

Table 5 A Comparison of Different Individual Human Chromosomes in Four Cell Lines

chromosome number	ratio (# of cells bearing a specific human chromosome / total # of cells from each cell line)				total
	MHCG 1A	MHCG 9	MHCG 10	MHCG 11	
8	1/22	0	5/19	0	6/54
12	8/22	0	0	0	8/54
14	20/22	3/4	0	0	23/54
22	20/22	0	5/19	0	25/54
der 2	0	0	7/19	0	7/54
t(Xq)	0	0	4/19	0	4/54
t(der 2q)	18/22	3/4	4/19	9/9	34/54

the only two human chromosomes retained in MHCG 9 (Figure 4).

MHCG 10: The chromosomes of human origin were the derivative 2 (intact and part) in 11 out of 19 cells, the translocated Xq in 4 cells, chromosome 8 in 11 cells, chromosome 13 in 1 cell and chromosome 22 in 5 cells (Figures 5-8).

MHCG 11: Nine cells were examined in this cell line. All of them contained a single chromosome which had both human and hamster staining materials as showed in Figure 9. It was identified to be a translocation between the human derivative 2 and a Chinese hamster chromosome. This was the only chromosome containing human genetic material detected by G-11 staining in MHCG 11 clone.

3. Rearrangement of the human derivative 2 in four clones:

As shown in Table 5, the derivative 2 was found in 41 out of total 54 spreads from all four clones. In 34 spreads it was translocated to different Chinese hamster chromosomes (Figures 10, 11, 12, 13 and 14) while in seven cells, it was found intact.

The break points of the derivative 2 were also different from cell line to cell line. In MHCG 1A and MHCG 9, the break points of the derivative 2 seemed to be identical and was located within the region near its centromere between 2q11.1 or 11.2 and 2p11.1 or 11.2 (Figures 10 and 11). The exact position is difficult to determine by G-11 staining

alone because the centromeres of the human chromosome 2 is not usually stained pink. Therefore both centromeric regions of the hamster chromosomes and the human derivative 2 display a pale blue color. If this specific rearranged chromosome carried human derived centromere, the breakpoint would be then assigned at 2p11.1 or 11.2; but if the centromere is Chinese hamster oriented, the derivative 2 would be at 2q11.1 or 11.2.

The breakpoint in MHCG 11 was also located on the long arm of the derivative 2, but at 2q23 (Figure 14).

In cell line MHCG 10, three kinds of karyotype were found. Seven cells had the intact derivative 2 (Figure 15). Four cells contained a part of the long arm of the derivative 2 which was broken at 2q21 or 22 and translocated to a Chinese hamster chromosome (Figure 13). The third type (including four cells) involved a translocation between a Chinese hamster chromosome and the distal part of the long arm of the human X. The breakpoint was determined at Xq22 (Figure 12). The origin of this rearranged Xq could be either from the normal X chromosome or from the derivative 2. It is not possible to distinguish one from the other by karyotypic analysis alone, but the identification will become possible when the restriction fragment length polymorphism technique is employed.

4. <u>Cells missing the locus for the Menkes gene</u>:

Nine out of 54 cells were found to carry neither the

human derivative 2 nor the derivative or normal X chromosome. These included 4 cells in MHCG 1A, 1 cell in MHCG 9 and 4 cells in MHCG 10. In addition, four more cells in MHCG 10 retained the translocated Xq distal to the Menkes locus with the breakpoint at Xq22.2. Therefore, the Menkes gene was apparently missing from 13 out of 54 cells analyzed.

5. Chinese hamster chromosome numbers in four cell lines:

Chinese hamster has 22 (11 pairs) chromosomes, of which 10 pairs are autosomes and 1 pair is the sex chromosome. In our four hybrid clones, 35-45 chromosomes were found per cell from hamster origin in MHCG 1A. Most cells (19/22) had near tetraploid chromosomes varying from 39 to 44. They were probably the result of either fusion of two hamster cells with a single human cell (Grzeschik et al, 1973[24]) or the failure of mitosis in the hybrid cells at early stage followed by random loss of an individual chromosome from the same cell later. In contrast, the numbers of hamster-derived chromosomes were close to normal (22) in the other three clones: for example, 20-23 hamster chromosomes were found in every cell in MHCG 9. The origin of the extra chromosome in the cell with the total number of 23 could possibly come from a breakage of a hamster chromosome into two, therefore, the total number of chromosomes increased to 23 instead of the normal value of 22. In fact such a breakage was observed in MHCG 9 as one segment of the hamster chromosome was rearranged with the human

Table 6 Numbers of Hamster Chromosomes in Each Cell Line

cell lines	# of hamster chromosome/cell	# of cells	total
MHCG 1A	35	1	22
	38	1	
	39	3	
	40	3	
	41	5	
	43	3	
	44	5	
	45	1	
MHCG 9	20	1	4
	22	2	
	23	1	
MHCG 10	15	1	19
	19	2	
	20	1	
	21	9	
	22	4	
	43	1	
	45	1	
MHCG 11	20	2	9
	21	1	
	22	5	
	38	1	

derivative 2 to form a new chromosome. The similar phenomenon was observed in MHCG 10, in which the majority of cells (14/16) retained 19-22 hamster-derived chromosomes except one which had 45 from the hamster. This specific spread might represent that either two separate sets of chromosomal contents from two metaphases overlapped with each other during the process of making slide or a non-disjunction occurred at a certain stage during cell division. Two intact human derivatives 2 were found in this mitosis; In MHCG 11, most spreads (8/9) reserved 20-22 hamster chromosomes. The remaining cell was found to have 38 hamster chromosomes which might be explained by the same reason as discussed above.

DISCUSSION

An X-linked recessive disorder affects males while females are unexpressed carriers of the gene. Therefore, it was unusual that full expression of the Menkes Syndrome was observed in the female patient, C.G., carrying an X-autosomal translocation. It is likely that the normal X chromosome was non-randomly inactivated in this patient while the rearranged X remained active and the gene for Menkes was interrupted by the translocation. As a result, expression of the Menkes gene in this patient was defective. Support for this hypothesis comes from comparative mapping studies from an animal model of Menkes disease and linkage data in humans. The Mo locus ('mottled' in mouse) is equivalent to the Menkes gene in human and was mapped to X chromosome between loci for Pgk-1 and Ags, and closely linked to Pgk-1. The gene order for these loci with respect to the centromere was already known to be highly conserved between mice and humans. The human PGK locus has been mapped to band Xq13 (Horn et al, 1984). This band was where the translocation breakpoint was located in the patient, C.G.. Based on these findings, it is hypothesized that full expression of Menkes disorder in this patient was due to disruption of normal functions of the gene by the translocation. The gene for Menkes was, therefore, most likely

located at band Xq13. Recently, this case has been utilized by investigators at the University of Michigan to precisely map the Menkes gene in humans. Molecular DNA analyses using a large number of probes from the human X chromosome confirmed that the breakpoint in this patient was located in the region Xq13.2-13.3, proximal to the PGK1 locus (Verga et al[46], 1990). However, another possibility exists that the locus for Menkes disease might be somewhere on the rearranged X chromosome and due to non-random inactivation of the normal X, this defective gene was expressed. Under this hypothesis, the translocation and dysfunction of the gene by a separate mutation are two independent events that occurred in father's germline since the rearranged chromosome was identified as paternal origin. However, the possibility for two events arising independently on the same chromosome is extremely low.

■X Somatic cell hybridization has provided a powerful experimental approach to map the human genome. By growing hybrid cells in a selective HAS media, we are able to isolate clones retaining the human X chromosome(s) on which the selective marker HPRT gene resides. Since one X chromosome is usually randomly inactivated in females and, therefore, the HPRT gene, the opportunity will be increased for the random loss of the inactivated X chromosome with retention of the active one, provided cultures are kept in the selective medium for a prolonged period. Whether a gene of interest on X chromosome is able to be retained in cells depends on the

distance between this gene and the selective marker and also the length of the time which the clones are allowed to grow in the selective medium. Previous studies have shown that chromosome breakage was a frequent outcome when somatic hybrid cells were kept in HAT selective medium for an extended period of time (Siniscalco[47], 1974), and it occurred more often between two gene loci which were far apart than two loci which were close to each other. Such a breakage would result in mitotic separation of the gene of interest from HPRT locus on the same X chromosome. Subsequently intra- or inter-species chromosome rearrangements will result in the loss of a particular gene despite the retention of the selective marker HPRT in the clone. HPRT has been assigned at Xq26 and the gene for Menkes is assumed to be located at Xq13. In the case of the patient C.G., the long arm of the X distal to the breakpoint Xq13 was rearranged with chromosome 2 to form the derivative 2. Therefore, the der 2 contained HPRT gene and presumably a part of or the whole gene for Menkes. Based on cytogenetic analysis, the der 2 was present in cells in two forms: the intact and the translocated chromosome derived from the broken der 2 containing loci for both Xq13 and Xq26. Either one of the two forms of the der 2, but not both, was found in each clone of MHCG 1A, 9 and 11, which probably indicated that the breakages and rearrangements of the der 2 occurred independently at an early stage of cell fusion in each of those cell lines. However, it was observed that in

addition to the intact and translocated der 2, a few cells in MHCG 10 did carry the distal part of long arm of a broken chromosome X with the breakpoint at Xq22.2 between the presumed gene loci for Menkes and HPRT. This finding suggested retention of the selective marker HPRT and loss of Menkes gene from these cells. It is assumed that this piece of broken X should bear the activated HPRT gene for HAT selection because it was the only copy of X found in these cells. X chromosome inactivation is known to be quite stable in humans and there is little possibility for an inactive gene to become reactivated (reviewed by Gartler et al[48], 1990). This means that the inactivated normal X in this patient was unlikely to be reactivated. It is reasonable to postulate, therefore, that the distal Xq carried in MHCG 10 was likely from the der 2 broken at Xq22.2. Since a total of 8 out of 19 cells in MHCG 10 were found missing the Menkes gene, this cell line would not be useful for the purpose of mapping the locus for Menkes gene. The absence of the normal X in each cell line indicates that it has been preferentially lost from the cultures due to non-random inactivation of the entire chromosome including HPRT gene.

Identification of human chromosomes against a mouse chromosomal background by various staining methods is a prime requirement for genetic analysis via somatic cell hybridization. Use of G-11 staining technique is a reliable, reproducible, fast and easy way to achieve this goal. Giemsa

banding followed by G-11 staining allowed us to compare different banding patterns on the same chromosome so that the origin of an individual chromosome can be distinguished. It is especially useful in characterization of a chromosomal translocation involving two different species. Under this condition, the two-colored chromosome will be observed in a well-stained metaphase, indicating the rejoining of the different specie-specific DNA materials into one chromosome.

The mechanism of G-11 banding is not clear. Two components of Giemsa are required to obtain the differential staining. One is azure A or B, the other is eosin Y. It was observed that G-11 banding was highly dependent on the concentration and ratio of two dyes, the pH of the stain solution and the buffer composition (Wyandt et al, 1976). Since the banding was achieved only when methylene blue and eosin were used in the combined solution, it indicated that the formation of a azure-eosinate complex accounted for the striking magenta color of chromosome bands (Sumner et al[48], 1973). The role of alkalinity in stain solution is not clear. It can only be speculated that the raised pH of the stain will result in an increased amount of human chromatin losing the capacity of forming magenta dye complexes (Borrow[49], 1974). The phenomenon to support this theory is that both mouse and human chromosomes in hybrid cells are stained bright magenta at low pH, but turn uniformly blue at high pH. Clearly the formation a of red color is not because of a particular property of

constitutive heterochromatin or of highly repetitious DNA sequences since mouse heterochromatin is stained the same color as human euchromatin. The color difference on species-specific chromosomes seems likely due to an alteration in the structure of particular regions of chromatin by the effect of pH on associated structural proteins rather than the direct influence of pH on the process of dye binding (Borrow, 1974). However, the real mechanism for the differential staining is not well understood.

G-11 banding is a relative sensitive technique. Many influences can be attributed to the staining quality, for example; the type and lot number of Giemsa stain, the banding method used, the age of slides and the manner they are aged, the densities of cells on the slide, the type and pH of different buffers, the length of time of staining and of pre-soaking slides in alkali before the staining, etc. Erratic staining results were observed by using, not only different brands of Giemsa but also different bottles of the same brand and lot number of the stain solution (Wyandt[50], 1976). The pH of the buffer has the most notable influence on staining quality of a slide and its optimum varied between different cell types and different slides. Generally speaking, newly-made slides need higher pH than aged slides to achieve maximum differentiation; room temperature aged slides require longer staining time than slides aged in 60°C oven; in order to obtain an optimal banding pattern, slides made from different

clones may also need slightly different pH. The G-11 banding procedure used in this research is modified from Wyandt's method (1976). The Giemsa stains that Wyandt suggested were Harleco, azure blend, type 42476 and MCB type GX85 B527. With these blood stains, the best G-11 banding patterns on the human chromosomes were observed at pH 11.3-11.6 and the staining time around 10 minutes on the slides aged from 3 days to one month. However, the optimal color differentiation on hybrids MHCG 1A, 9, 10 and 11 in this experiment was not obtained until pH of CAPs buffer raised to 11.98-12.03. This difference may be explained by several reasons. The first is that the Giemsa powder used to stain MHCG cell lines was Fisher G-146, which was different from the Giemsa powder that Wyandt utilized. This difference relied on the composition and ratio of several common components, which often influence G-11 staining quality. Secondly, G-11 banding was thought to be involved in denaturation of a specific satellite DNA known to be localized to magenta stained regions (Jones[51], 1973). Different species have different satellite DNA composition, therefore a different pH was needed to achieve the structural alteration required for color differentiation.

SUMMARY

Utilization of human strains with an reciprocal translocation has become a valuable tool for mapping the human genome via somatic cell hybridization. G-11 staining technique has provided a useful approach to characterize the human chromosome contents in human-mouse hybrid clones. Under an appropriate pH of stain solution, human DNA materials can be distinguished by its light blue color against dark magenta-stained mouse or Chinese hamster chromosome background. Four cell hybrids were tested by G-11 sequential staining technique in this study to identify the translocated human chromosome Xq for further mapping Menkes gene in humans. The majority cells from MHCG 1A and 9 and all the cells from MHCG 11 were found carrying this piece of X chromosome including the speculated locus for Menkes (Xq13). They were, therefore, usable for the further gene mapping. In contrast, MHCG 10 is not an appropriate cell line because the locus Xq13 was found to be missing from 42% of the cells in this cell line. Neither the derivative nor the normal X was observed in all four clones on the basis of cytogenetic analysis.

G-11 staining technique is relatively inconsistent. Modification may be needed on the pH of the buffer and the length of staining time for different slides and different

cell lines. It is strongly recommended that a minimum number of slides made from each clone and cells photographed from each slide is required to guarantee enough well-stained metaphases for further analysis.

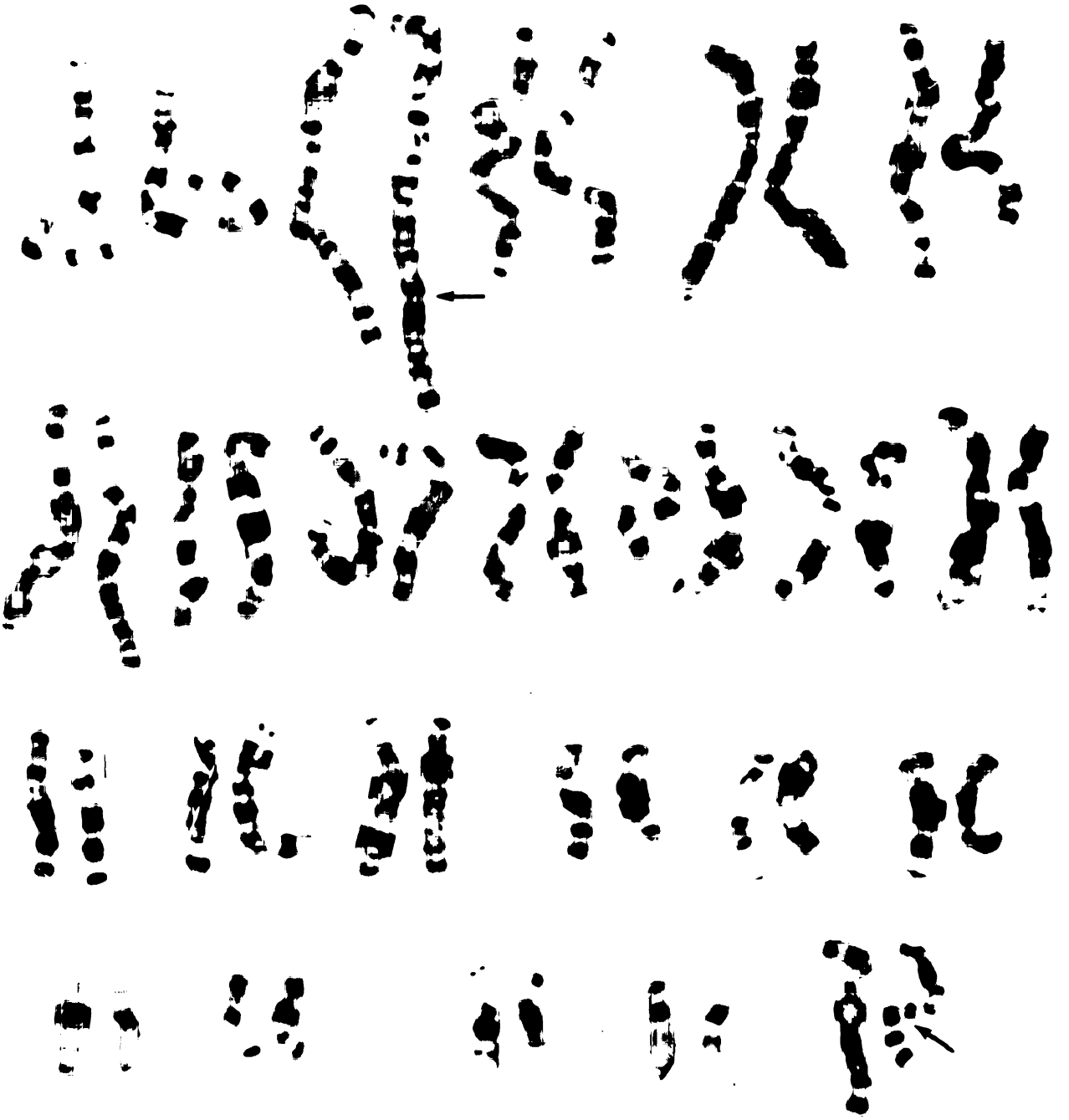


Figure 1 Blood karyotype of the patient C.G.
(Arrows indicates the breakpoints of the chromosomes 2 and X)

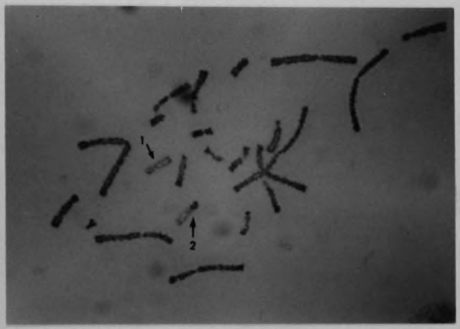


Figure 2 Chromosomes stained by G-11 technique
(Arrow 1 indicates an entire human chromosome. Arrow 2 marks a translocated chromosome. The part to the left of the arrow is of human origin and the part to the right of the arrow is of mouse origin)

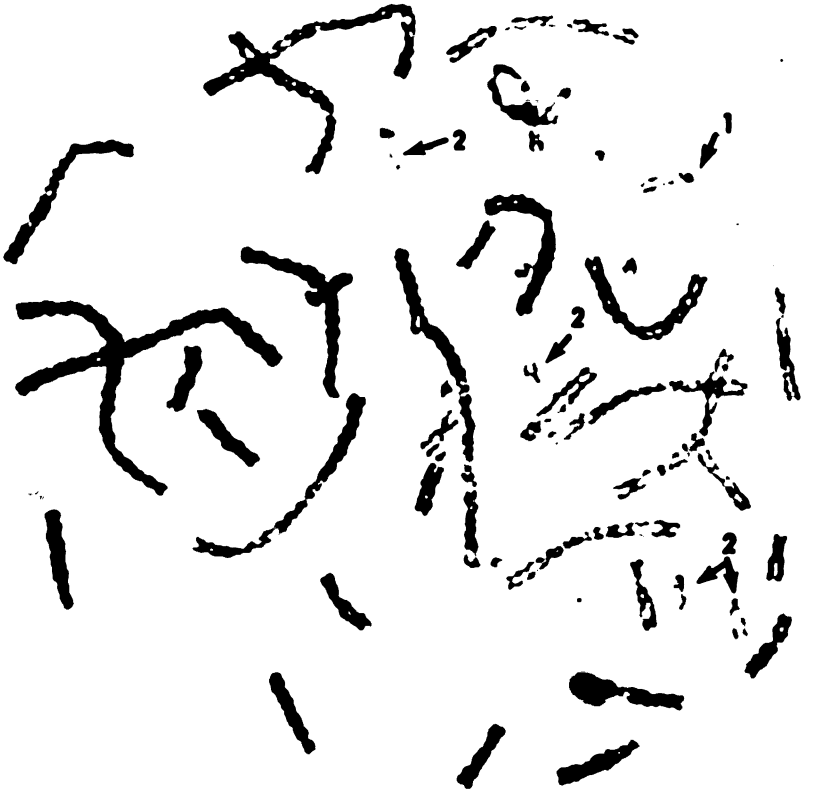


Figure 3a G-11 stained metaphase showing human chromosome composition in MHCG 1A
(Arrow 1 marks the rearranged mouse/human derivative 2. The portion to the right of the arrow represents part of the translocated human der 2. Arrows 2 indicate other human chromosomes)

Figure 3b Giemsa-banded metaphase showing human chromosome composition in MHCG 1A
(Arrow 1 indicates the rearranged mouse/human Derivative 2; Arrows 2 show the human chromosomes 14. Arrows 3 mark the human chromosomes 22)

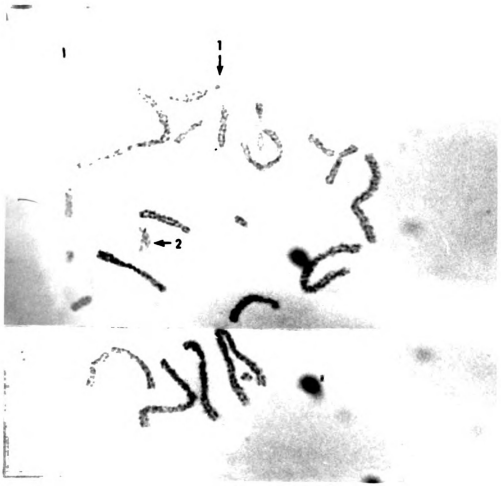


Figure 4a G-11 stained metaphase showing human chromosome composition in MHCG 9
(Arrow 1 indicates the rearranged mouse/human Derivative 2. The portion to the right of the arrow is part of the derivative 2. Arrow 2 indicates the human chromosome 14)

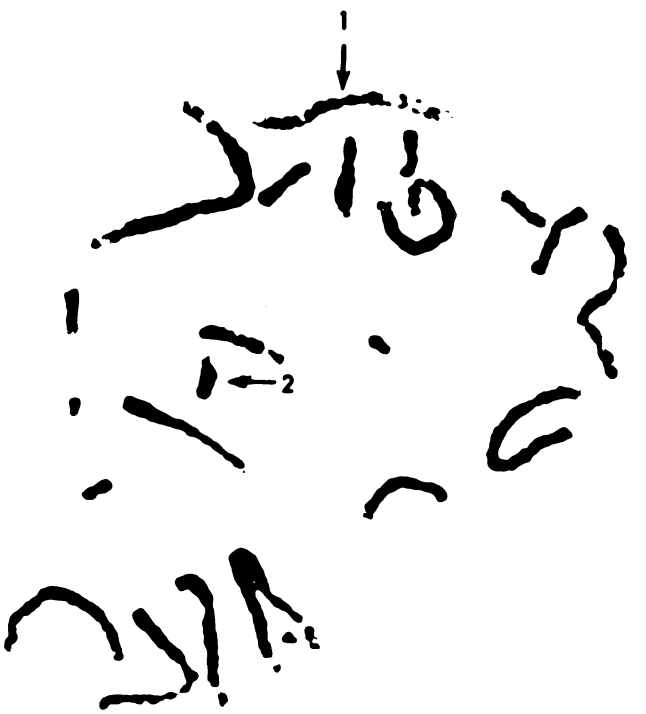


Figure 4b Giemsa-banded metaphase showing human chromosome composition in MHCG 9
(Arrow 1 marks the translocated mouse/human chromosome. The derivative 2 is the part to the right of the arrow. Arrow 2 indicates the human chromosome 14)

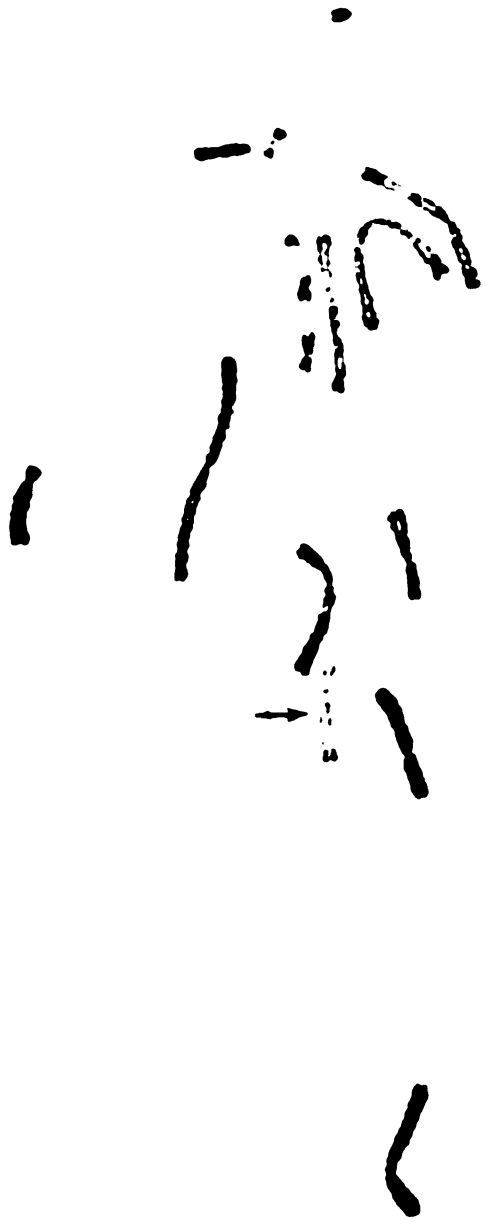


Figure 5a G-11 stained metaphase showing the intact human derivative 2 present in MHCG 10
(Arrow indicates the human derivative 2)

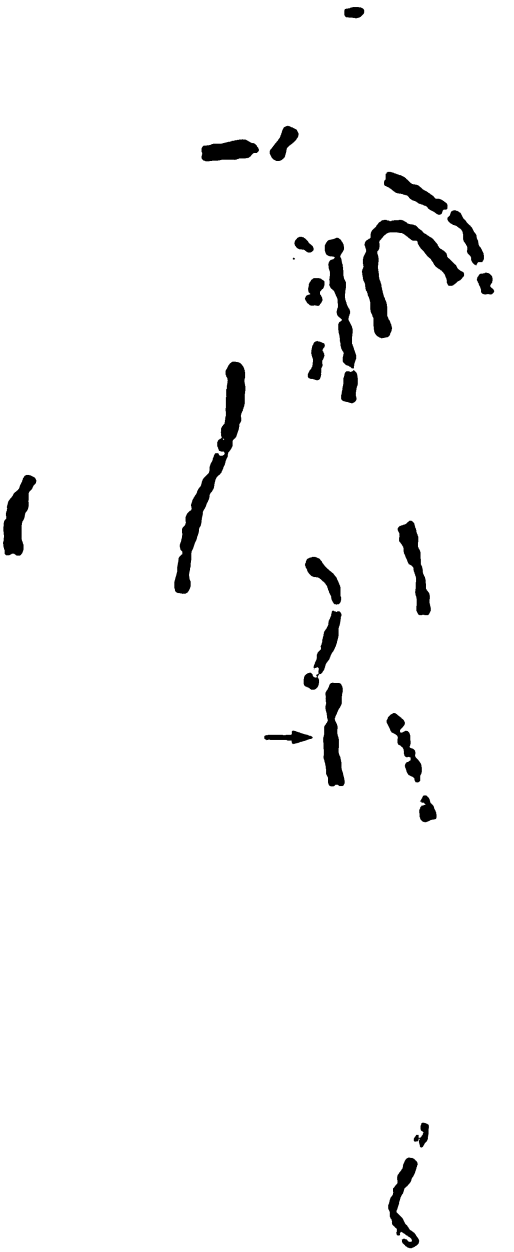


Figure 5b Giemsa-banded metaphase showing the intact human der 2 present in MHCG 10
(Arrow indicates the human derivative 2)

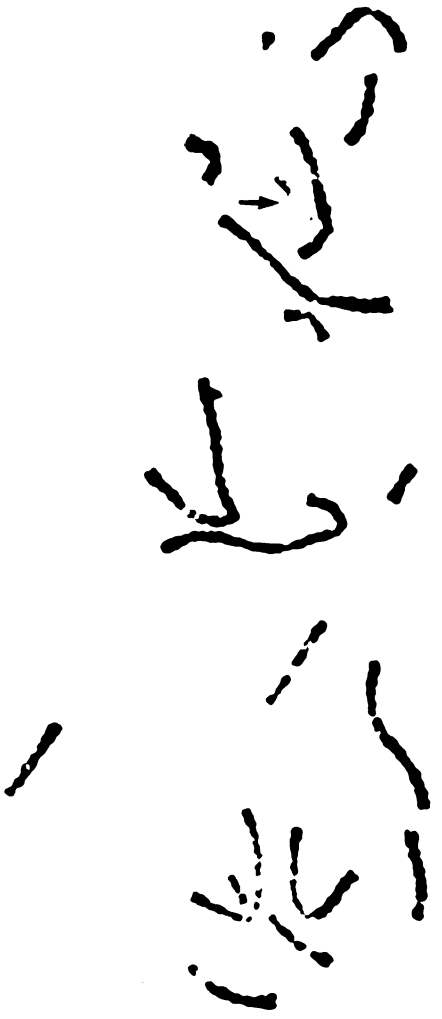


Figure 6a G-11 stained metaphase without the derivative 2 from MHCG 10 (Arrow indicates the human chromosome 8)

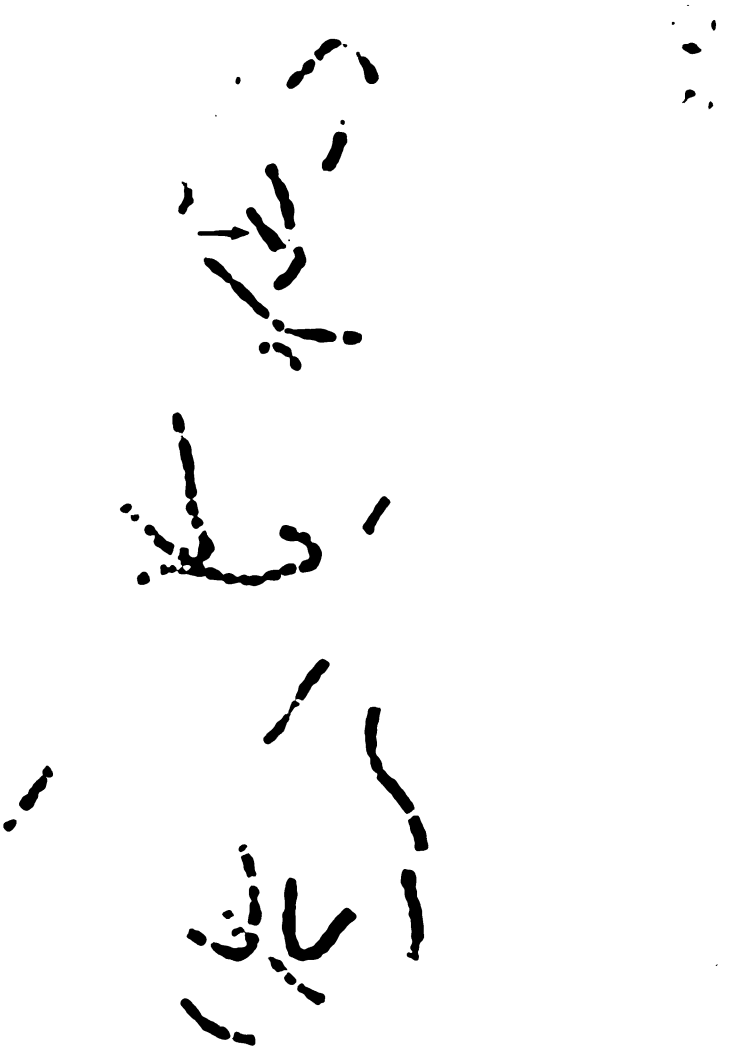


Figure 6b Giemsa-Banded Metaphase without the derivative 2 from MHCG 10 (Arrow indicates the human chromosome 8)

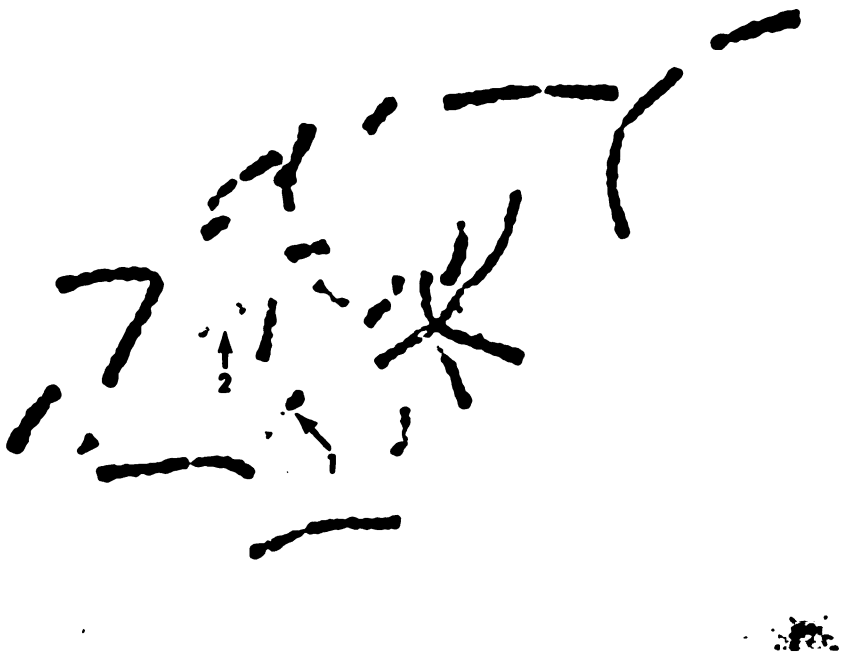


Figure 7a G-11 stained metaphase showing the translocated der 2 in MHCG 10
(Arrow 1 indicates the translocated mouse/human derivative 2. The light portion to the left of the arrow is of human origin. The dark portion to the right of the arrow is of mouse origin. Arrow 2 marks the human chromosome 8)

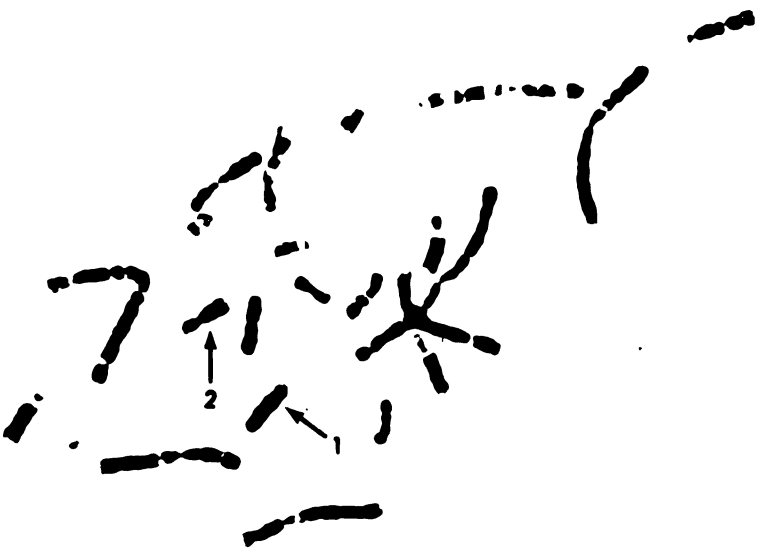


Figure 7b Giemsa banded metaphase showing the translocated der 2 in MHCG 10
(Arrow 1 indicates the translocated mouse/human der 2. The portion to the left of the arrow is of human origin. The portion to the right of the arrow is from mouse. Arrow 2 indicates the human chromosome 8)

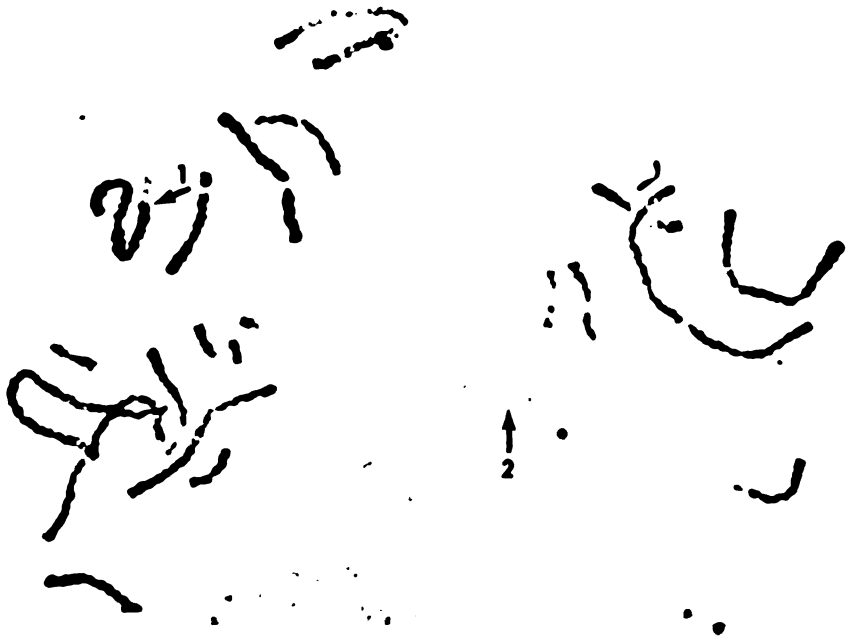


Figure 8a G-11 stained metaphase showing the translocated Xq in MHCG 10
(Arrow 1 indicates the translocated mouse/human X. The light portion above the arrow is of human origin and the dark portion below the arrow is of mouse origin. Arrow 2 marks the human chromosome 8)

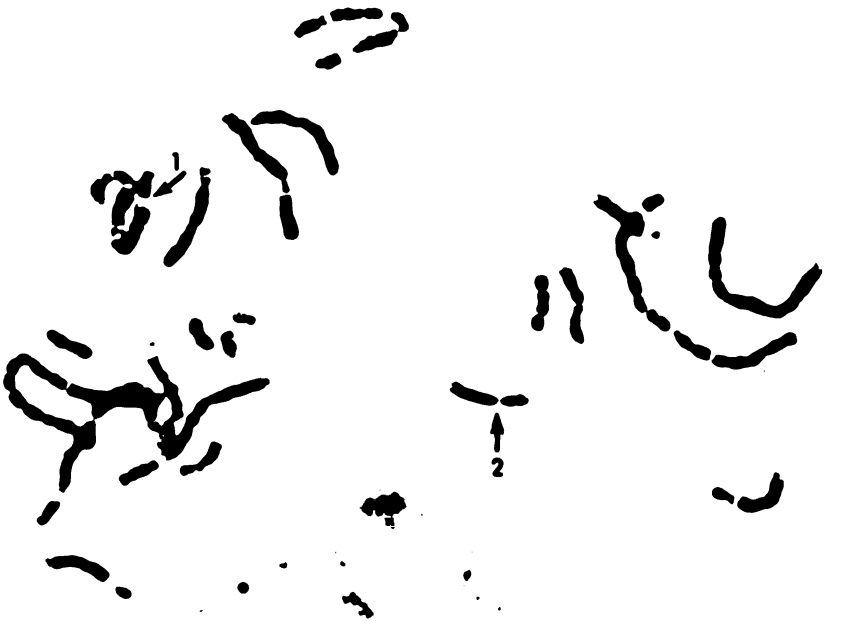


Figure 8b Giemsa banded metaphase carrying the translocated Xq in MHCG 10
(Arrow 1 marks the rearranged mouse chromosome/human X. The part above the arrow is from the human chromosome X. The remaining part is of mouse origin. Arrow 2 indicates the human chromosome 8)

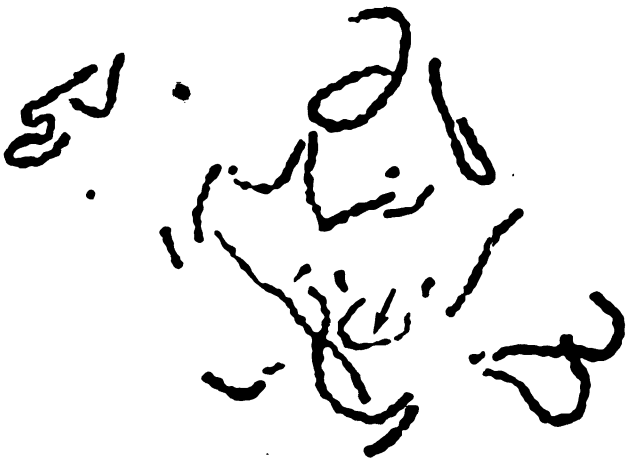


Figure 9a G-11 stained metaphase showing human chromosome composition in MHCG 11
(Arrow marks the translocated mouse/human derivative 2. The chromosome portion to the right of the arrow is of human origin and the portion to the left of the arrow is of mouse origin)

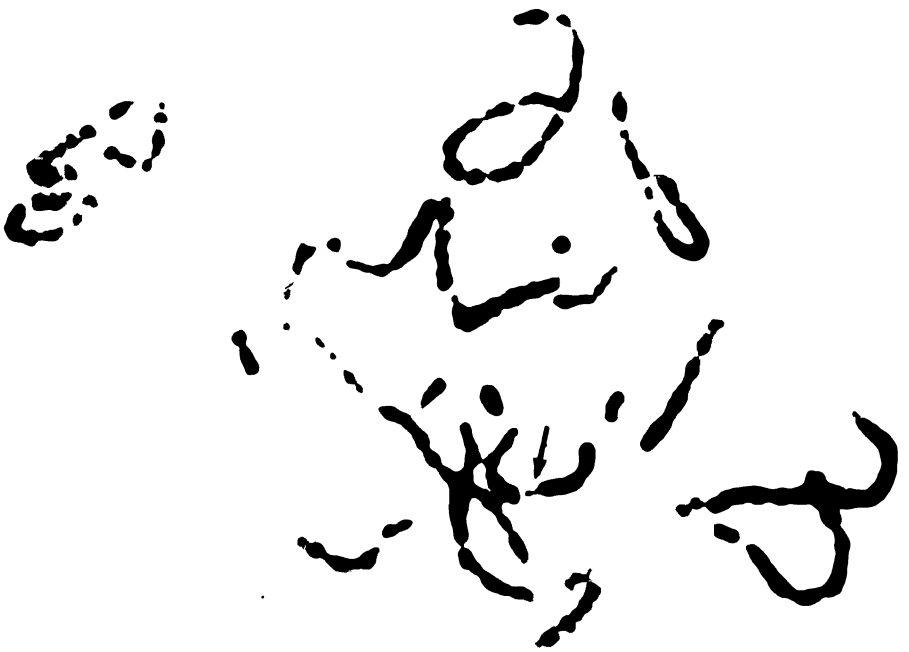
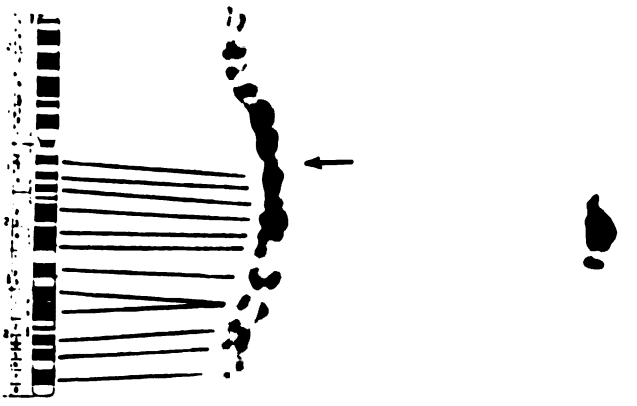


Figure 9b Giemsa banded metaphase showing human chromosome composition in MHCG 11
(Arrow marks the rearranged mouse/human derivative 2. The part of the chromosome to the right of the arrow is of human origin and the portion to the left of the arrow is of mouse origin)

Figure 10 Karyotype of MHCG 1A
(The translocated derivative 2 and the human chromosomes 14 and 22 are shown at the top of the karyotype. The mouse chromosomes are at the bottom and arranged in order of the length. Arrow marks the junction point between the mouse and human chromosomes)



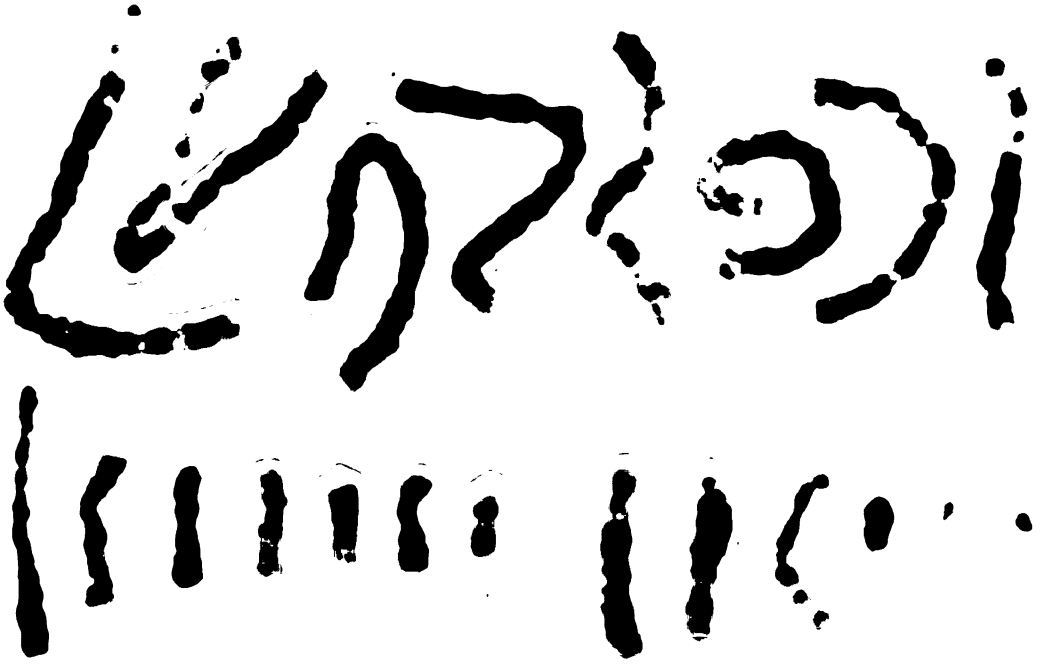


Figure 11 Karyotype of MHCG 9 showing the translocated derivative 2
(The chromosomes below the double-line are of mouse-origin. The human chromosomes are above the double-line. Arrow indicates the junction point between the mouse and the human derivative 2. The part below the arrow is of human origin and the part above the arrow is from the mouse)

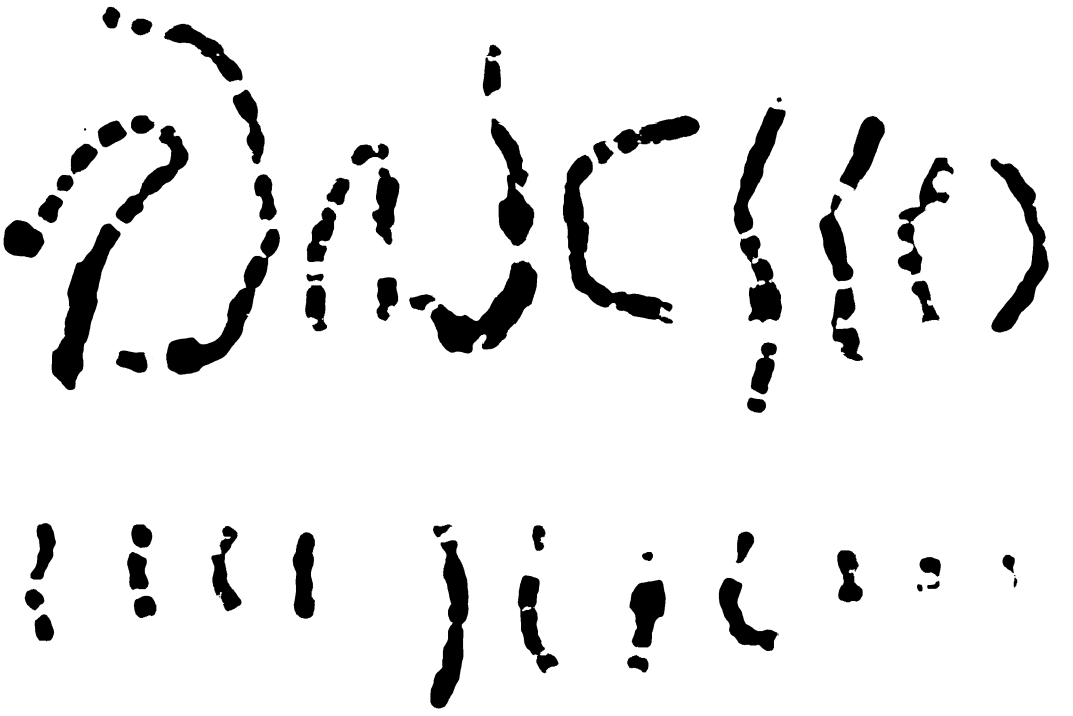
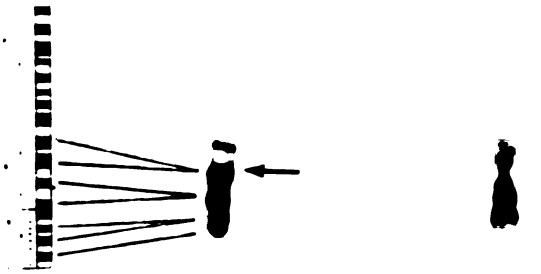


Figure 12 Karyotype of MHCG 10 showing the rearranged human Xq
(The human chromosomes are at the top of the karyotype. Arrow indicates the junction point between the mouse and human chromosome. The chromosome portion below the arrow is from the human X)



der 2 t(der 2) 8

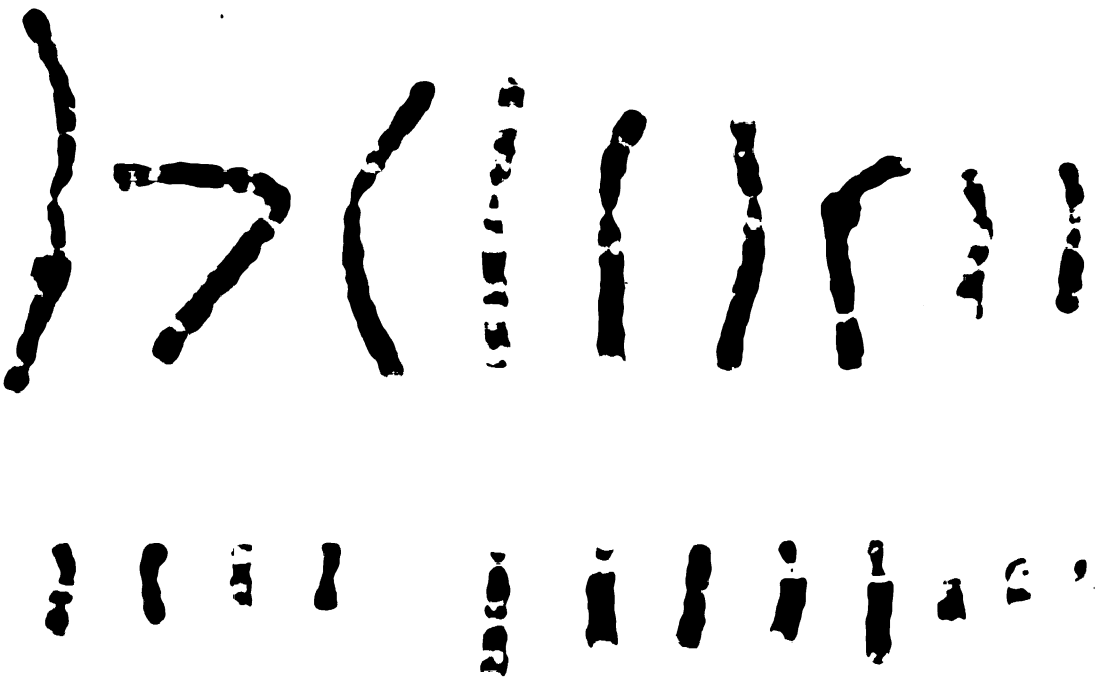


Figure 13 Karyotype of MHCG 10 carrying the translocated human derivative 2
(The derivative 2 is showed as the portion of the chromosome below the arrow. The mouse chromosomes are under the double-line)

der 2 t(der 2)

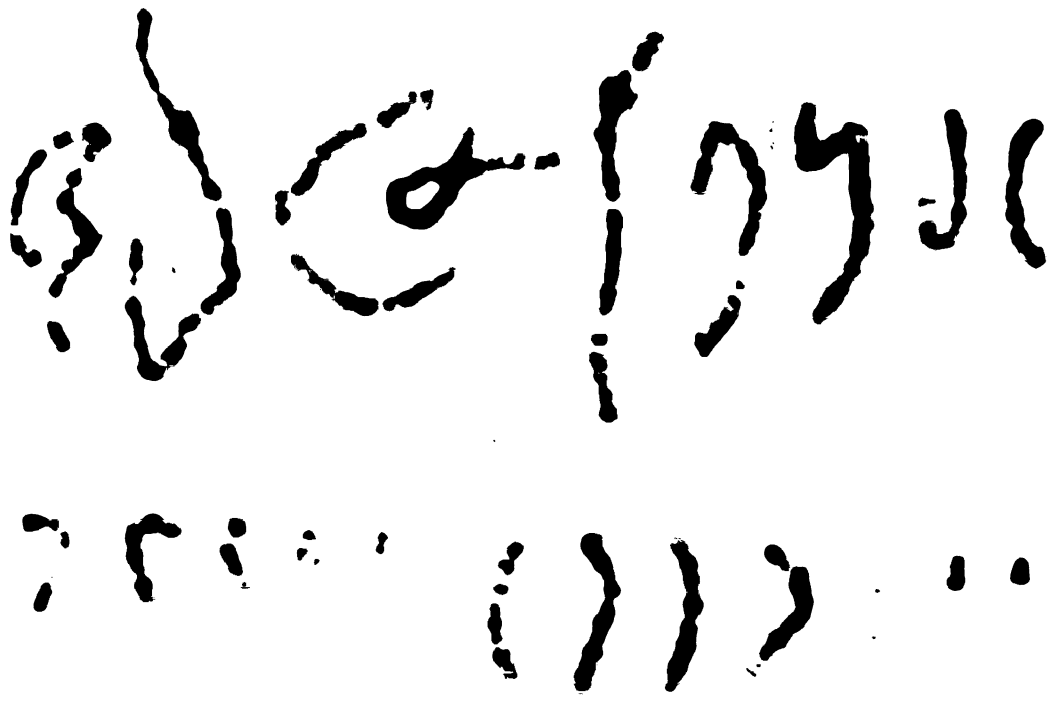


Figure 14 Karyotype of MHCG 11 showing the translocated derivative 2
(The human chromosome is at the top of the karyotype. The portion below the arrow is part of the human der 2. The mouse chromosomes are under the double-line)

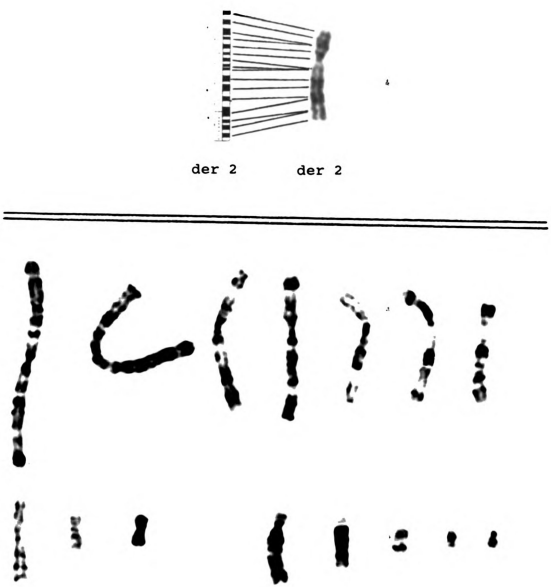


Figure 15 Karyotype of MHCG 10 carrying the intact derivative 2
(The human chromosome is above the double-line and the mouse chromosomes are below the line)

LIST OF REFERENCES

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