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MICRODISSECTION AND MOLECULAR CLONING OF EXTRA SMALL RING CHROMOSOMES OF HUMAN

by

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Thesis submitted for the degree of

Doctor of Philosophy

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Australia

January, 1998

Errata for Thesis of Yu-Yan Fang

Page 6, line 5-6
delete "and they are chromosome."

Page 19, Paragraph 2
replace "0.65-1.5‰ with "0.065-0.15%"
replace "0.1-0.72‰ with "0.01-0.072%"
replace "6‰ with 0.6%"

Page 55, Paragraph 2, line 6
replace "since they also mapped to CY120" with the phrase "since they mapped to CY170 but not CY120"

Page 55, Paragraph 2, line 10
replace "The other three clones (Y42, Y73 and Y87) were negative for CY120 ..." with "The other three clones were positive for CY120 and CY170 (Fig. 3-4)..."

Page 56, line 6-7
replace "cosmid 176F1" with "cosmid 177C6"

Page 56, line 10
replace "cosmid 177C6" with "cosmid 176F1"

Page 57,Table 3-3
In this table replace the cosmids labelled 177C6 and 176F1 with 176F1 and 177C6 respectively.

Fig. 3-6
At 4pter, replace "177C6" with "176F1"
At 4q12, replace "176F1" with "177C6"

Page 65, line 2
replace "His" with "Her"

Fig. 4-10
replace "devision" with "division"

Page 73, line 7
replace "since FISH study showed ..." with "since FISH study with the probe in the region of 15cen→q11clearly showed defined euchromatic region between the two FISH signals, and on the basis of the abnormal phenotype in this patient it is also suggested that the PWS/ AS region was involved in this marker."

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SUMMARY

The changes of the dosage of normal genes, either by addition or deletion, can result in phenotypic abnormality. Constitutional small ring chromosomes accessory to the normal diploid karyotype change the dosage of genes by partial trisomy and such rings have been found in individuals with abnormal development. These small ring chromosomes have been relatively poorly characterized. From most reported cases, the ring chromosomes were identified by fluorescence *in situ* hybridization (FISH) with specific-centromere probes. However, the exact origin of the euchromatin of these ring chromosomes cannot be determined by this procedure.

To precisely identify their origins and genetic content and therefore provide a better groundwork for genetic counselling, extra small ring chromosomes from nine normal or abnormal carriers have been characterized by a combination of microdissection, FISH and molecular cloning. With microdissection and reverse hybridization, the origin of six ring chromosomes have been identified as from chromosome 1 (one case), chromosome 4 (two cases), chromosome 8 (two cases), and chromosome 15 (one case). All these ring chromosomes included the centromeric region of their original chromosomes and in addition, the ring chromosome 1 contained the heterochromatic region of chromosome 1q12; one ring chromosome 8 contained 8p11 and ring chromosome 15 contained 15q11.

A ring chromosome 4 was found to contain the centromere and portion of band 4q31 by reverse hybridization. Further investigation with molecular cloning revealed that this ring also contained the 4p13-14 region. Using isolated cosmid DNA, a third ring chromosome 4 was identified which contained a segment from the centromere to band 4q12. Using the same method, a ring chromosome 15 was further confirmed to contain the euchromtin from the most proximal area of long arm of chromosome 15 (15q11). Microdissected DNA from this ring chromosome 15 was used for FISH to characterize two inv dup(15)s. One inv dup(15) was composed entirely of alphoid

centromeric heterochromatin and the other one probably included inverted duplication of Prader-Willi syndrome (PWS)/Angelman syndrome (AS) region (15q11→13). Two other ring chromosomes, a ring chromosome 1 and a ring chromosome 20, were also studied by microdissection and cloning of the products. However, this study was unsuccessful as the initial microdissected products were contaminated with chromosome 6 and chromosome 5, respectively.

The comparison of these results demonstrated that molecular cloning is an effective method in the characterization of extra small ring chromosomes. With this method, one small ring 4 was found to originate from three discontinuous regions of chromosome 4 and this allowed us to hypothesize a new mechanism of ring chromosome formation, i.e. ring chromosome may be formed from an initial large ring by subsequent interlocking, breakage and fusion occurring during cell division to generate a smaller ring.

Another type of gene dosage change is chromosome deletion which forms a partial monosomy and result in abnormality. Wolf-Hirschhorn Syndrome (WHS) is caused by a deletion of the band 4p16.3 and patients with this deletion have growth and mental retardation, characteristic facies and seizures. In the present study, a subtle interstitial deletion of 4p16.3 in a patient has been characterized with FISH. This small interstitial deletion of 4p16.3 contributed to the refinement of the critical deletion region of WHS.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

March 26, 1998

Yu-yan Fang   Date

ACKNOWLEDGMENTS

This project was carried out in the Department of Cytogenetics and Molecular Genetics at the Women's and Children's Hospital, North Adelaide, South Australia, from September 1994 to December 1997. I gratefully acknowledge the financial support provided by Women's and Children's Hospital Scholarship.

I would like to express my sincere gratitude to my supervisors, Associate Professor David Callen and Professor Grant Sutherland, for offering me the opportunity to study in their department, for their enthusiastic and inspiring supervision throughout this work and for their help and encouragement in many aspects.

I am very grateful to Drs. Sui Yu and Jozef Gecz for providing valuable ideas, encouragement and methodological details and Drs. Jozef Gecz and Oliva Handt for critical reading of this thesis.

I would like to thank Scott Whitmore and Sinoula Apostalou for technical assistance; Helen Eyre and Sharon Bain for carrying out part of the FISH work; Liz Baker for scanning some FISH scanning images.

I would like to thank the members of "routine lab" for cell culture, chromosome preparation and karyotyping. They are Anothony Correll, Jeckie Beerworth, Wendy Barratt, Fiona Frickleton and Trudy Hocking.

Thanks must go to "research groups" and my fellow postgraduate students for providing technical help, "trouble-shooting" and a delightful atmosphere for this study. They are Ingrid Lensink, Sharon Lane, Joanna Crawford, Kathy Friend, Marie Mangelsdorf, Annette Orsborn, Shirley Richardson, Andrew Donnelly, Jean Spence, Agi Gedeon, Hilary Phillips, Robyn Wallace, Lynne Northcott and Julie Nancarrow.

My sincere thanks also extend to the various collaborators who have made this project viable: Drs. Stefan Bohlander, Xin-Yuan Guan, Ronald Hill and Andreas Houben for their performance of microdissection on the ring chromosomes; Dr. Olaf

Riess for screening cosmid library and providing cosmids for ring chromosome 4 and Wolf-Hirschhorn Syndrome studies.

Finally, I would like to thank Zan-Min, my husband, for his unwavering support, assistance in computer technique, figure editing and critical reading of my paper works throughout my study. Thank you for always being there. Many thanks to my loving parents, my maternal uncle and my two sisters, without whose and Zan-Min's encourage and support this thesis would never have been completed. My thanks must also go to my baby, Allen, who brings so much enjoyment and flavour to my life. All my love to all of you.

PUBLICATIONS

Full research papers:

Fang Y-Y, Eyre HJ, Bohlander SK, Estop A, McPherson E, Träger T, Riess O, Callen DF (1995) Mechanisms of small ring formation suggested by the molecular characterization of 2 small accessory ring chromosomes derived from chromosome 4. Am J Hum Genet 57:1137-1142.

Fang Y-Y, Bain S, Haan EA, Eyre HJ, MacDonald M, Wright TJ, Altherr MR, Riess O, Sutherland G, Callen DF (1997) High resolution characterization of an interstitial deletion of less than 1.9 Mb at p16.3 associated with Wolf-Hirschhorn Syndrome. Am J Med Genet 71:453-457.

Fang Y-Y, Guan X-Y, Houben A, Eyre HJ, Trent JM, Callen DF (1998) Detection of extra small ring chromosome 1, 4 and 8s by microdissection and FISH (in preparation).

Fang Y-Y, Guan X-Y, Eyre HJ, Trent JM, Callen DF (1998) Characterization of a ring chromosome 15 and two inv dup(15) with microdissection, microcloning and FISH (in preparation).

Published abstracts and conference presentations:

Fang Y-Y, Bohlander SK, Riess O, Callen DF (1995) Determination of genetic content of a marker chromosome 4 by molecular cloning. Proceedings of the 17th annual conference on the organisation and expression of the genome Genome, Lorne, Victoria, Australia.

Fang Y-Y, Bain S, Haan EA, Sutherland G, Callen DF (1996) High resolution characterization of 4p16.3 deletion resulting in Wolf-Hirschhorn Syndrome. Proceedings of the 4th international chromosome 4 workshop in Bochum, Germany. Cytogenet Cell Genet 74:57-69.

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ABBREVIATIONS

AMCA	amino methyl coumarin acetic acid
AS	Angelman syndrome
BSA	bovine serum albumin
CMT	Charcot-Marie-Tooth disease
DAPI	diamidino phenylindole dihydrochloride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOP-PCR	degenerate oligonucleotide-primed PCR
EDTA	ethylenediaminetetra
ESAC	extra structurally abnormal chromosome
FGFR	fibroblast growth factor receptor
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
IDUA	a-L-Iduronidase
inv dup(15)	inverted duplication chromosome 15
ISCN	International System for Human Cytogenetic
mar	marker
MYL	myosin light chain
NOR	nucleolus organizer region
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDEb	b-subunit of rod cGMP phosphodiesterase
PEG	polyethylene glycol
PHA	phytohemagglutinin
PI	propidium iodide

PRD	Pitt-Roger-Dank syndrome
PRINs	PRimed in situ labelling
PVP	polyvinylpyrrolidone
PWS	Prader-Willi syndrome
SDS	sodium dodecyl sulphate
STS	sequence-tagged site
TBE	Tris-base, Boric acid and EDTA
TE	Tris-HCl and EDTA
TRITC	tetramethyl rhodamine isothiocyanate
UPD	uniparental disomy
WHS	Wolf-Hirschhorn syndrome
YAC	yeast artificial chromosome
ZNF	zinc-finger gene

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Historical aspects of human genetics*

Interest in heredity is as old as mankind. Described in "Genesis", human being is created by God. I was taught by my great grandfather, human being mainly consistents of five elements: gold, wood, water, fire, and earth. If this is true, then what is the magic power making the distinction between one species of living things from the others? How does one individual human being have a unique appearance? Why do some people suffer from physical or mental aberrations while others appear normal? One of the main secrets can be summarized in one word— **Genetics,** which was coined by the English zoologist William Bateson in 1906.

As early as 500 to 300 B.C., Pythagoras theorized that human life originated from a blend of male and female fluids (Wynbrandt and Ludman 1991). In Aristotle's opinion, males were primarily responsible for passing on hereditary characteristics. Hippocrates, the great founder of medicine, pointed out that the semen is produced by the whole body, healthy by healthy parts, sick by sick parts. This parallels a Chinese idiom: "dragon begets dragon, phoenix begets phoenix, and the kids of the hamster can dig a hole". The basic pattern that males provide the seed and females provide the field in which the seed is planted and grown was not found until the first centuries by establishment of the first agricultural model of heredity at Institutes of Manu in India. Pedigrees, in which traits are transmitted from generation to generation, were recognized in 1768 when De Maupertuis observed that

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* Much of the pre-twentieth century historical literature has been cited from several more recent authors. In most cases I wasn't able to obtain the original publications, and have therefore listed the original article and where it was cited from, in "Citations in Introduction" at the end of this chapter.

polydactylly exhibited in several generations of a family. By crossing two species of tobacco plants, Joseph Gottlieb Kölreuter created the first hybrid plants in 1760, and surmised that each parent contributed an equal hereditary element to their offspring.

It is not exaggerating to state that the 1800s was a century of scientific ferment. In 1814, a remarkable masterpiece "A treatise on the supposed hereditary property of disease," was published by Joseph Adams. In this book the recessive and dominant conditions were distinguished, hereditary predisposition in some disorders and the role of environmental influence in their development were mentioned, and the theory that higher rates of familial diseases discovered in isolated populations could be due to inbreeding were proposed. Even the concept of new mutations was invoked. When Charles Darwin's "The origin of species on the basis of natural selection" was published in 1859, it catalysed a shift of interest from studying different species to looking at variations within a given species. The question of why various members of a species were not all alike was raised. Gregor Johann Mendel's (1822-1884) famous experiments with garden peas, and his elegant interpretation of the transmission of characteristics to their offsprings by statistical analysis of their subsequently appearing traits, marked the dawn of classical genetics. To compare the influence of heredity and environment, Francis Galton (1876) examined identical twins as a model referred to as "nature versus nurture."

Another line of study of genetics arose from the study of cytology. As early as 1665, Robert Hooke had named the "cell" by his observation of tissue structures under a primitive microscope, giving birth to cytology. With the development of the microscope, studies of the cell made great progress during 1800-1850. Subsequently Louis Pasteur published his "germ theory of disease" in 1865 and described the concepts of inoculation, pasteurisation and fermentation, the latter led to the discovery of enzymes (Garrison 1929). Distinction between organic and inorganic compounds had been established in 1810 and the compounds were recreated in laboratories,

which led to another discovery—synthetic dyes in the mid-1850s. The use of synthetic dyes has been and is still invaluable in studies of the various cellular structures, including chromosomes. Using synthetic dyes, Robert Brown (1833), a Scottish botanist, identified the "nucleus", located at a central area of the cell and revealed that cells contain carbohydrates, lipids and proteins. In 1871, Friedrich Miescher, a Swiss chemist, reported extracting a new substance named "nuclein" from the nucleus of the cell. Since nuclein was found to have the characteristics of an acid this later became termed "nucleic acid". Subsequently tiny thread-like bodies that readily absorbed dyes were revealed within the nucleus and termed chromosomes (Waldeyer 1888). Walther Flemming published drawings of these structures based on his observations in 1879, showing them in a cycle of splitting and replication during cell division. This process was dubbed "mitosis", originated from the Greek "formation of threads". Pondering the doubling of genetic material that accompanied mitosis, German physician August Weismann (1834-1914) surmised that there must be a mechanism for reducing the genetic material in sex cells — so called “meiosis", which was confirmed by Eduard Van Beneden in 1883.

Once the ideas of the chromosome, mitosis and meiosis were established, scientists focused their interests on the relationship between chromosomes and heredity. Sutton (1903) and Theodor Boveri (1903) of Germany independently proposed that chromosomes were the carriers of genetic information and that they occurred in pairs, one inherited from the father and the other from the mother. American zoologist and geneticist Thomas Hunt Morgan published his monumental work on the constitution of the chromosomes and their relationship with heredity in 1911, formally ushering in the era of the "chromosome theory" of heredity. In 1909, Wilhelm Ludwig Johannsen, a pharmacist's apprentice from Copenhagen, defined a "gene" as a counting or calculating unit of heredity and introduced "genotype" to describe an individual's genetic make-up and "phenotype" to describe an individual's physical appearance, which may or may not reflect his or her genotype. Recognition

of cell, chromosome, genotype and phenotype was the groundwork for the advent of genetics.

1.2 Development of cytogenetics and molecular cytogenetics

Since 1923 it had been considered that humans have 48 chromosomes. The development of hypotonic treatment techniques for the preparation of human chromosomes by Hsu in 1952 and the establishment of the correct diploid number of 46 chromosomes for humans by Tjio and Levan (1956) marked the beginning of cytogenetics. Improved methods of chromosome preparation made chromosomal analysis easier. Phytohemagglutinin (PHA) was used to stimulate the division of lymphocytes from peripheral blood and the use of colchicine allowed the dividing cells in lymphocyte culture to be halted at various stages of mitosis. With the growing abilities in chromosome visualisation, it became possible to photograph human chromosomes and the individual chromosome can be cut and arranged into pairs according to their sizes and the positions of their centromeres. This karyotyping is a standard method of arranging human chromosomes, it was adopted at a meeting of cytogeneticists in Denver (Denver Conference, 1960).

1.2.1 Chromosomal structure and classification

Morphologically, the basic structure of a chromosome consists of telomeres, nucleolar organizing regions (Norse), short arm, centromere, and long arm (Fig. 1-1). Each chromosome contains two broad types of DNA sequence: euchromatin and heterochromatin. Euchromatin is a functional description of the chromosome containing genes and is less condensed than heterochromatin. Heterochromatin is enriched with repeated sequences, is highly condensed, and tends to be transcriptionally inactive and replicate late in S-phase. Telomeres, centromeres, and NORs contain mainly repeat DNA. There are multiple copies of ribosomal RNA genes found in NORs. Telomere DNA sequences are essential components in the

control of chromosome integrity and prevent shortening of the chromosome at each round of cell division. The primary structure of the telomere is a conserved G/C -rich repeat unit of the general formula $(T/A_{1-4}dG_{1-8})$ (Blackburn 1991) forming unusual folded structures in solutions. The centromere is permanently contracted due to the a lack of coiling of the 250-nm chromatin fiber (Rattner 1991) and consists of various kinds of repetitive DNA and kinetochore proteins which control chromosome separation during cell division (Skibbens et al. 1993). The predominant satellite family in centromeres is alpha satellite DNA, characterised by multiple copies of a highly diverse basic repeat unit of approximately 171bp (Willard 1990). Tandem repeats of this alpha satellite DNA are further organised into macro repeat units ranging from 0.5 to 10 Mb in length. Other families of human satellite DNA, including classical satellite DNA (Moyzis et al. 1987) and beta satellite DNA (Waye and Willard 1989), appear to be localized to the pericentromeric region. However, the exact nature of the structure of these repetitive DNAs is unknown.

A somatic cell from a normal human contains 23 pairs of chromosomes: 22 pairs of autosomal chromosomes, which are the same in male and female and one pair of sex chromosomes, XX in a female and XY in a male. Using cytogenetic nomenclature, the letter "n" represents the haploid gametic number, i.e. the number of chromosomes in an egg or a sperm and "2n" indicates the number of chromosomes in a zygotic cell, in which there are two sets of homologous chromosomes: one maternal, the other paternal. Therefore, the karyotype is 2n=46,XY for males and 2n=46,XX for females (Paris Conference 1972). Chromosomes are arranged and numbered according to their size, becoming shorter with increasing chromosomal number, such that chromosome 1 is the longest while chromosome 21 is the shortest. Chromosomes are classified into seven groups from A to G group according to their length (Table 1-1).

Table 1-1 Classification of human chromosomes according to their length (adapted from (Hamerton 1971)

Group	A	B	C	D	E	F	G
Chromosome	1, 2, 3	4, 5	6, 7, 8, 9, 10 11, 12, X	13, 14, 15	16, 17, 18	19, 20	21, 22, Y

Chromosomes can also be classified as metacentric, submetacentric, and acrocentric according to the positions of the centromere and the relative length of the chromosome arms (see Vogel and Motulsky 1996). Metacentric chromosomes (1, 3, 16, 19, 20) have the centromere located centrally, acrocentric chromosomes (13, 14, 15, 21, 22 and Y) have the centromere closed to the end of the short arm and they are chromosome . All the remaining chromosomes (2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18 and X) are submetacentric chromosomes.

1.2.2 Banding

When chromosomes are stained with some nonfluorescent or fluorescent dyes, the whole lengths are not uniformly stained but some deep stained bands are interspersed with nonstained bands. This is called chromosome banding. Each chromosome in the human somatic cell complement can be uniquely identified following a number of different banding procedures. The common bandings include G-banding (Sumner et al. 1971), C-banding (Sumner et al. 1971, Salamanca and Armendares 1974), Ag-NOR staining (Goodpasture and Bloom 1975), and fluorescent banding with Distamycin A-DAPI (Schweizer et al. 1978).

G-banding: It produces a characteristic light and dark banding pattern along the chromosomes by a pretreatment with enzymes such as trypsin and subsequent staining with Giemsa or Leishman (Sumner et al. 1971, Drouin et al. 1991a, b, Holmquist et al. 1982). As a tool in the routine analysis of human chromosomes, it

allows the identification of individual chromosomes and the recognition of structural abnormalities in the case of disrupted banding pattern.

C-banding: Staining with Giemsa following heat and /or alkali denaturation results in dark staining of the heterochromatic regions at the centromeres and the pericentromeric region of all chromosomes with lightly staining chromosome arms. An exception is the Y chromosome which has an additional distinctive block of heterochromatin towards the end of the long arm. This banding method has been used to define constitutive heterochromatin at the centromeric regions of all human chromosomes and pericentromeric heteromorphisms of chromosome 1, 3, 4, 9, 13-15, 16, 21-22 and the long arm of Y chromosome (De Braekeleer et al. 1986, Verma et al. 1988).

Fluorescent banding: Multiple fluorochromes have been used simultaneously on the same metaphase spread to enhance the banding pattern by increasing the apparent contrast. They include Actinomycin D combined with 4,6-diamidino-2-phenylindole (DAPI) staining and chromomycin A3 or distamycin A (DA) combined with DAPI staining. Using these banding methods, the centromeric heterochromatin regions of chromosomes 1, 9, 15, 16, and Yq can be distinguished. DA/DAPI staining particularly highlights the heterochromatin region on the proximal short arm of chromosome 15.

Ag-NOR: Ribosomal RNA associated with the nucleolus organizer regions (NOR) can be stained by silver. This method allows the identification of the satellite stalk region of the acrocentric chromosome, which contains multiple copies of 18s and 28s RNA sequences. It is likely that the silver technique stains an acid protein associated with the chromosomal satellite in the region of human D and G group rather than rRNA itself (Goodpasture and Bloom 1975, Howell and Black 1978).

These staining techniques have been used in each case of this study and some results will be shown in Chapters 5, 6 and 7.

1.2.3 Molecular cytogenetics

A further development in chromosome identification methodology was *in situ* hybridization. It permitted the unequivocal visual identification of specific chromosomes and chromosomal segments through light microscopy (Pardue and Gall 1969, Jones 1970, John et al. 1969). The principle of this technique involves the labelling of a single-stranded DNA (probe) and annealing to its complementary sequence (target) from denatured nuclei or chromosomes. Detection of the probe allows identification of the site of hybridization and thus the region of chromosomal DNA complementary to the probe. The probes can be labelled by either radioactive isotopes (Gall and Pardue 1969, John et al. 1969, Buongiorno Nardelli and Amaldi 1970) or fluorophores (Rudkin and Stollar 1977, Bauman et al. 1980). Isotopic *in situ* hybridization with highly repetitive (satellite) DNA as a probe was first used to map the centromeric regions of mouse chromosomes (Pardue and Gall 1970). Moderately repetitive ribosomal RNA was mapped to the NORs in the polytene chromosomes of Diptera (Pardue and Gall 1970). This approach was soon used to study human chromosomes (Henderson et al. 1972, Jones and Corneo 1971).

The search for nonisotopic alternatives to label probes for *in situ* hybridization led to the introduction of biotin (Langer et al. 1981), acetylaminofluorene (Landegent et al. 1984), mercury (Bauman et al. 1983) and digoxigenin (Heiles et al. 1988, Seibl et al. 1990). Biotin and digoxigenin have been shown to be excellent and are used in most cases. Fluorescence *in situ* hybridization (FISH) was introduced in 1977 (Rudkin and Stollar 1977). Compared with isotope labelling, fluorescence labelling has several advantages: no exposure to radioactive isotopes for researchers, more precise localisation of the signal, longer storage of labelled probes. Furthermore a combination of several probes can be used for simultaneous multi-colour FISH (Dauwerse et al. 1992, Ried et al. 1992). The fluorochromes commonly used include fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), amino methyl coumarin acetic acid (AMCA), and Texas red. To detect small targets,

amplification of the signal can be achieved by the application of several layers of fluorochrome-conjugated antibodies.

Various DNA probes have been used to resolve particular cytogenetic problems in diagnosis and research, including chromosome-specific centromeric probes, chromosome-specific painting probes, and single-copy DNA sequence probes.

(1) Centromeric probes, either cloned from the alphoid-repetitive DNA of different human chromosomes (Vogt 1990, Waye and Willard 1987) or produced directly from somatic cell hybrids containing a single human chromosome (Koch et al. 1983), hybridize to the repetitive sequences of a specific centromere. These probes produce not only very strong but also relatively punctate signals in interphase nuclei. They are the probes of choice for the rapid diagnosis of aneuploidy. However, chromosomes 13/21 and 14/22, share indistinguishable sequences.

(2). Chromosome-specific painting probes are made from several sources including chromosome-specific genomic libraries (Cohen et al. 1993), single chromosome-interspecific somatic cell hybrids, flow-sorted chromosomes (Gray et al. 1975) and microdissected chromosomes (Meltzer et al. 1992). Chromosome painting covers the whole chromosome or large chromosomal segments, therefore it has advantages in the analysis of chromosome structural rearrangements such as translocations (Pinkel et al. 1988, Speleman et al. 1991, Speleman et al. 1992, Kohler et al. 1994).

1.3 Molecular biology

1.3.1 The building blocks of molecular biology

Genetic studies at a molecular level were reported from early this century. In 1928, an English microbiologist, Frederick Griffith (1928), isolated a material from the bacterium pneumococcus (type II) that could influence heredity. Mixing it with

another bacterium pneumococcus (type III) resulted in a change of the hereditary characteristics of the type III to type II. This was the first isolation of DNA and the first example of the "transformation" principle. In 1941, George Beadle and Edward L. Tatum demonstrated the one to one relationship between genes and proteins. DNA, as the vehicle of hereditary transmission, was established in 1944 by Oswald Avery. This was demonstrated by a study of characteristics transmitted from type III pneumococci to another type using DNA. At that time, the study of hereditary disease also reached a molecular level. Sickle cell disease is a common hereditary hematologic disorder known in humans. As early as 1910, Herrick had noted peculiarly shaped sickle cells in the peripheral blood of a black West Indian student. However, the molecular era for the study of sickle cell disease did only begin when Dr. Linus Pauling (Pauling 1949) found out that the sickle-cell behaved differently from normal red blood cells in an electrical field and that the molecule's electrical charges in these sickle-cells were altered. He proposed that hemoglobin S was abnormal and that this resulted in sickle-cell anemia. When Watson and Crick's (1953) ground-breaking publication about the "Molecular structure of nucleic acids. A structure for deoxyribose nucleic acid." and Wilkin's (1953) paper about the "Helical structure of crystalline deoxypentose nucleic acid." DNA structure, function and its replication were understood and this provided a framework for all subsequent research in genetics.

1.3.2 Molecular cloning

The discovery of restriction endonucleases by Smith and Wilcox (1970) made DNA manipulation possible. With restriction endonuclease digestion, DNA could be cut at defined sites and this allowed investigators to take specific fragments of DNA from one cell and "re-combine" it with DNA of another. This method is known as "molecular cloning" (Cohen et al. 1973) and it has been used in Chapter 3, 4 and 6.

1.3.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was introduced in 1985 (Saiki et al. 1985), has made possible to amplify a specific DNA fragment a million fold. This in vitro procedure includes three steps per single PCR cycle: separating double strand DNA by denaturation, annealing of two sequence specific oligonucleotides and DNA synthesis by extension of these primers. The initial studies that applied PCR (Mullis et al. 1986, Saiki et al. 1985) utilized the Klenow polymerase I from Escherichia coli to amplify specific targets from human genomic DNA. However, this required the addition of enzyme after the denaturation step of each cycle since the high temperatures necessary for strand separation inactivate this polymerase. This has been changed with the discovery of thermostable DNA polymerases, such as Taq DNA polymerase which was isolated from the thermophilic bacterium, *thermus aquaticus* (Brock and Freeze 1969). The isolation of such heat-stable DNA polymerases has allowed the automation of PCR, the amplification reaction can be carried out in a single tube.

PCR is widely used in research, including (1) Physical and genetic mapping and the sequencing in the Human Genome Project (Rose 1991) (2) Reconstruction of the evolutionary history of species (Vigilant et al. 1989); (3) Analysis of gene expression (Acha-Orbea et al. 1988); (4) Identification of mutations and new members of multi-gene families (Orita et al. 1989). In Chapters 3-6 of this thesis, microdissected DNA was amplified by PCR and insert DNA of clones was recovered by colony PCR. In addition to research studies, PCR is also applied in clinical diagnosis such as the detection of sickle cell anaemia and ß-thalassemia (Saiki et al. 1988) and in a forensic setting, PCR has allowed the genetic typing of biological evidence ("DNA fingerprinting") found at the crime scene by amplifying polymorphic sequences (Kasai et al. 1990).

1.3.4 Combination microdissection with microcloning and PCR

Microdissection:: This approach was developed by Edström (1964), its application allowed the generation of recombinant genomic DNA clones from microdissected *Drosophila melanogaster* polytene chromosome (Scalenghe et al. 1981). Genomic clones were also obtained using similar techniques from mice (Rohme et al. 1984, Brockdorff et al. 1987, Greenfield and Brown 1987, Weith et al. 1987) and humans (Bates et al. 1986, Kaiser et al. 1987, Martinsson et al. 1989).

Microdissection involves the following preparation of chromosomes and equipment. Chromosome preparation from cell culture to spread metaphase on a slide is essentially the same as treatment for conventional cytogenetic banding. However, for microdissection chromosomes are fixed with the shortest possible exposure to acetic acid since long treatment with acid causes depurination and nicking of DNA. Another important point is that metaphases are freshly spread and uniformly stained (or unstained) just before microdissection since "aged" chromosomes result in DNA degradation. The basic equipment includes an inverted microscope, micromanipulator, glass needles, needle holders and needle pullers. Before dissection, the needle is pulled to produce a tip diameter less than 0.5 μm (Hagag and Viola 1993) to avoid touching other chromosomes during microdissecting. After microdissection, chromosomal fragments are collected, treated with restriction enzymes and amplified by PCR. At this stage, microdissected products are ready for molecular cloning or for FISH studies.

Microdissection provides a great opportunity to directly analyze DNA from specific chromosome regions of interest. It has been applied to the following areas: 1) Gene mapping (Cotter et al. 1991); 2) Detection of the sites of chromosome rearrangements and deletions (Lengauer et al. 1991, Meltzer et al. 1992); 3) Recombinant DNA libraries for physical mapping (Davis et al. 1990, Yu et al. 1992); 4) Analysis of regulatory and coding regions of genes (Raju 1986). Microdissection,

combined with FISH and molecular cloning, has been used throughout the present study.

The quality of the DNA sequences of recombinant clones generated from microdissection and microcloning depends on chromosomal preparation, microdissection manipulation, and optimal PCR amplification. Usually the inserts in recombinants from microdissected DNA are smaller than 1 kb. Hagag and Viola (1993) have found that not all fragments can be recovered and only a small fraction (<5%) of the total DNA sequences in the microdissected region can be cloned. The limitation may be due to the following factors: (1) Depurination and nicking of DNA occurring during chromosome fixation (Tamm et al. 1953, Arrighi and Hsu 1965, Holmquist 1979); (2) Contamination by other chromosomes or adjacent chromosomal bands during the dissection process; (3) Some recombinant clones can be contaminants derived from other sources such as bacteria on slides and glassware.

Laser microdissection offers a greater accuracy than micromanipulator microdissection in obtaining extremely small fragments. Laser microdissection with a blue-green argon ion laser microbeam was first introduced for subcellular microsurgery by Berns et al. (1969, 1970) and a typical laser microbeam apparatus was described by Monajembashi et al. (1986), consisting of an excimer laser as a primary source of laser light. The principle of this method is that optically active biological material can break down or melt on irradiation with light at very high photon densities (Djabali et al. 1991, Hadano et al. 1991). Using this technique, libraries of clones have been generated including regions of the human fragile X site, the Huntington disease region on chromosome 4, and a chromosomal translocation between chromosome 1 and 7 (Djabali et al. 1991, Hadano et al. 1991, Lengauer et al. 1991).

Microcloning and PCR: Initially in the development of microdissection-microclone methods, microdissected product were directly cloned into λ phage vector

following treatment with restriction enzymes (Rohme et al. 1984, Scalenghe et al. 1981). However, PCR amplification of microdissected chromosomal DNA, with either universal primers (Wesley et al. 1990) or Alu sequence primers (Guan et al. 1992), has proved simpler and faster than direct cloning methods. Generally 50-200 copies of microdissected fragments are required for λ phage cloning (Hagag and Viola 1993) while only 5-20 copies of each target region are needed for PCR amplification (Guan et al. 1995). Furthermore, time-consuming steps in direct cloning such as restriction enzyme digestion, ligation, and subcloning can be omitted. Microdissected DNA directly amplified with PCR can be used as a probe either to screen for human genes in complex DNA mixtures (Bicknell et al. 1991, Nelson et al. 1989) or to perform FISH (Meltzer et al. 1992). Ideally, the PCR reaction should be symmetrical and unbiased, and the amplification product should represent a heterogeneous population of DNA sequences. Nevertheless, PCR has its disadvantages such as contamination with small amount of DNA from other sources which can be amplified and lead to false positive results, and bias in the amplification procedure depending on the sequence of the template resulting in over-representation of certain sequences (discussed in more detail in Chapter 6). The advantages of microcloning are that the DNA of interest can be stored for a long time, and problems of contamination in the PCR amplification can be avoided.

1.4 Chromosomes and disease

1.4.1 Chromosome aneuploidies

Chromosome dosage alterations (aneuploidies) associated with diseases mainly include trisomy and monosomy. A few typical and common examples are: Down syndrome, which was first described by Down (1866) and identified as trisomy 21 by Lejeune and his collaborators (Lejeune 1959) in autosomal trisomy; Edwards syndrome with trisomy 18 (Edwards et al. 1960), and Patau syndrome with trisomy

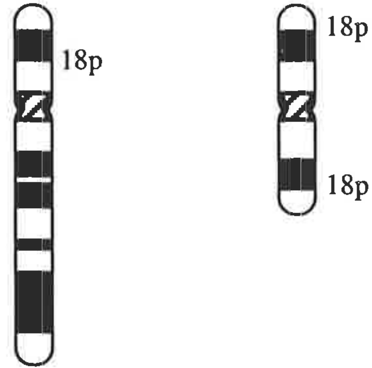
13 (Patau et al. 1960); sex chromosome aneuploidies including Klinefelter syndrome (47, XXY) (KLlinefelter et al. 1942) and Turner syndrome (45, X) (Turner 1938). The above examples involve the addition or deletion of a whole chromosome. Another type of dosage change is caused by the addition of only part of a chromosome. This can be attached to one of the other chromosomes or be present as an additional chromosome and is termed an extra structurally abnormal chromosome (ESAC). This thesis deals mainly with the characterization of ESACs.

1.4.2 Extra structurally abnormal chromosomes

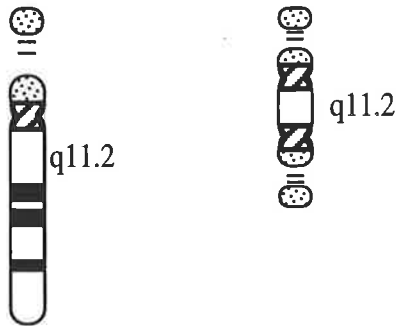
An extra chromosome of unknown origin is referred to as a marker chromosome (Paris Conference 1972, Harnden and Klinger 1985). It is also called a supernumerary, or accessory chromosome. In order to avoid ambiguity, Hook and Cross (Hook and Cross 1987) coined the term "extra structurally abnormal chromosome", (ESAC) and this terminology will be used in this thesis. ESACs can be either familial or *de novo*. ESACs are common types of isochromosomes, inverted duplication chromosomes (inv dup), and ring chromosomes (or ring ESAC) (Fig. 1-2). They can also have other origins, either derivatives from translocation or large deletions giving small acrocentric chromosomes.

ESACs have been found not only in patients with various anomalies including mental retardation (Frøland et al. 1963, Tangheroni et al. 1973) and but also in normal individuals (Fried and Rosenblatt 1979, Ridler et al. 1970). Specific phenotypic effects of ESACs are largely unknown except in a few well-established instances where the ESAC can be specifically characterized, i.e. Pallister-Killian syndrome associated with isochromosome 12p, isochromosome 18p syndrome, and cat eye syndrome with inverted duplication of chromosome 22 (inv dup 22). Inv dup(15) can result in normal or abnormal phenotypes depending on the size.

1-2a
Isochromosome 18
Karyotype: 47,XY, +i(18p)



1-2b
Inverted duplication 22
Karyotype: 47,XY, +inv dup(22)



1-2c
Ring chromosome 4
Karyotype: 47,XY, +r(4)

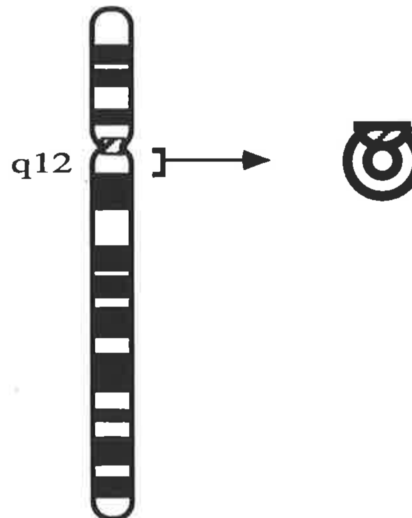


Fig 1-2. Ideogram and karyotype of three types of ESAC (a and b were adapted from Ferguson-Smith and Andrew 1997).

1.4.2.1 ESACs and defined clinical syndromes

Pallister-Killian syndrome: This syndrome was first described by (Pallister et al. 1977) and subsequently reported by Teschler-Nicola and Killian (1981). The clinical feature consists of profound mental retardation, seizures, craniofacial anomalies including coarse face with pigmentary skin, localized alopecia or hair sparse in the frontal bossing area, hypertelorism, epicanthal folds, flat nasal bridge, large mouth with downturned corners and abnormal ear. Affected newborns can also have hypotonia, congenital heart defects, diaphragmatic defects, and supernumerary nipples. Adults with the disorder can develop epilepsy and macroglossia (Schinzel 1991). The syndrome has been correlated with the presence of an isochromosome of the short arm of chromosome 12, i(12p) (Peltomaki et al. 1987). It is noteworthy that the cytogenetic abnormality appears in a high proportion of cells from bone marrow and fibroblasts from the skin, lung, and testes but not in neonatal peripheral blood lymphocytes.

Isochromosome 18p syndrome. Isochromosome 18p, a small metacentric ESAC, was first suggested by Frøland et al. (1963) to be of this origin on the basis of G-banding and similar cases reported by several other authors (Balicek et al. 1976, Condron et al. 1974, Ogata et al. 1977, Taylor et al. 1975). This small metacentric chromosome was definitively identified as isochromosome 18p with a radioactively labelled single-copy probe from the short arm of chromosome 18 (Mattei et al. 1985). The origin was further confirmed with a chromosome 18 alpha satellite probe in combination with a probe mapping to 18p11.3 (Callen et al. 1990). The signal of the 18p11.3 probe was present on both arms of the marker as expected for an isochromosome (Callen et al. 1990). The general phenotype is characterized by microcephaly, mental retardation from the moderate to severe range, low birth weight, hypotonia, camptodactyly or adducted thumbs. The baby can have feeding problems in the first year with hypotonia and develop limb spasticity in the following year. Nearly 50% patients suffered from seizures. Recognizable facial dysmorphism

includes dolichocephaly, an oval face with low set and /or malformed ears, high eye-brows, short palpebral fissured, strabismus, small nose, long philtrum, high arched palate and small mouth (Schintzel, 1994). Prognathism develops with increasing age and the mouth and nose will be normal in size (Batistal et al. 1983, Callen et al. 1990, Rivera et al. 1984).

Cat eye syndrome. This syndrome was first described and associated with an abnormal extra chromosome in 1965 (Schachenmann et al. 1965). The name was derived from the "cat eye-like" appearance of the pupil, which results from coloboma of the iris. The incidence of transmission of the ESAC from parents to child is high (Ing et al. 1987, Luleci et al. 1989, Mears et al. 1994, Schachenmann et al. 1965, Schinzel et al. 1981). The phenotype varies and includes anal atresia, coloboma, a down-slanting palpebral fissures, hypertelorism, preauricular pits or fistulas, cardiac malformations, renal malformations, and mild to moderate mental handicap. Some patients have a normal intelligence but emotional disturbance (Schinzel et al. 1981). This small additional G-like chromosome was originally suggested to be derived from chromosome 22 (Buhler et al. 1972), and then described as a bisatellited isodicentric chromosome 22-derivate (Schinzel et al. 1981). *In situ* hybridization study utilizing a single-copy probe localized to 22q11 confirmed the existence of four copies of 22q11 in the patients (McDermid et al. 1996).

Inv dup (15). The inv dup (15) is the most frequent ESAC accounting for approximately 50% of the total number of markers ascertained. It usually appears as dicentric and bisatellited but its shape can be metacentric/submetacentric or acrocentric. Cytogenetic studies show that the marker is positive for DA/DAPI staining. The size of this ESAC can be larger or smaller than a G-group chromosome. Individuals with inv dup (15)s can have phenotypes varying from normal (Knight et al. 1984, Stetten et al. 1981) to abnormal (Schreck et al. 1977, Wisniewski et al. 1979). The clinical features of the abnormal phenotype, or inv dup

(15) syndrome, include mental retardation, seizures, behavioural problems and mild dysmorphology (Gillberg et al. 1991, Kirkilionis and Sergovich 1987, Mohandas et al. 1985, Schmid et al. 1986, Schreck et al. 1977, Wisniewski et al. 1979). The presence of a normal or abnormal phenotype is correlated with the presence of the Prader-Willi/Angelman syndrome (PWS/AS) region in the inv dup(15). Recently molecular characterization of inv dup(15)s has shown that when the breakpoints of small inv dup(15)s are proximal to the PW/AS region it is associated with a normal phenotype. The larger inv dup(15)s contain the PW/AS regions and are consistently associated with the phenotypic abnormalities and mental retardation as described above (Battaglia et al. 1997, Huang et al. 1997).

1.4.2.2 Familial ESACs and de novo ESACs

ESACs can either be inherited from a parent or form *de novo* within individuals. Familial ESACs account for approximately 30-40% of the total number ESACs (Brondum Nielsen and Mikkelsen 1995, Buckton et al. 1985, Sachs et al. 1987, Stetten et al. 1992) and no preferential inheritance from either father or mother has been found. No discernibly increased risk for fetal abnormality exists when a carrier parent is non-mosaic for an ESAC and is phenotypically normal (Brondum-Nielsen and Mikkelsen 1995, Tsukahara et al. 1986). On the other hand, when the parent has an ESAC in a mosaic state, it will be difficult to prognosticate whether the fetus is expected to have a normal phenotype. In this situation, Table 1-2 provides a guide for the risks of congenital abnormality. It is likely that the child will have serious phenotypic abnormality if the ESAC is found to be derived from 3:1 malsegregation of a parental balanced translocation (Stamberg and Thomas 1986).

For *de novo* ESACs, however, empirical risk figures indicate an average risk of 10-15% for congenital abnormality (Brondum-Nielsen and Mikkelsen 1995) with confidence limits 6.9%-19.1% (Warburton 1991). The frequency of *de novo* ESACs increases with maternal age (Hook et al. 1983) and this has been confirmed in a

separate study (Crolla et al. 1992). In two studies, about 75% of ESACs were no-mosaic and 25% of ESACs mosaic, although there is no significant different for the rate of abnormalities between these two types (Buckton et al. 1985, Warburton 1991). The frequency of *de novo* ESACs in different groups of patients with various disorders has been surveyed (Table 1-3). However, the chance of phenotype abnormality for a fetus with a *de novo* ESAC is not easy to quantify and depends on the composition and origin of the ESAC.

The incidence of ESACs is between 0.65-1.5‰ fetuses in prenatal investigations (Benn and Hsu 1984, Ferguson-smith and Yates 1984, Hsu 1986, Hook and Cross 1987, Blennow et al. 1994b, Brondum Nielsen and Mikkelsen 1995, Sachs et al. 1987, Warburton 1984, Warburton 1991) and 0.1-0.72‰ in newborns (Buckton et al. 1980, Hamerton et al. 1975, Jacobs 1974, Jacobs et al. 1990, Nielsen and Rasmussen 1975, Nielsen and Wohlert 1991), compared with the incidence of all constitutional chromosome abnormalities of approximately 6‰ (Hamerton et al. 1975, Jacobs 1974, Nielsen and Rasmussen 1975).

1.4.3. Ring ESACs (small extra ring chromosomes)

Ring ESACs constitute approximately 10% of all ESACs (Blennow et al. 1994b) and represent the most poorly characterized group (Callen et al. 1990b, 1991, 1992a, Hoo et al. 1974, 1980, Kaffe et al. 1977). Ring ESACs have not yet been studied in detail for the following reasons. Firstly, they are unstable, often gradually disappearing in vivo and eventually only cells with normal karyotype are observed (Hoo et al. 1980). Secondly, they give rise to different degrees of mosaicism, making the analyses more difficult to perform and to interpret (Blennow and Tillberg 1996). Thirdly they can be derived from any chromosome (Callen et al. 1991, 1992a, Michalski et al. 1993, Plattner et al. 1993a, 1993b, Blennow et al. 1993, Blennow and Tillberg 1996, Brondum-Nielsen and Mikkelsen 1995).

Table 1-2 Estimate risks of fetal abnormality following prenatal detection of an ESAC, depending upon level of its characterization. Adapted from Gardner and Sutherland (1996)

ESAC	**Risk**
i(18)p	100%
i(12)p	100%
idic(22)	100%
der(X) without XIST	100%
Multiple ESACs	<95%
Large der(15), breaks distal to q11.2(> No. 21 in size)	<95%
Distamycin A/DAPI-negative rings	<80%
Satellited acrocentrics with Euchromatin	<80%
Without satellites	<15%
Any, not further characterized	<13%
Satellited	<11%
Small, mostly C-band positive	<5%
Smaller der(15), break proximal to q11.2 (< No. 21 in size)	<5%
der(Y)	<5%
"dots"	<5%
Small distamycin A/DAPI-positive rings	<5%
der(X) with XIST	<5%
Small bisatellited, single centromere	<2%

Table 1-3. Frequency of *de novo* ESAC carrier in various patients Adapted from Buckton et al. (1985)

Population with disorder (No. cases)	ESAC carrier (‰)
Mental subnormality (3,673)	3.27
Congenital abnormalities (1,142)	2.63
Abnormal sexual development (683)	2.93
Subfertile patients (2,565)	1.95
Cancer (3,295)	0.91

1.4.3.1 Characterization of ring ESACs with conventional cytogenetics

Ring ESACs are usually revealed by conventional cytogenetic methods but their origin cannot usually be identified. With G-banding, although the size and number of ring ESACs can be determined, it is generally impossible to identify any banding patterns due to the small size of the rings. Using C-banding, the relative amount of heterochromatin in ring ESACs can be assessed but with C-banding alone, the origin of the ring ESAC cannot be positively identified. Ag-NOR staining is useful to identify the satellites of ESACs and therefore their acrocentric origin. However, ring ESACs rarely have satellites. Ring ESACs originating from chromosome 1, 9, 15 and 16 will be suggested when DA/DAPI staining is positive.

1.4.3.2 Characterization of ring ESACs with molecular cytogenetics

The identification of the chromosomal origin became possible with the development of fluorescece *in situ* hybridization (FISH). Probes to the repetitive alpha-satellite sequence can be specific for the centromere of a chromosome. Using such centromeric probes for the X and Y chromosome, the origins of ESACs in Turner syndrome have been determined (Cooper et al. 1991, Crolla and Llerena 1988, Jacobs et al. 1990). In other studies, the chromosomal origins of 42 small ESACs have been identified with autosomal centromere specific probes (Callen et al. 1990b, 1991, 1992a).

Other probes for identification of ESACs are either whole chromosome-specific painting probes or region-specific painting probes that are produced from degenerate oligonucleotide-primed PCR (DOP-PCR) amplification (Telenius et al. 1992), from flow-sorted chromosomes (Gray et al. 1975, Blennow et al. 1992) or microdissected chromosomes (Guan et al. 1993, Meltzer et al. 1992, Thangavelu et al. 1994, Viersbach et al. 1994).

1.4.3.3 Origins of ring ESACs

In total only about 40 ring ESACs have been reported. These ring ESACs have originated from all chromosomes except for chromosomes 5 and 11. This was established using FISH with centromere specific probes (Blennow et al. 1993, 1995, Blennow and Tillberg 1996, Callen et al. 1990b, 1991, 1992a, Estop et al. 1993, Rauch et al. 1992, Wiktor et al. 1993, Michalski et al. 1993, Daniel et al. 1994, Melnyk and Dewald 1994, Pezzolo et al. 1993a, Plattner et al. 1993a, Voullaire et al. 1993, Batista et al. 1995, Begleiter 1996, Brondum-Nielsen and Mikkelsen 1995, Chen et al. 1995a, Crolla et al. 1995, James et al. 1995, Lanphear et al. 1995, Morrison et al. 1997, Rosenberg et al. 1995, Sun et al. 1995). The origin of ring ESACs and associated phenotype findings are summarized in Table 1-4. Because the sequence homology of chromosome 13 and 21 or 14 and 22 are very high and differentiation is generally impossible, the exact origin of these ring ESACs is not easily determined. The phenotype in patients extends from normal to abnormal, including mental retardation, developmental delay, and dysmorphic features. In these reported cases, there are only a few examples of ring ESACs that are derived from the same chromosome. Although the origin of most ring chromosomes can be accurately defined, their composition is largely unknown. The information is too limited to explain why patients with similar chromosomal defects develop different phenotypes. The situation may be improved with additional knowledge about the origin of small ring ESACs or their composition.

TABLE 1-4. ORIGINS OF REPORTED RING ESACS AND ASSOCIATED PHENOTYPES

1	2	3	4	6	7	8	9	10	12	13/21	14/22	15	16	17	18	19	20	X	Y	References
N							N						N							Callen et al. 1990
DF		M*		M*					ToP* DD	N			Top*		N		DF	M*		Callen et al. 1991
			MR/ DF				N					N								Callen et al. 1992a
										DF (21)										Rauch et al. 1992
										DD (21)										Sun et al. 1995
																			M/ DF	Pezzolo et al. 1993a
N																N				Michalski et al. 1993
						DD/ DF														Melnyk and Dewald 1994
N	MR					DD/ DF			M*	M* M*	M* M*	M*			M*					Plattner et al. 1993
												MR								Crolla et al. 1995
MR/ DF																				Chen et al. 1995
DD/ DF																				Lanphear et al. 1995
	N					DD/ DF												DD		Daniel et al. 1994
			MR/ ToP		DF	DF	DD										DD			Blennow et al. 1993
			MR /DF																	Estop et al. 1993
				UPD																James et al., 1995
								DD												Voullaire et al. 1993
								MR/ DF												Blennow et al. 1996
														DD	MF/ ToP					Brøndum-Nielsen & Mikkelsen 1995
																ToP*				Blennow et al 1995
														M*				M*		Wiktor et al. 1992
														DD						Rosenberg et al. 1995
														DD/ DF						Morrison et al. 1997
																	DD/DF			Batista et al. 1995
																		DF		Begleiter et al. 1996

*Phenotype cannot be evaluated, DD = developmental delay, DF = dysmorphic features, M = multiple extra chromosomes, MF = malformations, MR = mental retardation,; N= normal, Top = termination of pregnancy, UPD= uniparental disomy

A subtype of ring ESACs causing phenotypic abnormality is associated with uniparental disomy (UPD) (Robinson et al. 1993a, Temple et al. 1995). The symptoms may be caused by UPD of the two normal homologs rather than any effect of the ESAC itself, e.g. a ring chromosome 6 has been found to be associated with UPD for chromosome 6 (James et al. 1995). It is suggested that the presence of an ESAC increases the probability of non-disjunction and therefore UPD.

1.4.3.4 Multiple ring ESACs

Patients with multiple ring ESACs in the same metaphase have been reported and up to 5 rings have been found in different cells (Callen et al. 1991, Mascarello et al. 1987, Pezzolo et al. 1993b, Plattner et al. 1993b, Tozzi et al. 1988). These rings can originate from different chromosomes in the same cell. For example, in one patient with two rings, one ring was derived from chromosome 6 and the other from chromosome X (Callen et al. 1991). In another individual with multiple rings these rings were derived from chromosomes 8, 14/22, 15, and X (Plattner et al. 1993b). Multiple rings were found in 22% of patients with ring ESACs and since they originate from different chromosomes, it is difficult to make phenotypic correlations (Blennow and Tillberg 1996).

1.4.3.5 Clinical relevance of ring ESACs

When ring ESACs are ascertained in prior postnatal diagnosis, the potential risk for physical and mental problems cannot be defined precisely. Sachs et al. (1987) suggested that a *de novo* DA/DAPI positive ESAC probably carries a low risk of fetal anomalies. This conclusion was supported by Callen et al. (1990b, 1992a) analyzed 42 patients with ESACs and particularly pinpointed that small ring ESACs derived from both alphoid and satellite II or III pericentric heterochromatin of chromosomes 1, 9, 15, and 16 are all associated with a low risk of phenotypic abnormality. In contrast, when ring ESACs do not contain satellite II or III pericentric heterochromatin, or satellited acrocentric ESACs derived from chromosome 22, an

association with a high risk of phenotypic abnormality has been observed (McDermid et al. 1986).

A normal phenotype may be expected when the ring ESAC is found to gradually disappear in the prenatal stage (Hoo et al. 1974, Michalski et al. 1993). A case was reported (Michalski et al. 1993) with a ring derived from chromosome 1 and during the development of the fetus the percentage of metaphases with a ring chromosome decreased. The proportion of cells with a ring chromosome was approximately 25% in the second trimester but was 16% in cord blood and further reduced to 8% at 9 months. He reported another case where the percentage of cells with ring was 60% in amniocentesis but 46% in lymphocytes when the child was 10 month old. Michalski et al. (1993) suggested that if the small ring ESAC did exert a functional ill-effect, it may be minimized as the percentage decreased with development .

1.4.3.6 Mechanisms of ring formation

Rings which are found as accessory chromosomes i.e as 47,+r karyotype, are generally small. These rings might arise from one break at the centromere and the other break adjacent to the centromere on either the short arm or long arm with subsequent reunion between the two broken sister chromatids, i.e. a "U-type" exchange (Callen et al. 1991) , as illustrated in Figure 1-3a.

Some patients are found with a karyotype of 46 chromosomes with one of the chromosome as a large ring with partial loss of the distal ends of the short and/or long arm. These patients have abnormal phenotypes due to monosomy of genetic material or "ring syndrome" caused by the instability of the ring during mitosis (Gordon and Cooke 1964, Bobrow et al. 1973, Gardner et al. 1984, Kjessler et al. 1978, Wolf et al. 1967). These rings are proposed to be the result of breaks occurring on either side of the centromere and subsequent fusion of the broken ends. These rings consist of a

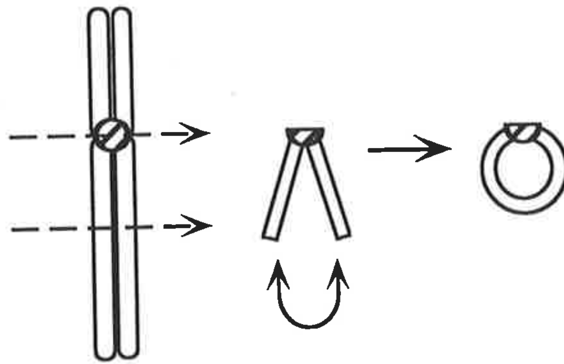


Fig 1-3a.

Chromosome breaks at both the centromere and either p or q arms. A U-type chromatid reunion forms (adapted from Blennow 1994a).

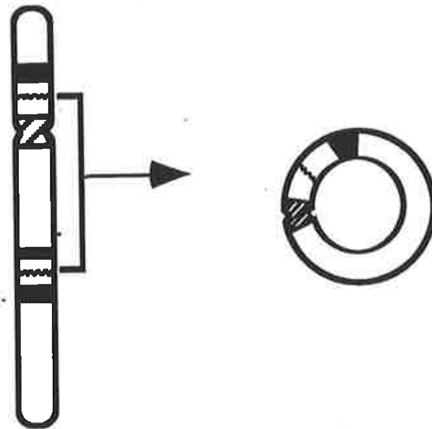


Fig 1-3b.

Chromosome breaks at two sites with reunion at the sebreaking sites. The two distal segments are lost.

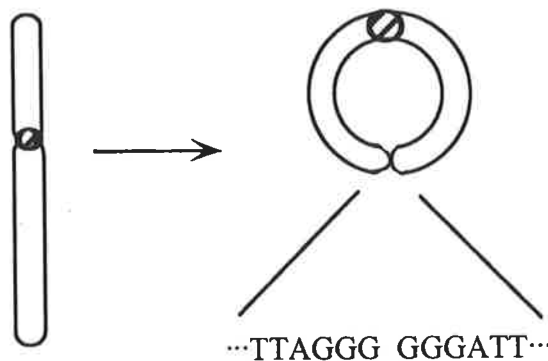


Fig 1-3c.

End to end fusion of chromosome without the loss of genetic material.

large part of the respective chromosome and always contain the centromere (Hamerton 1971), as illustrated in Figures 1-3b.

The third type of ring chromosomes is formed by end-to-end fusion of palindromic DNA base sequences at the chromosome ends without loss of genetic materials (Cote et al. 1981, Dutrillaux et al. 1978, Zuffardi et al. 1980) (Fig. 1-3c). There is an abnormal phenotype associated which is called "ring syndrome". This was confirmed by Pezzolo et al.(1993b) based on their FISH study of three ring chromosomes (ring 4, ring 16 and ring 20) with telomere probes $(TTAGGG)_n$. Hybridization revealed that the fluorescence signals were present at the ends of all chromosomes and in the rings. All patients had failure to thrive and minor dysmorphic signs. Two patients also had moderate mental retardation while one had normal intelligence, this patient had the ring in mosaic form with a normal cell line.

1.4.4 Chromosome rearrangements

The above dosage change in chromosomal material dealt with mainly extra small ring chromosomes. In the following part, the dosage change in chromosomal material dealt with duplication and deletion. Duplications lead to trisomy for the segments concerned. Charcot-Marie-Tooth disease (CMT) type 1A, an example of the partial duplication of a chromosome, is a 1.5 Mb duplication of the short arm of chromosome 17 (17p11.2). It results in affected individuals carrying three copies in this region instead of the normal two (Chance et al. 1992a, Chance et al. 1992b, Lupski et al. 1991, Lupski and Garcia 1992, Raeymakers et al. 1991).

The common deletion syndromes are Cri Du Chat syndrome with chromosome 5p deletion (Lejeune et al. 1963) and Wolf-Hirschhorn syndrome (WHS) with chromosome 4p deletion (Hirschhorn et al. 1965, Wolf et al. 1965). The WHS will be discussed in detail.

Since Wolf and Hirschhorn first described this syndrome (Hirschhorn et al. 1965, Wolf et al. 1965) , up to 200 cases have been reported (Dallapiccola et al. 1993, Johnson et al. 1976, Lurie et al. 1980, Preus et al. 1985, Fagan et al. 1994, Wilson et al. 1981, Altherr et al. 1991, El-Rifai et al. 1995, Gandelman et al. 1992, Reid et al. 1996, Wheeler et al. 1995, White et al. 1995, Wyandt et al. 1993) In these reports, abnormal findings involve the central nervous system, circulatory system, craniofacial dysmorphism and growth development retardation. Disorders in the central nervous system consisted of mental retardation, seizures and hypotonia. Aberrations in circulatory systems included congenital heart defects and atrial septal defects. Craniofacial dysmorphism involve eye abnormalities such as strabismus, mouth abnormalities such as cleft palate and fused teeth, beaklike nose and simple ears.

Cytogenetic studies showed this syndrome is associated with deletions of the distal short arm of chromosome 4. These deletions can occur either as an isolated deletion (Lurie et al. 1980) or as a result of segregation of a reciprocal translocation (Altherr et al. 1991, Dallapiccola et al. 1993) and range in size from one-half of the short arm (Lurie et al. 1980, Wilson et al. 1981) to cytogenetically undetectable (Preus et al. 1985) or only detectable by FISH (Altherr et al. 1991).

An approximately 2 Mb deletion interval between D4S43 and D4S142 within 4p16.3 has been localized as the critical WHS region (Gandelman et al. 1992) and six known expressed genes have been found within this region. These are a zinc-finger gene (ZNF141) (Tommerup et al. 1993), the b-subunit of rod cGMP phosphodiesterase (PDEb) (Collins et al. 1992a), myosin light chain (MYL5) (Collins et al. 1992b), a-L-iduronidase (IDUA) (Scott et al. 1990), HDA1-1 (McCombie et al. 1992) and fibroblast growth factor receptor 3 (FGFR3) (Thompson et al. 1991). Recently phenotypic mapping and correlation of physical traits with chromosomal regions, has been performed on 11 patients with chromosome 4p deletion

(Estabrooks et al. 1995). In Chapter 7, a study to further define the critical region for WHS is presented.

1.5 Aims of the thesis

Ring ESACs represent the most poorly characterized group of ESACs. When they are encountered at prenatal diagnosis or in a young child, ring ESACs, especially *de novo* ring ESACs, pose a difficult problem for the clinical doctor and genetic counsellor since it is difficult to give precise information regarding the long-term prognosis for the child. This may be due to several reasons. Firstly the ring ESACs are usually unstable in nature, giving a variable degree of mosaicism or it may gradually disappear completely. Secondly ring ESACs can be derived from any human chromosome. Thirdly patients with a ring ESAC derived from the same chromosome can have phenotypes which vary from normal to abnormal.

Throughout the literature approximately 40 small ring ESACs have had their origins determined, usually by FISH with centromere specific probes or whole chromosome painting probes. However, the exact origin of the euchromatin of these ESACs has not been determined. It is usually concluded that the euchromatin originates in pericentromeric regions. An origin from other regions of the chromosome or in fact other non-homologous chromosomes is possible. In this thesis an approach was used to investigate in detail the origin of the euchromatin content of nine ESAC chromosomes. This involved the development of a methodology based on microdissection, molecular cloning, isolating unique probes from cloned, microdissected ring ESACs, screening cosmids or phage library of chromosome 4 or genomic DNA, and FISH. Microdissection-FISH (reverse painting) was also used to identify the origin of ring ESACs (Chapter 5). The specific aims for this thesis are as follows:

(1) To precisely define the origin of ring ESACs.

(2) To obtain more information about the exact origin of the chromatin of ring ESACs

(3) To relate the genetic content of different ring ESACs derived from the same chromosome with the patient's phenotype.

(4) To generate probes for diagnostic use.

(5) To refine the critical region of Wolf-Hirschhorn syndrome. A small interstitial deletion of 4p16.3 in a patient was characterized by FISH with a battery of cosmid DNAs in that region.

Citations in Introduction:

These original publications were cited in many of the articles from which the pre-twentieth century history was drawn, and I did not actually have access to them, nor would I have been able to translate them. The papers in which they were cited are indicated in brackets.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

Methods applied in molecular cytogenetics and molecular genetics were used to characterize several ring ESACs and a case of Wolf-Hirschhorn syndrome. The materials and methods described in this chapter are general methods used throughout the project and no details will be given in individual chapters, unless specific techniques or modifications were required.

The microdissection and amplification of ring ESACs has been accomplished in collaboration with Dr Bohlander (Section of Hematology/Oncology, The University of Chicago); Dr Guan (Laboratory of Cancer Genetics, National Center for Human Genome Research, National Institutes of Health, MD, USA); Dr Hill (CSIRO Division of Biomolecular Engineering, Laboratory for Molecular Biology, New South Wales, Australia), and Dr Houben (Department of Genetics, The University of Adelaide, Australia). The screening of a cosmid library of chromosome 4 was undertaken by Dr Riess in the Department of Molecular Human Genetics, Ruhr-University, Bochum, Germany. Details of these specialized techniques will be described in the relevant chapters.

2.2 Fluorescence *in situ* hybridization (FISH)

2.2.1 Preparation of metaphase chromosome spreads from cultured lymphocytes

Lymphocyte culture: Five to six drops of heparinized peripheral venous blood were grown in 5 ml culture medium at 37°C for 3 days (Moorhead et al. 1960). The culture medium was either MEM-FA (Sutherland et al. 1979) or RPMI 1640 (GIBCO) containing 5% fetal calf serum (CSL), 0.5 units/ml heparin, and 2% (v/v)

phytohaemagglutinin (PHA) (GIBCO). The following two different protocols were used to prepare prometaphase chromosomes to allow high resolution banding: cell synchronization ("TdC" method) and treatment of lymphocyte cultures with ethidium bromide.

Cell synchronization ("TdC" method): Twenty-two hours before harvest, cells were blocked in the S phase by the addition of 0.3 mg/ml thymidine to the cultures. Five and three quarter hours before harvest the block was released by replacement with fresh medium and the addition of 30 μg/ml deoxycytidine and 10 μg/ml BrdU (Wheater and Roberts 1987). For synchronization, 55 μl colchicine (0.1 mg/ml) was added to lymphocyte cultures 20 min before harvest.

Ethidium bromide-treated lymphocyte cultures : The method of ethidium bromide-treated lymphocyte cultures (Ikewchi and Sasaki 1979) will elongate chromosomes to allow high resolution studies. The ethidium bromide intercalates with the chromosomal DNA, thus preventing the binding of certain chromosome proteins responsible for chromosome condensation. Ethidium bromide (0.5 ml) was added to 5 ml lymphocyte culture 2 hours prior to harvest, this was followed by the addition of 55 μl colchicine (0.1 mg/ml) 15 min before harvesting.

Chromosome harvest: Cultures were centrifuged at 1000 rpm for 5 min, the supernatant was removed, and the cell pellet was resuspended in 9 ml of 0.075M KCl at room temperature. After 25 min, the suspension was mixed with 1 ml fixative (methanol:glacial acetic acid in ratio of 3:1) and centrifuged. The supernatant was removed and the pellet resuspended in 10 ml fresh fixative. The centrifugation and resuspension procedures were repeated two more times. Harvested cells were either directly spread on the clean slide or stored in fixative at -20°C until needed (Moorhead et al. 1960).

2.2.2 Preparation of slides for FISH

Fixed cell suspensions were brought from -20°C storage and allowed to warm to room temperature for 1 hour. A single drop of cell suspension, at an appropriate concentration which was determined visually under an inverted microscope, was dropped onto a dried, ethanol-cleaned glass slide in a controlled atmosphere of 46-48% relative humidity at 24°C. Slides with cell spreads were stored for 1-4 days in a box with desiccant prior to FISH.

2.2.3 Solutions required for FISH

The recipes of solutions required for FISH are given in Table 2-1.

Table 2-1 Contents of solutions for FISH

Working solution	**Contents of solution**
1× TE buffer (pH7.5)	10mM Tris-HCl pH8.0, 1mM Ethylenediaminetetra (EDTA)
1× SSC (pH7.0)	1M NaCl, 1M Sodium citrate, prepared as 20× stock,
Hybridization mixture (pH 7.0)	10% dextran sulfate, 2× SSC, 50% deionized formamide, 0.1% Tween 20, stored at -20°C
Blocking solution	4× SSC, 1% Bovine serum albumin (BSA), stored at 4°C
Antifade solution	1 part 20mM Tris, (pH7.5) plus 9 parts glycerol containing 2% of diazabicyclooctane (DABCO), mixed for 2 hours on a rotating wheel (10 rpm), stored at 4°C

2.2.4 Nick translation of probes

Genomic DNA (1 μg) was nick translated with biotin 14-dATP using the BIO-nick labelling system (GIBCO) in 50 μl as recommended by the supplier and incubated at 16°C for 1 hour. After the labelling reaction 25 μl of sonicated salmon sperm DNA (10 mg/ml) was added as carrier DNA. The labelled DNA was

precipitated by adding 220 μl of ice-cold 100% ethanol and 8 μl of 3M sodium acetic acid, pH 5.2 and kept for at least 3 hours at -70°C before it was centrifuged and resuspended in 1× TE buffer to a final concentration of 20 ng/μl. Biotin labelled probes were stored at 4°C.

2.2.5 In situ hybridization (ISH)

RNase treatment and denaturation of target DNA: Slides with chromosomal DNA were incubated in DNase-free RNase A (100 μg/ml) in a moist chamber with 2× SSC at 37°C for 1 hour, followed by 4 washes in 2× SSC for 1 min each, slides were dehydrated in a series of 70%, 95%, and 100% ethanol for 1 min each and airdried in a vertical position. A Coplin jar filled with 70% formamide/2× SSC, pH7.0 was heated in a waterbath until the temperature of the solution reached 70°C. To allow the denaturation of chromosomal target DNA, slides were soaked in the solution at 70°C for 3 min, followed by either an immediate application of the denatured probe mixture (as detailed later) or by a rapid quench through an ice-cold dehydrating alcohol series (70%, 95%, and 100% ethanol) for 1 min each. The slides were airdried in a vertical position and were then ready to be hybridized.

Probe mix preparation and denaturation: Labelled probes used in these studies included two types: repetitive DNA consisting of cloned alpha specific-centromeric DNA or single-copy DNAs from PCR-amplified microdissected DNA, cosmids, YACs or PACs. To prepare the probes of repeated DNA, usually 1 μl of DNA (20 ng/μl) was directly added to 9 μl hybridization mixture (Table 2-1). To prepare the probes from PCR products or single-copy DNA, 20 ng of labelled DNA was combined with 5 μg of unlabelled human placenta DNA. The ratio between the labelled probe and the human placenta DNA was varied from 200:1 to 20:1 to achieve the best signal/noise ratio. Mixed DNAs were dried in a vacuum centrifuge and resuspended in 10 μl hybridization mixture. After 5 min denaturation at 70°C, the 10 μl probes were applied to denatured target DNA on the slides, covered with a

coverslip which was sealed with rubber cement. The slides were incubated overnight at 37°C in a moist chamber.

Post hybridization washes: Two Coplin jars filled with 50% formamide/2× SSC (pH7.0) were heated to 42°C in a waterbath. Coverslips were gently removed and the slides were washed twice for 10 min in Coplin jar with agitation at the first 3 min. Slides were then washed twice in 2× SSC (pH7.0) at room temperature for 5 min each, and once in 1× SSC (pH7.0) for 10 min.

Detection & amplification: Slides were equilibrated in 4× SSC/0.05% Tween 20 for 3 min, and incubated in a blocking solution at room temperature (Table 2-1) for 10 min (Kievits et al. 1990). For the detection of biotin-labelled alphoid repeat probes, slides were incubated in FITC-conjugated avidin/blocking solution (5 μg/ml) under parafilm in a moist chamber at room temperature for 20 min and washed twice in 4× SSC/0.05% Tween 20 for 5 min each. For the detection of biotin-labelled single-copy probes, amplification steps were usually needed. In this case, biotinylated anti-avidin/blocking solution (1 μg/ml) was applied to the slide for 20 min followed by a further incubation of avidin-FITC as described above. Finally the slides were rinsed once in 2× SSC for 2 min, twice in 1× PBS for 2 min each, and mounted in 20 μl of antifade solution (Table 2-1) with the addition of 0.08 μg/ml propidium iodide (PI) and 0.6 μg/ml diamidino phenylindole dihydrochloride (DAPI) for chromosome identification.

For probes generated from orange-spectrum labelled PCR products of microdissected DNA, only post hybridization washes were needed. They were followed by number rinses with 2× SSC and two rinses with 1× PBS. Slides were mounted in 20 μl of antifade solution containing DAPI (1 μg/ml).

2.2.6 Analysis of FISH and photography

Slides were viewed under an Olympus BX 40 microscope fitted with single pass filters for UV, FITC, and TRITC and dual pass filter for FITC/TRITC

(Chroma). At least 20 cells were analyzed for each slide. Photographs were taken with KODAK Ektachrome 160T colour slide film. Alternatively, the fluorescence images of FITC (green), orange-spectrum, and DAPI counterstain (blue) were captured with an Applied Imaging CCD camera and merged using the Cytovision Ultra image collection and enhancement system (Applied Imaging).

2.3 Molecular cloning

The method of molecular cloning mainly includes three steps: preparation of insert and vector DNA (plasmids were used in this study), ligation of insert and vector DNA, and transformation. PCR products from microdissected ring ESACs were used as insert. The microdissection was carried out by several collaborators (section 2.1). The details and the subsequent ligation will be described in more detail in Chapters 3, 4 and 6. Transformation involved the preparation of competent cells and introducing the ligated DNA into the competent cells which were either purchased from commercial sources (Chapter 3) or prepared by myself (Chapters 4 and 6).

2.3.1 Materials required

Luria Bertani Broth (LB-broth): Five g yeast extract, 10 g Bacto-tryptone, 10 g NaCl, and 1000 ml distilled water were mixed, the pH adjusted to 7.0 and solution was autoclaved.

AMP-LB-plate (50 μg/ml): Fifteen grams of Bacto Agar (DIFCO MICHIGAN) were added to 1000 ml of autoclaved LB-broth. The medium was reautoclaved and cooled to 55°C in a waterbath. One ml of ampicillin (50 mg/ml) was added. Approximately 20 ml of media was poured into each Petri dish. The plates were kept at 4°C for up to 1 month.

TSB solution: One gram polyethylene glycol (PEG) 3350, 0.5 ml dimethyl sulfoxide (DMSO), 0.1 ml of 1M $MgSO_4$, 0.1 ml of 1M $MgCl_2$, LB-broth was added up to 10 ml, and kept in an ice bath.

TSS solution: As to TSB solution but without $MgCl_2$.

0.1M IPTG: The stock was made by adding 23.83 mg IPTG in 1 ml distilled water, and sterilized by filtration. The solution was stored at -20^oC.

6% X-gal: 60 mg X-gal was added in 1 ml dimethylformamide and stored at -20^oC.

Bacterial strains: All strains were streaked from stocks kept at -70^oC in LB+15% (v/v) glycerol.

2.3.2 Preparation of competent cells

The method of (Chung et al. 1989)was used. One colony of E. coli (XL-1-Blue or JM 109) was inoculated into 25 ml of LB-broth, and incubated at 37^oC with shaking for 12 to 16 hours. From this culture, 200 µl was taken and added to 10 ml of LB-broth and incubated at 37^oC with shaking until the optical density (OD) reading was 0.3 when read at 600 nm in a spectrophotometer (OD_{600} = 0.3). Generally this was achieved after 3 hours of incubation. The culture was centrifuged in a Jouan CR3000 centrifuge at 4^oC at 2000 rpm for 10 min and the supernatant was discarded. Cells were resuspended with 1 ml of ice cold TSB (or TSS) solution and kept on ice prior to transformation.

2.3.3 Transformation

Fifty ng of ligated insert and plasmid vector DNA was added to 100 µl of cell suspension, and the mixture was kept on ice. After 30 min 0.9 ml TSB (or TSS) and 20 µl of 1M glucose was added, mixed gently by inverting the tube, and incubated at 37^oC for a further 60 min with shaking. Each AMP-LB- plate was evenly spreaded with 40 µl of 0.1M IPTG and 6% X-gal and dried. The transformation culture was quickly centrifuged, approximately 950 µl of the supernatant discarded and the remainder was resuspended and evenly spread on the plate. After the surface of the AMP plate was airdried, plates were inverted and incubated at 37^oC for 12-16 hours.

If the transformation was successful, hundreds of colonies could be observed on the plate. The majority of colonies were white and therefore contained insert DNA. A small number of colonies appeared blue and contained only vector DNA. During the transformation procedure, appropriate controls were used in case transformations were not successful. When transformation efficiencies were low, a heat shock of the mixture of the cell and ligation DNA at $42^{o}C$ for 45 second after 30 min incubation in ice successfully increased the frequency.

2.4 Polymerase chain reaction (PCR)

In this study, PCR was used for two purposes. The first was to amplify microdissected DNA as detailed in chapters 3, 4, and 6. The second was "colony PCR" in which cloned inserts were amplified. PCR reactions were performed in a Perkin-Elmer Cetus Thermal Cycler 480.

2.4.1 PCR reagents

Two hundred nM each of primers, 200 μM of each dNTP, 0.025 - 0.05 U/μl Taq polymerase, 1.5 mM $MgCl_2$ in either 1× Taq buffer (Boehringer Mannheim) or 1× PCR solution (Adapted from GIBCO) which contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA.

2.4.2 PCR conditions

Colonies were picked, added to 50 μl mineral oil each and incubated at $100^{o}C$ for 10 min prior to adding the PCR reagents. Each PCR cycle consisted of $94^{o}C$ for 30 seconds, $55^{o}C$ for 30 seconds, $72^{o}C$ for 2 minutes, a total of 30 cycles was carried out.

2.5 Purification of PCR products

PCR products were usually purified with different commercial kits including Prep-A-Gene (Bio-Rad), QIAquick PCR purification kit (QIAGEN), and Magic^{TM} PCR preps DNA purification system (Promega). Purification steps were according to protocols supplied by the manufactures. The purified PCR products were used for molecular cloning, sequencing, mapping studies by Southern blot (Chapters 3, 4, and 6), or for FISH (Chapters 3 to 5).

2.6 Restriction digests

Most restriction digests were performed overnight using 4× the recommended amount of enzyme for an hour digest. To avoid inhibition by glycerol, the amount of enzyme added did not exceed 1× the volume of the reaction.

Rodent/human hybrids were used for mapping studies (Chapters 3, 4 and 6). The digest condition were as following: Hybrid DNA (10 μg) was added to 50 μl solution containing 5 μl 10× buffer (Biolabs), 40 units of enzyme [*Hind*III, *Eco*RI, and *Pst*I (Biolabs)], 5 μg of BSA, and 0.25M spermidine. The reaction mixture was centrifuged and incubated at 37°C overnight.

2.7 Electrophoresis of DNA

Solutions for electrophoresis:

1× TBE solution: 1M Tris-base, 1M Boric acid, 0.5M EDTA, pH8.0

10× loading buffer: 1 g sarcosyl, 7.5 g ficoll 400, 50 mg bromphenol blue, 50 mg xylene cyanine, 20 ml of 0. 5M EDTA, 5 ml of 1M Tris, added distilled water to 50 ml, mixed on a rotating wheel (10 rpm) for overnight.

To prepare agarose gels for electrophoresis, from 50 ml to 150 ml of 0.8% to 2.5% agarose in 1× TBE were poured in 11 cm × 14 cm gel trays with the comb

positioned. The concentration of agarose depended upon the size of DNA fragment to be analyzed. After the gel was set, it was placed in the electrophoresis tank containing 1× TBE solution. The marker DNA (SPP-1 which was restricted with *Eco* RI or pUC19 which was restricted with *Hpa* II) and samples were separately mixed with 1/10 volume 10× loading buffer. The gel was run at 1~5V/cm according to the DNA size. For digested genomic DNA and hybrids DNA, the gel was run 1V/cm overnight to obtain maximum resolution of DNA fragments. The gel was stained with ethidium bromide in H_2O and viewed under UV light. Photographs were taken or images were captured with CCD camera (Gel Doc 1000, BIO-RAD).

2.8 Southern blotting

2.8.1 Solutions required

Denaturation solution for agarose gels: 2.5M NaCl, 0.5M NaOH

Neutralization solution for agarose gels: 1.5M NaCl, 0.5M Tris (pH7.5)

Denaturation solution for membranes: 0.5M NaOH

Neutralization solution for membranes: 0.2M Tris (pH 7.5), 2× SSC

2.8.2 Southern transfer

Gels were blotted using either one of two methods (based on the methods of Sambrook et al. 1989, or Reed and Mann 1985 respectively:

(1) Gels containing separated DNA fragments were given into denaturing solution and shaken for 30 min to denature double-stranded DNA. Subsequently they were placed in neutralized solution for 30 min. During the denaturation and neutralization steps, a blotting tray was prepared. Whattman 3MM paper was cut to fit the blotting tray, wetted with 10× SSC, and trapped air bubbles were rolled away. The blotting tray was covered with gladwrap until use. Additional 3 pieces of Whattman 3 MM paper and 1 piece of Amersham HybondN^{+} membrane were cut to

the size of 11 cm × 14 cm. The membrane was marked and soaked in 10× SSC for 2 min before proceeding with the blot. The gel was placed facing down on blotting tray without air bubbles. The wet membrane (marked side down) was put on top of the gel and air bubbles were removed with a plastic pipette. After the edges of the gel were sealed with gladwrap, and 3 pieces of Whattman paper blotting towels of at least 4 cm in thickness, a glass plate, and 500 gm of weight were added in turn on top of the gel and left overnight. Towels and Whattman paper were removed and the wells of the gel were marked on the membrane. The membrane was denatured and neutralized for 1 min each, then placed between two sheets of Whattman paper, airdried and baked at high setting in the microwave oven for 45 sec to immobilize the DNA on the membrane.

(2) Soaking the gel in 0.4 M NaOH for 20 min. The rest of the procedure was as described above except that the transfer tray contained 0.4 M NaOH.

2.8.3 Megaprime labelling of oligonucleotides

Genomic DNA: Total human DNA was labelled by ^{32}P with the Megaprime^{TM} DNA labelling systems (Amershan) in a final volume of 100 μl. According to the recommended protocol, 100 ng of total human DNA, 5 μl primer and 10 μl labelling solution containing dNTPs were mixed in a screwtop tube. The mixture was incubated at 100°C heating block for 10 min to denature the human DNA. After the addition of 2 μl DNA polymerase I Klenow (1 unit/μl) and 5 μl α-^{32}P-dCTP (specific activity 3000Ci/mmol), to the mixture, the tube was briefly spun and incubated at 37°C for 30 min. The labelled probe was denatured at 100°C for 10 min before it was applied to a hybridization bottle containing the membrane.

Single-copy DNA: Single-copy DNA (25-50 ng) and 2.5 μl primer were placed in 100°C heating block for 5 min for denaturation. After a quick spin, 5 μl labelling solution with dNTPs, 2.5 μl ^{32}P, and 1 μl enzyme were added to the above mixture to a total volume of 50 μl and incubated at 37°C for 10 min. If probes containing

repetitive sequences these were blocked by preassociation with human placental DNA (pre-reassociation, Sealey et al. 1985). Fifty µl human placental DNA (10 µg/µl) and 25 µl 20× SSC were added to above tube to the final volume of 100 µl . Following a quick spin, the tube was placed in 100°C for 10 min to denature the labelled probe and placental DNA, subsequent on ice for 1 min . The tube was then incubated at 65°C for at least 60 min prior to application to the membrane in hybridization bottle (see section 2.8.4).

2.8.4 Hybridization, post wash, and firm development

Solutions required for these procedures are list in Table 2-2.

Hybridization: Membranes were rolled into a hybridization bottle with immobilized target DNA side inwards. To wet the membrane, 5 ml of 5× SSC solution was added in Hybaid™ bottle, then discarded making sure that air bubbles did not get trapped underneath the membrane. About 10-20 ml of hybridization solution that had been preheated to 42°C was added to the hybridization mix and incubated at 42°C in a hybridization oven (Hybaid) to prehybridize for at least 3 hours. Then the P^{32} labelled probe was added to the bottle to hybridize to the immobilized target DNA at 42°C overnight.

Post wash and development: Membranes were washed with a series of 4 solutions under different conditions. Solutions 1, 2, and 3 all consisted of 2× SSC and 0.5% SDS and solution 4 consisted of 0.1× SSC and 0.5% SDS. The solutions 1 and 2 were prewarmed to 42°C in a waterbath and the membrane was washed at this temperature in each solution for 10-20 min with shaking. Solution 3 was prewarmed to 65°C but the membrane was washed in this solution in a 42°C waterbath for 10-20 min. Solution 4 was prewarmed to 65°C and the membrane was washed at 65°C for 10-20 min. After the first 10 min washing, the membranes in

Table 2-2 Preparation of hybridization solutions (1 liter)

Solution	Stock concentration	Working concentration	Stock/Liter
Deionized formamide[1]	100%	50%	500 ml
SSPE[2]	20×	5×	250 ml
SDS	10%	2%	200 ml
Denhardts[3]	100×	1×	10 ml
Dextran sulphate	Solid	10%	100 gm
Salmon sperm DNA[4]	10 mg/ml	100 μg/ml	10 ml
Distilled H_2O			30 ml

(1) Formamide was deionized by mixing 2 g mixed bed resin with 50 ml formamide and slowly stirred for 3 hour. Then the mixture was filtered through Whattman 3MM filter paper.

(2) 20× SSPE (pH 7.4): One liter of 20× SSPE stock contained NaCl (175.3 g), $NaH_2PO_4 \bullet 2H_2O$ (27.6 g), and EDTA (7.4 g).

(3) 100× Denhardts: Ficoll (5 g), polyvinylpyrrolidone (PVP) (5 g), BSA (5 g), and distilled water added to 250 ml. The stock was sterilized by filtering and stored aliquots (50 ml) at -20°C.

(4) Salmon sperm DNA: The DNA was dissolved in TE to a concentration of 10 mg/ml and sheared by boiling the solution in a beaker of water for approximately 30 min. The liquid level was topped up every 15 min. After the boiling, the size of the DNA fragments was checked by gel electrophoresis (aiming for an average size of 500 bp) and DNA concentration was measured by spectrophotometer.

solution 2 were checked by isotope-counts monitor (Series 900, mini-monitor, Mini-Instrument LTD, England) to determine whether non-specific background had been eliminated. When the membranes were washed to an appropriate degree, they were placed into a plastic bag. The bag was sealed , placed with an X-ray film in a cassette with an intensifying screen and exposure at -70°C for 1-7 days, The film was then developed for analysis.

2.9 Colony blotting

Colony blotting was used to distinguish colonies, which possessed plasmids with inserts of single-copy or very low repeat copy DNA from those containing insert of high repetitive DNA.

2.9.1 Materials required

Circular Hybond$^+$ charged nylon membrane (Amersham)

AMP-LB-plate (50 μg/ml) (see section 2.3.1)

Denaturing solution: 1.5M NaCl, 0.5M NaOH

Neutralizing solution: 1.5M NaCl, 0.5M Tris-HCl (pH7.2), 0.001M EDTA

2.9.2 Inoculation and immobilization of colonies

Pairs of AMP-LB-plates were used, one was for the nylon membrane to allow colonies to grow on the surface, the other was used as a stock plate. The same pattern of grids was marked (around 50) on the membrane with a 2B pencil and on the bottom of the stock plate with a waterproof marker. The membrane was put into a plate with the marked side facing up. Sterile tooth-picks or yellow tips were used to select appropriate colonies which were inoculated at the corresponding grid positions on both plates. Two positions on the grids were used for a positive control and a negative control. The plates were then inverted and incubated at 37°C for overnight. Colonies with diameter of 4-5 mm were ideal for obtaining a strong signal after

hybridization to a labelled probe. The stock plate was sealed with parafilm and kept at 4°C for later use. Two clean Petri dishes were prepared and denaturation solution was put into one dish and the neutralization solution into the other. The membrane was peeled away from the plate with colonies side up and soaked in denaturation solution for 7 min. This was necessary to allow the bacteria to lyse and denature bulk unfractionated ligated DNA. The membrane was then soaked in neutralization solution for 6 min, airdried and baked in a microwave oven set at full power for 45 sec. These steps immobilized the DNA on the membrane for subsequent hybridization. The procedures of labelling of probe, hybridization, and post wash were the same as for Southern blots (sections 2.8.3 and 2.8.4).

2.10 Isolation of DNA

2.10.1 Plasmid DNA and cosmid DNA

The detailed procedures were based on Sambrook et al. (1989). The bacteria E. coli containing either plasmid or cosmid were streaked to a plate containing 50 μg/ml ampicillin or 50 μg/ml kanamycin and incubated at 37°C overnight. A single bacterial colony was picked from the plate and inoculated in 200 to 400 ml L-broth containing ampicillin (50 μg/ml) or kanamycin (50 μg/ml) and incubated at 37°C with shaking (200 rpm) for 12-16 hours. The culture was transferred to a 400 ml autoclaved Beckman bottle and cooled on ice for 30 min, then placed in JA10 rotor of a Beckman J2-21 M/E centrifuge at 4°C and spun at 5000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 1.2 ml 1× TE/50 mM glucose and transferred to a 50 ml Oakridge tube. 120 μl freshly prepared lysozyme (40 mg/ml) was added, mixed gently and allowed to stand at room temperature for 5 min and on ice for 1 min. Freshly made 0.2 M NaOH/1% sodium dodecyl sulfate (SDS) (2.4 ml) was added, mixed gently by swirl-rotating tube horizontally, and placed on ice for 5 min. Ice-cold 3M potassium acetate (1.8 ml) was then added, mixed by inverting, and placed on ice for 10 min, prior to spinning in JA20 rotor in a high speed

centrifuge at 15000 rpm at 4°C for 10 min. The supernatant containing the DNA of interest was transferred to a 50 ml Oakridge tube and 2 volumes of absolute ethanol were added. After an incubation step at room temperature for 5 min, the tube was spun for 15 min at 15000 rpm at 4°C. The supernatant was discarded and each pellet was washed in 2 ml 70% ethanol by gentle mixing with 1 ml Gilson pipette. The sample was transferred to 4 Eppendorf tubes and spun for 5 min at 13000 rpm in a bench microcentrifuge. The supernatant was removed and the pellet was dried in a speed vacuum centrifuge for 5 - 10 min. To each pellet, 200 μl of 1× TE were added, left at room temperature for 60 min, then the pellet was resuspended gently with cut-off yellow tips. 10 μl RNAse (1 mg/ml) was added to the suspension and incubated for 30 min at 37°C on rotating wheel (10 rpm). A mixture of 100 μl 3× proteinase K buffer (10 mM NaCl, 50 mM Tris, 10 mM $MgCl_2$), 10 μl 10%SDS, 2 μl Proteinase K (10 mg/ml) was added to the tube, which was further incubated for 1 hour at 37°C. An equal volume of phenol was added, mixed, and centrifuged at 13200 rpm for 7 min in a microcentrifuge. The top layer was transferred to a new tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added. After mixing well, the tube was spun for 7 min in a microcentrifuge and the top layer transferred to a new tube. One third volume of 7.5M NH_4 acetate and 2 volumes absolute ethanol were added, and were left at -20°C overnight. The mixture was spun in a microcentrifuge for 15 min and the supernatant was discarded. To remove salts from the DNA solution, 1 ml of 70% ethanol was added and spun in a centrifugation for 3 min, and the supernatant was removed as much as possible. DNA was dried in a speed vacuum and resuspended in 1× TE or distilled water.

In some latter experiments, for isolation of plasmid, cosmid, and PAC DNA the following commercial kits were used as QIAGEN Plasmid Kit (QIAGEN) and RPM™ Kit (BIO 101). The use of these purification kits greatly reduced the time and avoided the tedious methods of isolation of nucleic acids as described above.

The procedures were followed as recommended by the supplier and will not be described here.

2.10.2 Yeast artificial chromosome (YAC) DNA

Preparation of AHC media and AHC plates:

AHC media (pH 5.8): *yeast nitrogen base (1.7 g), $(NH_4)_2SO_4$ (5 g), casein hydrolysate acid (10 g), adenine hemisulphate (20 mg), and glucose (20 g) were added to distilled water to 1 liter and autoclaved. [* If yeast nitrogenbase with $(NH_4)_2SO_4$, was used $(NH_4)_2SO_4$ (5 g) did not have to be added to this media].

AHC plate: Agar (17-20 g) was added to 1 liter of AHC media and autoclaved. When the autoclaved media was cooled to 55°C, 100 µl of tetracycline (40 mg/ml) was added and mixed. Approximately 20 ml of medium was poured into each Petri dish. After setting, the plates could be kept at 4°C up to 1 month.

Total yeast DNA was prepared using the method of Sherman et al. 1986, with some modifications. The yeast strain containing the appropriate YAC DNA were streaked on an AHC plate and grown at 30°C for 3 to 7 days. A single pink colony was inoculated into 400 ml AHC medium with 100 µl of tetracycline (40 mg/ml) or YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose) at 30°C for 3 to 4 days (or until turbid) with shaking. The culture was transferred to a 400 ml Beckman bottle (autoclaved) and cooled on ice for 15-30 min. The bottle was then placed in JA10 rotor and spun down with 5000 rpm at 4°C for 10 min. The pellet was resuspended in 3 ml mixture of 0.9M sorbitol and 0.1M EDTA, added with 50 µl of 40 mg/ml lyticase and incubated at 37°C for 60 min. The mixture was spun down at 5000 rpm for 5 min, the supernatant was removed and the pellet was resuspended in 5 ml of 50mM Tris, 20 mM EDTA. A further 0.5 ml of 10% SDS was added and the suspension was incubated at 65°C for 30 min. Subsequently, 1.5 ml of 5M potassium acetate was added and the suspension was put on ice for 60 min,

then spun at 10000 rpm to a JA20 rotor for 10 min. The supernatant was collected and 2 volumes of ethanol were added, mixed and spun at 5000 rpm for 15 min for precipitation. After the supernatant was removed, the pellet was dried at room temperature overnight, and resuspended in 3 ml of 1× TE. The suspension was spun at 10000 rpm in the centrifuge (JA20) for 15 min and the supernatant was transferred to a new tube into which 200 µl of 1 mg/ml RNase was added and further incubated for 60 min at 37°C. After the incubation, 1 volume of isopropanol was added and mixed gently by shaking. After spinning mixture at 10000 rpm for 15 min, discarding the supernatant, and drying pellet, 0.5 ml of 10mM Tris was added to resuspend the pellet.

2.11 DNA sequencing, oligonucleotide synthesis and purification

2.11.1 DNA sequencing

Cloned DNA was sequenced by using an ABI PRISM™ dye primer cycle sequencing ready reaction kit (Perkin Elmer). The reaction were analyzed on an Applied Biosytems Model 373A DNA sequencer. Analysis of sequence was performed using the BLASTN program (email address <blast@ncbi.nlm.ni.gov>) to search for sequence homology between isolated, cloned DNA and anything on the database.

2.11.2 Oligonucleotide synthesis and purification

The design of primers used the suggestions of Lowe et al. (1990). Briefly, the primers should: (1) be approximate 50% in GC content; (2) be at least 18 bp in length; (approximately 24 bp preferred); (3) not contain runs of identical bases; (4) not contain four contiguous base pair of inter-strand nor intra-strand complementarity. Primers were synthesized using an Applied Biosystems Model 391 PCRMATE EP DNA synthesizer and purified according to the protocol in the *Applied Biosystems User Manual* .

CHAPTER 3

MOLECULAR CHARACTERIZATION OF TWO SMALL ACCESSORY RING CHROMOSOMES DERIVED FROM CHROMOSOME 4

3.1 Introduction

Extra small ring chromosome arising from the same chromosome could be associated with different phenotypes because of the variation in the genetic contents of different rings. The development of *in situ* hybridization with centromeric probe allowed determination of the origin of such ring chromosomes and revealed considerable complexity (Blennow et al. 1993, Callen et al. 1990b, 1991, 1992a, Plattner et al. 1993a). However, the precise definition of the genetic content of ring chromosomes could not be made with this method.

In the past few years, the combination of microdissection, PCR amplification and FISH or reverse painting, has been applied to analyze the chromosome rearrangements in both cancer and hereditary disease (Guan et al. 1993, Meltzer et al. 1992). However, this method does not distinguish between repetitive DNA and single-copy DNA in the microdissected products.

In the present study, an extra small ring chromosome in a patient with phenotypic abnormalities was characterized in detail by microdissection and molecular cloning. The aim was to precisely determine the origin and genetic content of the ring chromosome, to elucidate phenotype-genotype relationship and therefore to provide a better basis for genetic counselling.

3.2 Patients, Materials and Methods

3.2.1 Patients and chromosome materials

Patient A: This patient has moderate mental retardation and minor anomalies consisting of macrocephaly, plagiocephaly, brachiocephaly, epicanthic folds, flat midface with relative prognathism, malocclusion, high arched palate, hypoplastic ala nasi, thin upper lip, short and broad neck, and small hands and feet (Callen et al. 1992a). The karyotype is 46,XY/47,XY,+mar with the ring being less than the width of a chromosome in size. Short-term lymphocyte cultures showed 30% of cells with the marker in 100 cells scored. Fibroblast cultures showed a similar proportion of the ring but the ring was lost during subsequent subculture. Variation in the size of this ring was not observed in any cultures. Parental karyotypes were normal.

Patient B: Pregnancy history and birth of this patient was unremarkable but motor development was delayed from birth. In his late teens, he was diagnosed with insulin-dependent diabetes mellitus. Assessment at age 27 years showed severe mental retardation with no development of language skills. Height was 160 cm, weight was 76 kg and head circumference was at 98th percentile. He had central obesity with gynecomastia and kyphosis. Minor dysmorphism includes narrow forehead and ridged occiput, downward slanting palpebral fissures, downturned mouth, short philtrum, narrow pinna, narrow fingers with bilateral clinodactyly of the fifth finger and syndactyly of toes 2 and 3. The karyotype was a 47,XY,+mar. The size of the ring was less than the width of a chromosome. The marker was *de novo* as parental karyotypes were normal.

3.2.2 Microdissection and amplification of chromosomal DNA

Short-term lymphocyte cultures from peripheral blood of patient A were harvested with minimal exposure to acetic acid and spread on glass coverslips. The

ring chromosome was stained with Leishman's stain and dissected under an inverted microscope with the use of fine glass needles controlled by an electronic micromanipulator (Eppendorf model 5170). Fifteen dissected chromosomes were PCR amplified by a sequence-independent amplification procedure as detailed by Bohlander et al. (1992). In brief, the procedure involves addition of buffer and the primer 5'-TGGTAGCTCTTGATCANNNNN-3' to the microdissected products. Two cycles of amplification with T7 DNA polymerase allow nonspecific amplification of DNA. The products of this amplification were added to a final volume of 30 μl with 1.5 μM of a universal primer: 5'-CUACUACUACUAGAGTTGGTAGCTCTTGATC-3', 200 μM each dNTP, 1.5 mM $MgCl_2$, 55 mM KCl, 6.6 mM Tris-HCl (pH 9.0), 0.1 mg gelatin/ml, and 1.5 U Taq polymerase. The reaction was performed for 15 cycles at 94°C for 1 min, at 56°C for 1.5 min, and at 72°C for 2 min. A no-DNA control was used to test for possible contamination. A Southern blot using total human DNA as probe showed that the further amplified PCR products were a smear in comparison with primary PCR products, and the no-DNA control lane was blank. The microdissection and initial PCR amplification were performed by Dr. Bohlander, Section of Hematology/Oncology, The University of Chicago, Chicago.

3.2.3 Cloning and analysis of microdissected products

PCR products were purified with the Magic™ PCR DNA Purification System (Promega) and cloned into the vector pAMP10 (BRL). Colonies were transferred to Hybond-N^+ nylon membrane (Amersham) and probed with ^{32}P labelled total human DNA to identify those clones containing high copy repeated DNA. For colonies that did not contain repeats the insert was amplified by PCR using the pAMP10 vector oligoprimers: forward, 5'-CACGACGTTGTAAAACGACGGCCAGT-3', and reverse, 5'-TAATACGACTCACTATAGGG-3'. Insert sizes were estimated after electrophoresis on agarose gels and those > 300bp were selected for further study.

PCR-amplified products from these selected clones were labelled with ^{32}P and used to probe filters from Southern blots of restricted DNAs from total human, mouse (A9) and mouse/human hybrids, CY120 and CY170 (Table 3-1). The mouse line A9 was also used as a control as it was the parental line of these somatic cell hybrids. Since these hybrids also contain human chromosome 16, a hybrid containing only chromosome 16 (CY18), was used as an additional control.

Table 3-1 Chromosome constitution of mouse/human hybrids

Hybrid line	Portion Autosome Present	Other Humans Present	References
CY120	4pter→q25	16	(Callen et al. 1990c)
CY170	4pter→q35	5, 16	(Callen et al. 1990c, d)
CY18	complete 16	absent	(Callen et al. 1986)

3.2.4 Screening of human chromosome 4 cosmid library

PCR-amplified products that were unique and mapped to chromosome 4 were individually kinase labelled with ^{32}P and used to screen filters of a high density arrayed chromosome 4 cosmid library (Riess et al. 1994). This work was kindly accomplished by Drs Träger and Riess, Department of Molecular Human Genetics, Ruhr-University, Bochum, Germany. Presumptive positive cosmids were confirmed by using the labelled DNA of the microdissected clones to probe filters of Southern blots of the restricted cosmid DNA.

3.2.5 Fluorescence *in situ* hybridization (FISH)

The FISH procedure was as described in Chapter 2. A fluorescein isothiocynate-labelled avidin/antibody step was used to amplify hybridization signal. Reverse painting was performed by FISH using PCR-amplified microdissected products labelled with biotin by nick translation. Biotin labelled DNA from cosmids

and a YAC was hybridized to metaphase spreads from the patients. The YAC clone, My884E7, was purchased from Research Genetics and was the most proximal member of the chromosome 4 short arm YAC contig. Counter-staining of the propidium iodide stained chromosomes with DAPI allowed identification of chromosomes and location of signal to chromosome bands. Images of metaphase preparations were recorded on colour slides or captured by a CCD camera and were computer enhanced.

3.3 Results

Reverse painting using the biotin-labelled microdissected PCR-amplified small ring chromosome 4 from patient A showed signal at the centromere of chromosome 4 and D group chromosomes (Fig. 3-1). In addition, signal was located at 4q31. The origin of the ring from patient B was chromosome 4 by FISH studies using a probe specific for the centromere of this chromosome (D4Z1, ONCOR) and by a whole chromosome 4 library, (pBS4, gift of Dr. Pinkel, University of California, San Francisco). A tentative diagnosis of partial trisomy 4q11 to q13 was established for this patient. This FISH study for patient B was performed by Dr. Estop, Human Genetics Department, Hahnemann University Medical College of Pennsylvania, Philadelphia.

The microdissected products were then used to construct a small library in pAMP10. In a total of 137 colonies, colony blots probed with total human DNA showed 7 clones were positive and therefore were considered to contain high or moderately repetitive DNA (Fig. 3-2). The remaining clones may contain single-copy DNA or low copy DNA, while some of them may have no insert. Colony PCR was performed to check which clones carried an insert and the results indicated that 53 colonies contained inserts (Fig. 3-3). Sixteen clones (Y7, Y37, Y40, Y42, Y51,

Y56, Y57, Y61, Y62, Y73, Y85, Y86, Y87, Y112, Y117, and Y136) with inserts of human DNA ranging from 300 to 600bp were randomly selected for further analysis.

Probing DNA from somatic cell hybrids (Fig. 3-4) demonstrated that 12 out of these 16 clones (Y37, Y40, Y42, Y51, Y62, Y56, Y57, Y73, Y85, Y87, Y112 and Y136) were located on chromosome 4 since there was hybridization to the somatic cell hybrid CY170 which contained an intact chromosome 4. Within these 12 clones, nine (Y37, Y40, Y51, Y56, Y57, Y62, Y85, Y112 and Y136) had a localization on the long arm of chromosome 4, distal to 4q25, since they also mapped to CY120. Moreover, clones Y56 and Y57 not only had a band (4.36 kb) on CY120, CY170 and total human DNA, but also had a band (4.69kb) on all hybrids, including A9 (mouse DNA), suggesting that these two clones might contain DNA homologous for both human and mouse. The other three clones (Y42, Y73 and Y87) were negative for CY120 indicating that they might have a localization on the short arm of chromosome 4.

The remaining 4 clones (Y7, Y61, Y73 and Y86), which were positive in colony PCR, were negative for all hybrids, total human DNA and mouse DNA. The failure of hybridization may be caused by the loss of inserts from these clones.

To allow localization of these microdissected probes by FISH, cosmids were screened from a gridded human chromosome 4 library by probing with PCR product for each isolated clone (Y37, Y51, Y62, Y87, Y112 and Y136). Four to seven cosmids were positive with each probe and some cosmids were positive with at least two probes (Table 3-2). The positive cosmids from initial screening were further checked by using the labelled DNA of each clone to probe filters of Southern blots of the restricted cosmid DNA (Table 3-2).

Finally confirmed positive cosmids, 142B3, 234C8, 35H2, 77G3, 269G2 and 69F1 were used for FISH studies and the results are illustrated in Figure 3-5A, B and summarised in Table 3-3. Additional FISH studies were undertaken with other

cosmids 177C6, 176F1, 15F9 and 8G3 and a YAC (My884E7) that map to various regions of chromosome 4 (Table 3-3). These results demonstrate that the ring chromosome 4 of patient A consists of three discontinuous regions of this chromosome: the centromere (probe to pericentromeric repeat, D4Z1); the region of euchromatin on the long arm at 4q31 (cosmids 142B3, 234C8, 35H2, 77G3, and 69F1); and a region of the short arm at 4p13 or 14 (cosmid 269G2). The cosmid 176F1, located proximal to the centromere on the long arm at 4q12, did not hybridize to the ring of patient A but did hybridize to the ring of patient B. The ring of patient B is likely to contain a contiguous region from the centromere to 4q12. These results are illustrated in Figure 3-5 (C and D). Cosmids 177C6, 15F9 and 8G3 and the YAC (My884E7 at 4p11) did not hybridize to the ring chromosomes of either of the two patients.

Table 3-2 Positive cosmids generated from initial screening and further confirmation by hybridization of clones with microdissected DNA

Clones	Positive cosmids from initial screening	Positive cosmids further confirmed
Y37	142B3, 174A2, 175H1, 268B3	142B3
Y51	**35H2, 57F11, 28D8,** 234C8, 235D4	28D8, 35H2, 234C8, 235D4
Y62	**35H2, 57F11, 28D8, 8C4,** 15F5,147F4, 152F6	8C4, 15F5, 28D8, 35H2, 57F11
Y87	70E8, 86C8, 175H1, 269G2	70E8, 86C8, and 269G2
Y112	**8C4**, 77G3, 142B2, 147E9, 249C8	77G3
Y136	69F1, 83D1, 241G11, 241G12	69F1, 83D1, 241G11, 241G12

Table 3-3 Summary of FISH to patients with small ring

Probe[a]	Cosmids	Location[b]	Ring Chromosome[c] patient A	Ring Chromosome[c] patient B
. . . .	177C6	4pter	–	–
Y87	269G2	4p13-14	+	–
My 884E7 ..	. . .	4p11	–	–
D4Z1	. . .	centromere	+	+
. . . .	176F1	4q12	–	+
. . . .	15F9	4q21	–	–
. . . .	8G3	4q25	–	–
Y37	142B3	4q31	+	–
Y51	234C8	4q31	+	–
Y51, Y62 ..	35H2	4q31	+	–
Y112	77G3	4q31	+	–
Y136	69F1	4q31	+	–

[a] The probes with the "Y" prefix are derived by microdissection from the ring of patient A.

[b] The location of the markers was determined from hybridization to normal chromosome 4s in metaphases of the patients.

[c] A plus sign indicates that probe hybridized to marker chromosome; a minus sign indicates that probe did not hybridize to marker chromosome.

3.4 Discussion

To investigate the structure of the accessary ring chromosome 4 in patient A, the ring was microdissected and DNA was amplified by degenerate-primer PCR. After cloning the microdissected products and mapping the clones to mouse/human hybrids containing human chromosome 4, six single copy clones were used to further isolate cosmids. FISH studies with these cosmids, and with additional markers on chromosome 4, demonstrated that the small ring in this patient consisted of three noncontiguous regions of chromosome 4. These regions were at the vicinity of 4p13 to 14 and the centromere, and at 4q31 (Fig. 3-6). Reverse painting with PCR amplified microdissected DNA demonstrated signal at the centromere and at 4q31. No signal could be seen at 4p13 to p14. Therefore, FISH with cosmid which were generated from cloned microdissected DNA is more sensitive than reverse painting. FISH studies of the ring from patient B were consistent with an origin from a contiguous region from the centromere to 4q12 (Fig. 3-6). However, in view of the results from patient A, it is possible that this marker may also contain non-contiguous DNA from this chromosome. This issue can only be resolved by microdissection of the ring and analysis of the cloned DNA. Both patients had in common mental retardation and similar minor dysmorphic features (brachiocephaly, downward slanting eyes, downturned mouth) which are evident in their facial resemblance. These shared phenotypic abnormalities can only have a common genetic basis if there is pericentric euchromatin in common between the two rings, which has not been detected by the present study unless it is the euchromatin immediately adjacent to the centromere. A previous approach to the analysis of marker chromosomes is by FISH using the PCR-amplified DNA from the microdissected marker as a probe (Thangavelu et al. 1994, Viersbach et al. 1994). However, in patient A this technique was not sufficiently sensitive to detect a small non-contiguous region of euchromatin present in the short arm of chromosome 4, although the larger region on

the long arm was detected. The microdissected DNA is a complex probe containing amplified products originating from the highly repetitive centromeric and pericentromeric repeats. The presence of these repeats may limit the sensitivity of this procedure in detecting small regions of euchromatin.

There are numerous patients reported with a 46 chromosome complement where one entire chromosome is a ring. These rings are thought to arise by breaks at both ends of the chromosome and subsequent fusion of the open ends (Kosztolányi 1987). FISH studies using the consensus telomeric sequences as probes have established that such rings can form without loss of genetic material, although the phenotype of patients with such rings can be abnormal, with failure to thrive and dysmorphism (Pezzolo et al. 1993b). This so-called "ring syndrome" is thought to be caused by ring chromosome instability continuously generating aneuploid cells, which are subsequently lost. Instability of ring chromosomes in *in vitro* culture has been well documented. For example, Carter et al. (1969) reported a ring chromosome 4 in a 46, XY karyotype. A variety of derivatives of this ring, ranging from dicentric rings to various smaller products were found, presumably generated by breakage and reunion of interlocked rings formed at cell division. It was found that among the different breakdown products of this large ring 4 there were two examples in 200 cells scored of small rings of similar size to that found in the two patients of this report (E. Baker, personal communication).

The small ring marker chromosomes found as accessary chromosomes were considered to arise from one break at the centromere, a second break in close proximity on either the long or short arm of the chromosome and with subsequent rejoining of the broken ends (Callen et al. 1991). This second break may be a "U-type" exchange. The evidence for this mechanism was based on the observation that ring chromosomes from chromosomes 1, 9, or 16 were either distamycin A/DAPI positive with a normal phenotype, or distamycin A/DAPI negative with an abnormal

phenotype (Callen et al. 1990b, 1991). The distamycin A/DAPI negative rings were considered to arise by one break at the centromere and a second within the euchromatin of the short arm, while the distamycin A/DAPI positive ring originated by one break at the centromere and a second within the pericentric heterochromatin on the long arm.

Consistent with this origin is a small chromosome marker derived from chromosome 9 which was characterized by sorting, molecular cloning and in situ hybridization by Raimondi et al. (Raimondi et al. 1991). This chromosome contained the centromere and adjacent proximal region of the short arm of chromosome 9. The patient B reported here has a small ring chromosome derived from the centromere and adjacent proximal region of the long arm of chromosome 4 (see Fig.1-2a in Chapter 1).

The marker of patient A characterized in this report shows a complex structure and contains three discontinuous regions of chromosome 4 DNA. The origin of such a chromosome is consistent with the breakdown product from an original 47,+r(4) karyotype where the ring 4 was a typical large ring chromosome. It is suggested that this large ring was involved in breakage and reunion cycles as a result of the formation of interlocked rings during cell division. As a consequence, complex deletions of DNA could have occurred until the stable form was generated (Fig. 3-7).

In conclusion, the unexpected complex nature of a small ring derived from chromosome 4 adds a further complication to any attempt to ascertain common phenotypes between patients known to have similar markers derived from the same chromosome.

3.5 Summary

Molecular cloning of a microdissected small accessary ring chromosome 4 from a moderately retarded and dysmorphic patient has been performed to identify the origin of the ring chromosome. FISH was performed with cosmids identified with the cloned, microdissected products and with other markers from chromosome 4. The present study clearly demonstrates that the small ring in this patient originates from three discontinuous regions of chromosome 4: 4p13 or 14, the centromere, and 4q31. It is suggested that the origin of the ring chromosome is a ring involving the entire chromosome 4, which has then been involved in breakage and fusion events, as a consequence of DNA replication generating interlocked rings. A second severely retarded and dysmorphic patient also had a small accessary ring derived from chromosome 4. FISH studies of this ring are consistent with an origin from a contiguous region including the centromere to band 4q12. It is apparent that there are at least two mechanisms for the formation of small ring chromosomes. That adds a further complication in any attempt to ascertain common phenotypes between patients known to have morphologically similar markers derived from the same chromosome.

Fig. 3-1. Partial metaphase from patient A after *in situ* hybridisation with microdissected DNA labelled by biotin. Signals appeared on the centromere, 4q31 of normal chromosome 4s (arrowheads), D-group chromosomes, and the ring (arrow) .

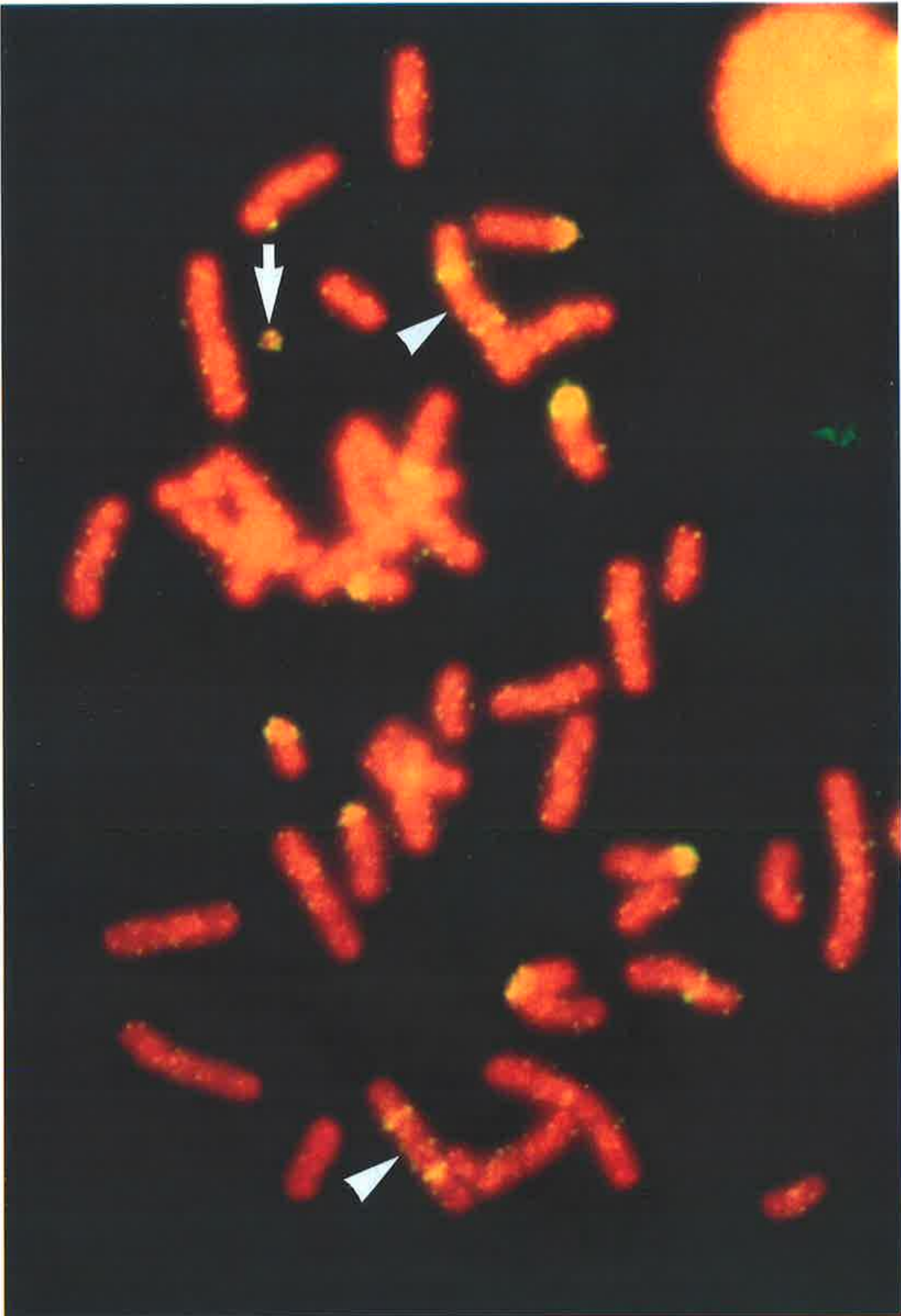
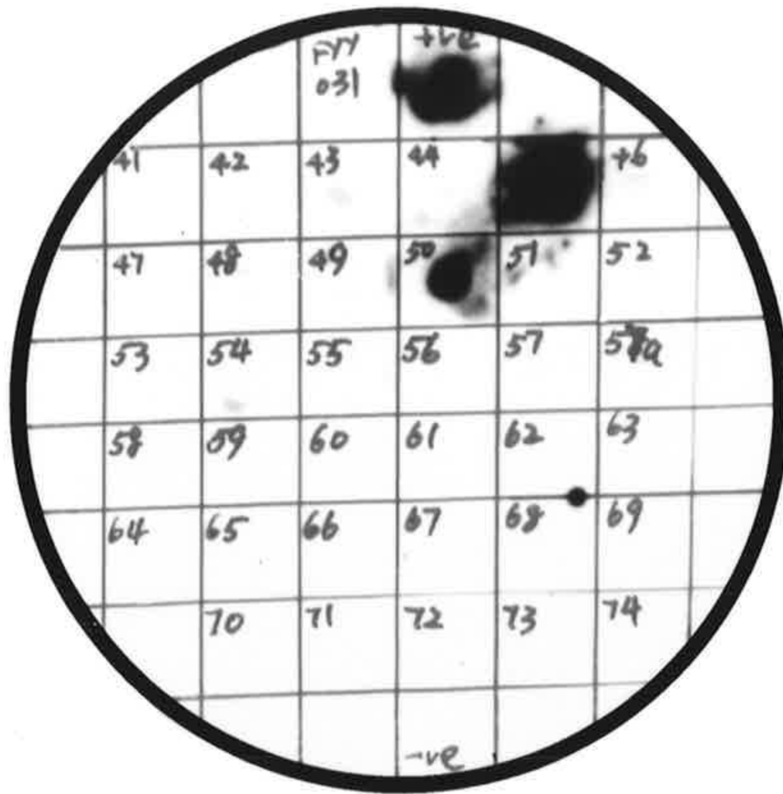


Fig. 3-2. Examples of colony blot, hybridization with total human DNA as a probe, showed positive clones 45, 50, 82 and 83 were positive and the remaining ones were negative in this library. The positive clone indicated that this one contained highly or moderately repetitive DNA while negative ones potentially contained single-copy DNA. "+ve" indicates positive controls in which total human DNA was used and "-ve" is negative controls in which plasmid containing the sequence of human chromosome 16 was used.


FYY 031
+ve
41
42
43
44
46
47
48
49
50
51
52
53
54
55
56
57
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
-ve

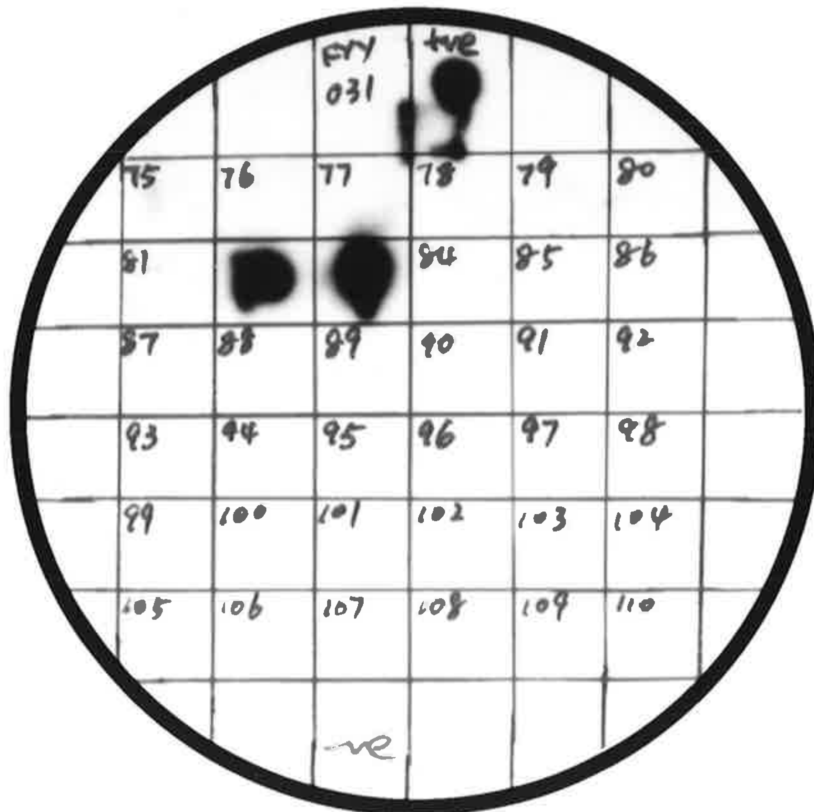

FYY 031
+ve
75
76
77
78
79
80
81
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
-ve

Fig. 3-3. Inserts recovered by PCR from 8 individual microclones (lanes 4-11 from left). The size of inserts were between 500bp to 800bp with included approximately 200bp vector sequence. The third lane is a control with vector sequence.

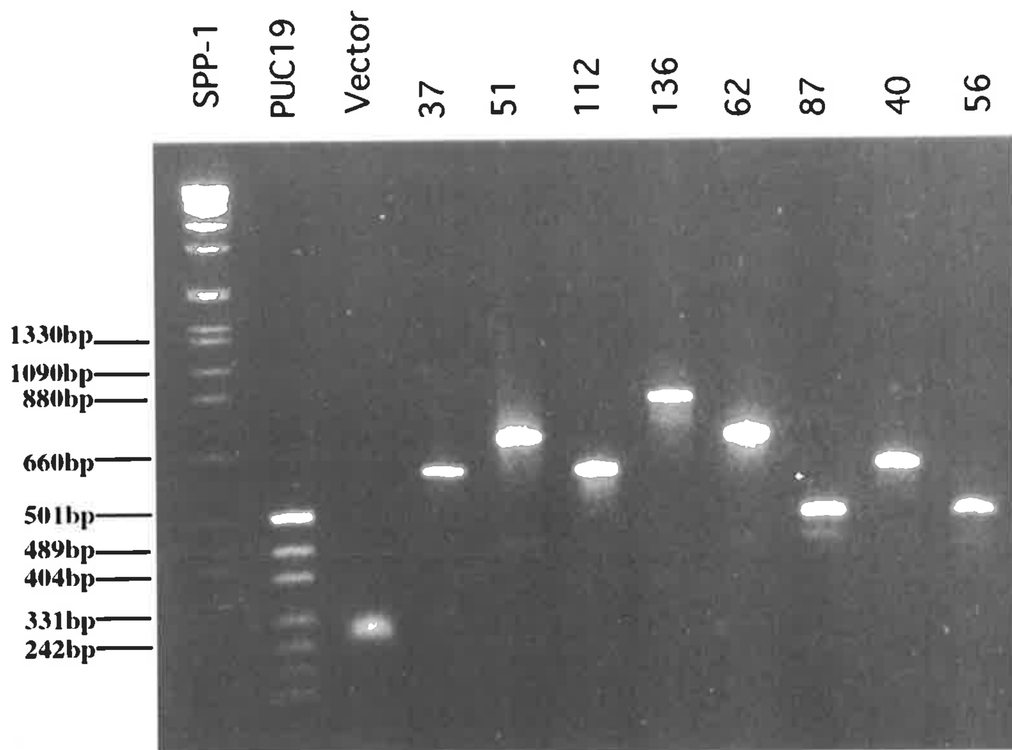
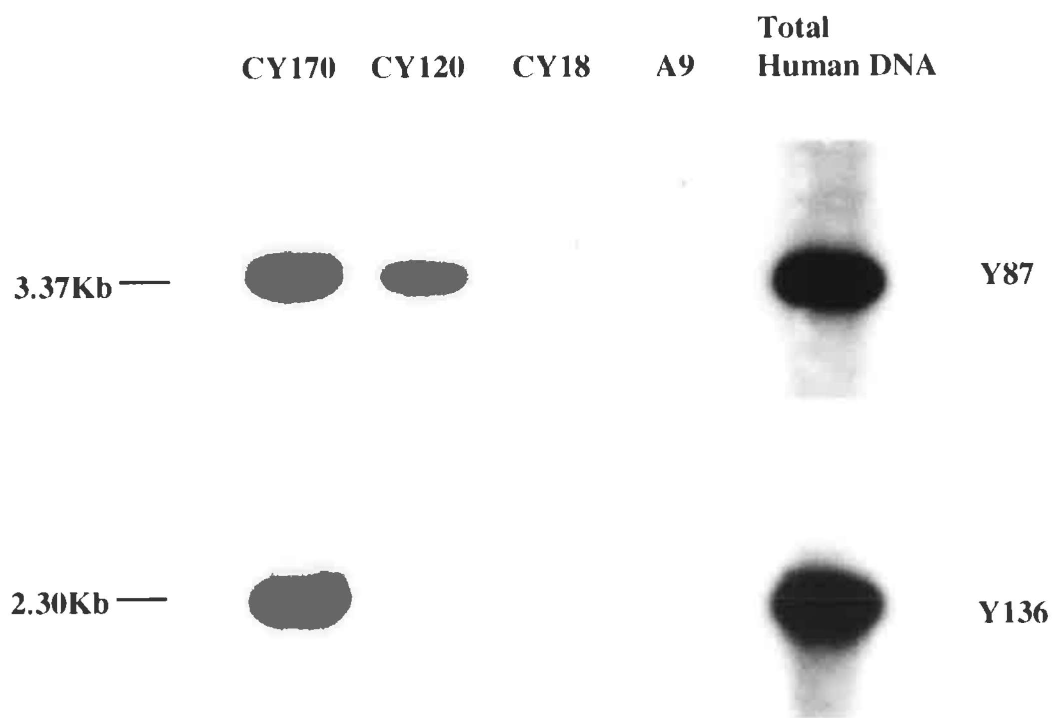
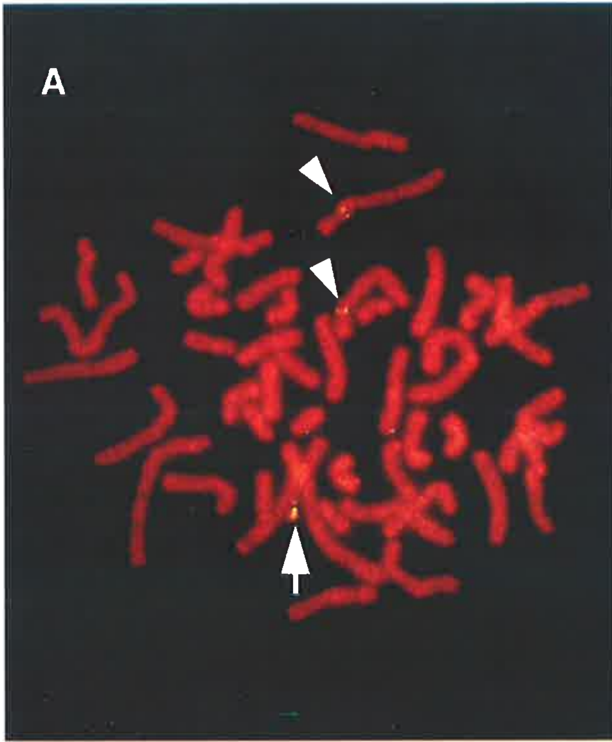

SPP-1
PUC19
Vector
37
51
112
136
62
87
40
56
1330bp
1090bp
880bp
660bp
501bp
489bp
404bp
331bp
242bp

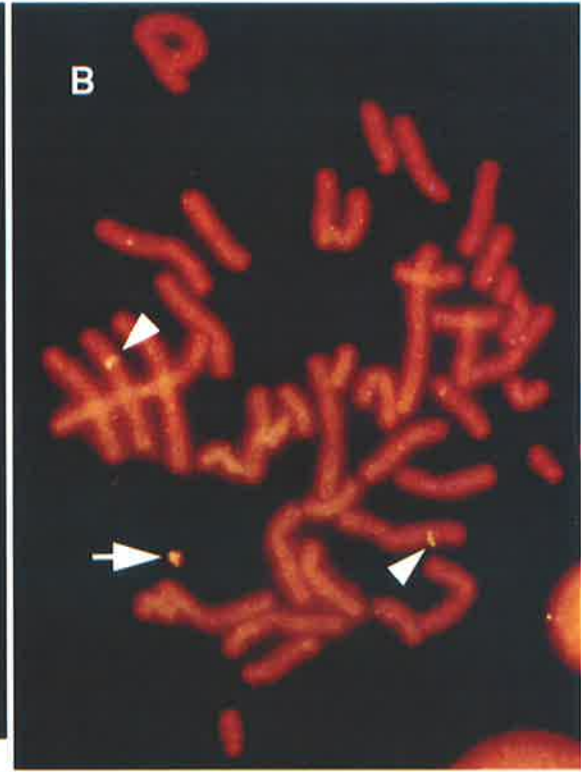
Fig. 3-4. Southern blots hybridization would use clones Y87 and Y136 as probes against Hind III-digested rodent/human hybrids CY170, CY120 and total human DNA. CY18 and A9 were as controls.

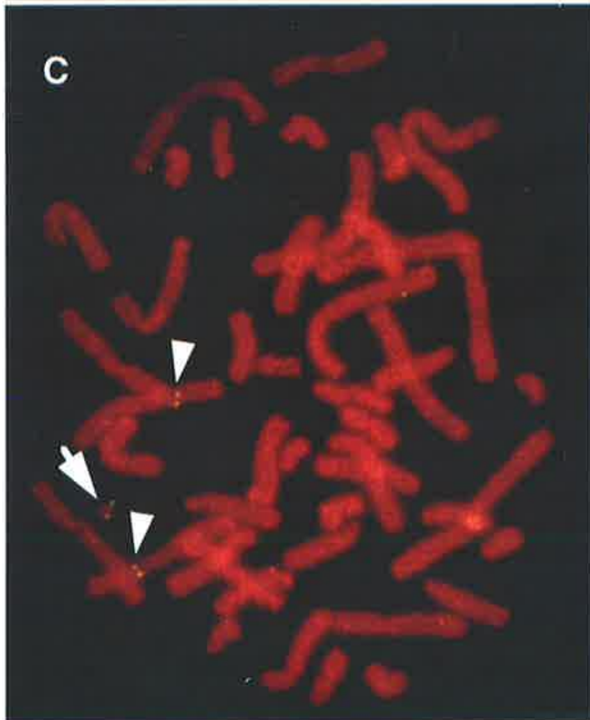


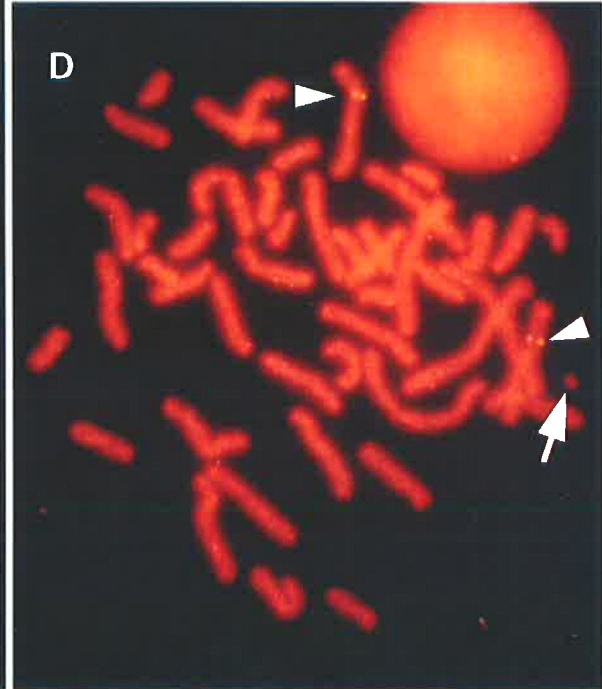
CY170
CY120
CY18
A9
Total Human DNA
3.37Kb
2.30Kb
Y87
Y136

Fig. 3-5. Two cosmids, 269G2 and 77G3, were separately located at 4p13-14 and 4q31.1 and also hybridized to the marker of patient A (**A, B**). Another chromosome 4 cosmid, 176F1, was located at 4q12 and hybridized to the marker of patient B (**C**), but did not hybridized to the patient A (**D**). The rings are indicated with arrows and the chromosome 4 homologues are indicated by arrowheads.


A


B


C


D

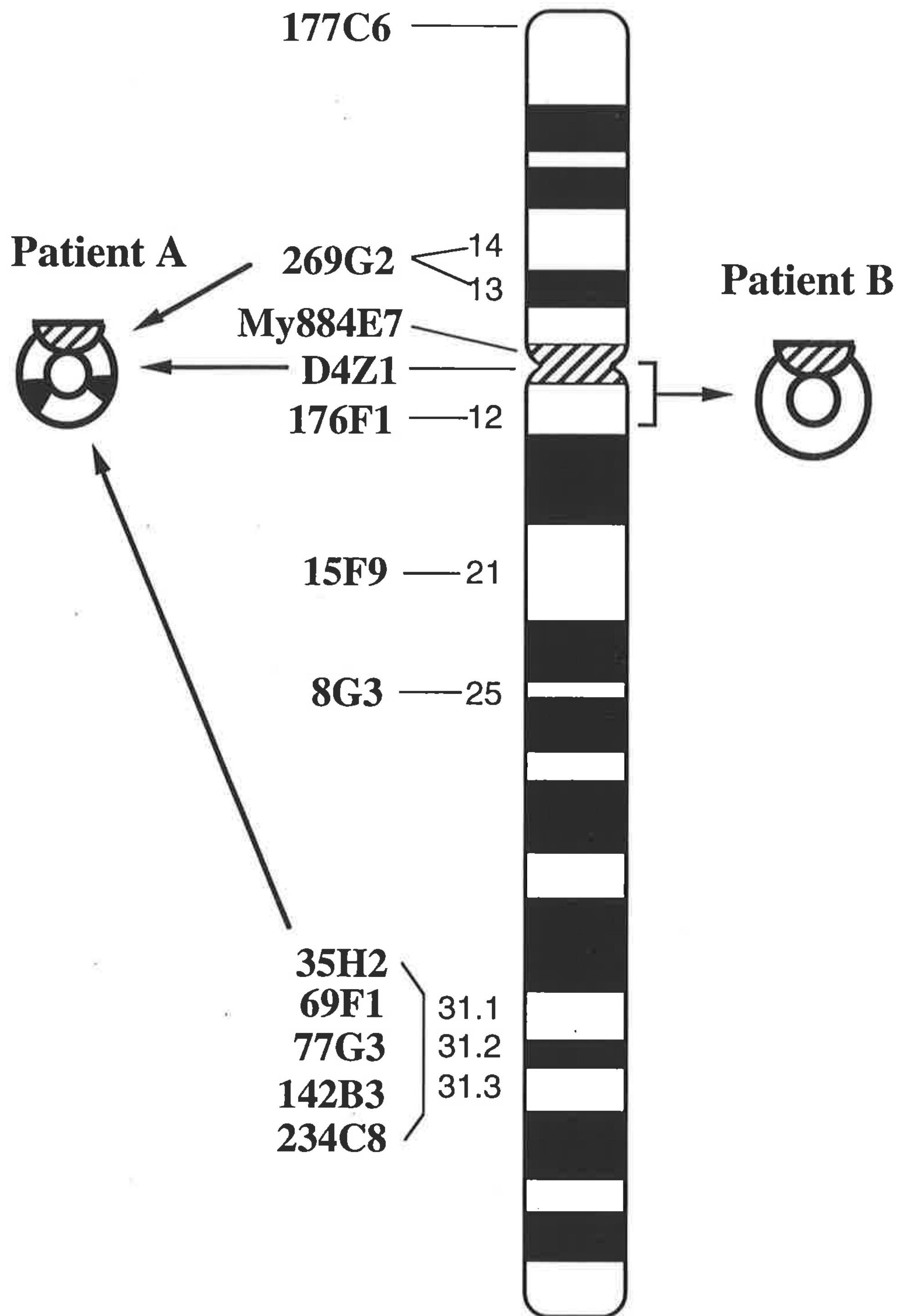


Fig. 3-6. Ideogram of chromosome 4 showing localization of cosmids and the YAC and origin of ring chromosomes in two patients.

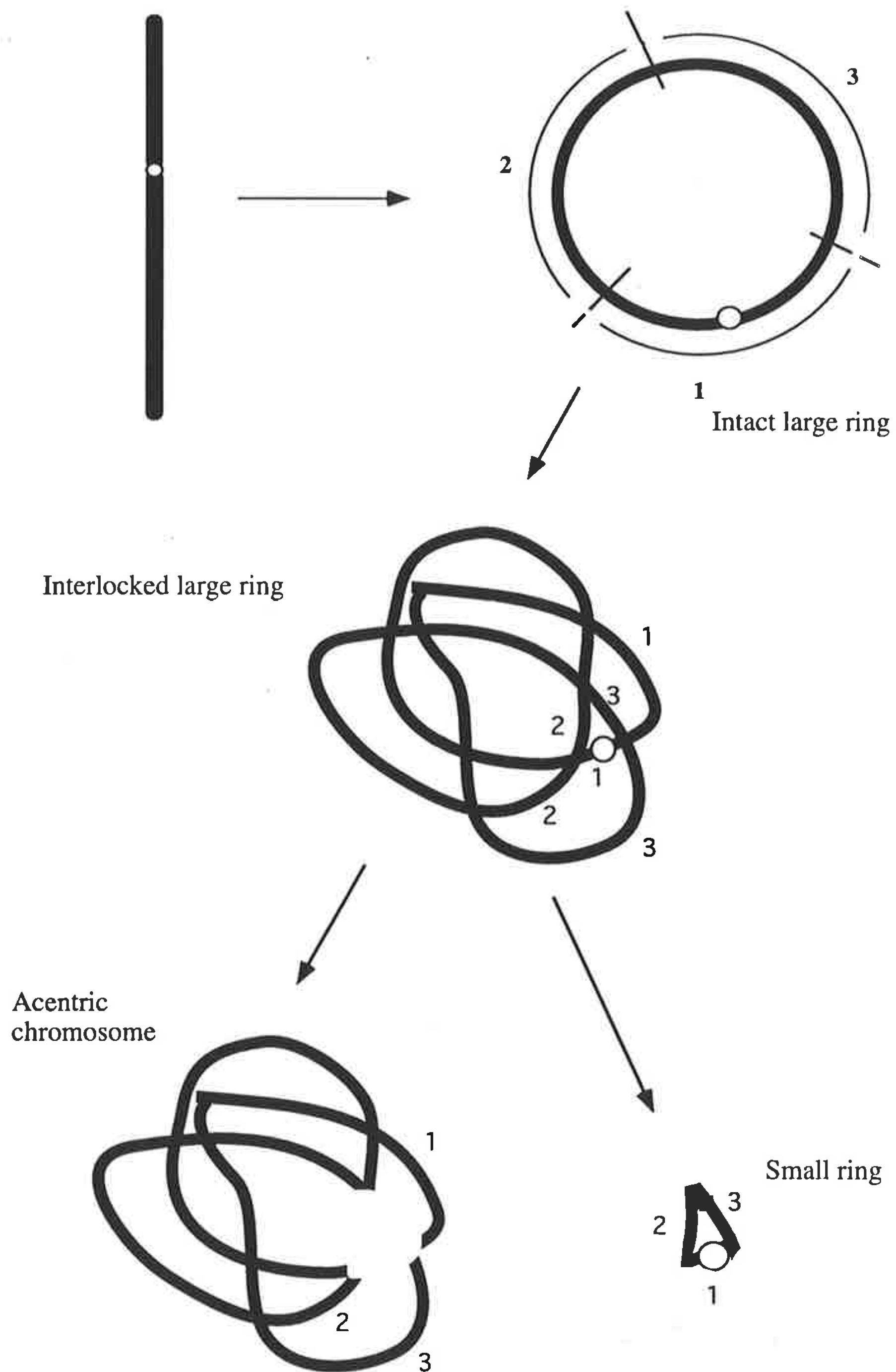


Fig. 3-7. A possible mechanism for the formation of small ring chromosome. The large ring formed and interlocked prior to breakage and reunion, resulting in the formation of a small ring with noncontiguous segments. The acentric fragment is presumed to be lost during cell division.

CHAPTER 4

CHARACTERIZATION OF MARKER CHROMOSOME 15s WITH MICRODISSECTION, MICROCLONING AND FISH

4.1 Introduction

Numerous cases of inv dup(15)s have been reported in the literature since the initial description by (Schreck et al. 1977). According to the International System for Human Cytogenetic Nomenclature (ISCN 1985), "inv dup(15)" is not the correct nomenclature to define these supernumerary markers. The terms "dic(15;15)", "psu dic(15;15)", iso(15p)" and "mar(15)" have each been proposed as an alternative. In order to avoid unnecessary confusion, here we retained the nomenclature of mar(15) since it is suitable for various extra small abnormal chromosomes derived from chromosome 15, including extra small ring chromosome 15s.

Mar(15)s are morphologically heterogeneous. Their sizes can be smaller or larger than G-group and their shapes can be either metacentrics, acrocentrics or ring-like. Their origins are usually from the region from centromere to 15q13. The region of 15q11-q13 has been known to be imprinted in humans (Hall 1990, Dahoun-Hadorn and Delozier-Blanchet 1990, Dittrich et al. 1993) and is associated with Prader-Willi syndrome (PWS)/Angelman syndrome (AS). For normal development, both a paternal and a maternal copy of that region is required. Loss of the paternal contribution by deletion or by maternal uniparental disomy for chromosome 15 can lead to Prader-Willi syndrome (PWS) phenotype (Donlon 1988). In contrast, loss of the maternal contribution by the same mechanisms results in Angelman syndrome (AS) (Nicholls et al. 1989).

To date, more than 80 cases of mar(15)s with metacentrics or acrocentrics have been reported (Buckton et al. 1985, Schreck et al. 1977, Wisniewski et al. 1979, Gillberg et al. 1991, Kirkilionis and Sergovich 1987, Mohandas et al. 1985, Plattner et al. 1993b, Schmid et al. 1986, Flejter et al. 1996, Crolla et al. 1995, Cheng et al. 1994, Mignon et al. 1996). Acrocentric mar(15)s have been found in patients with varying phenotypes, including mental retardation, seizures, behavioural problems, development delay, and dysmorphology. This type of mar(15)s can be found larger than a G-group chromosome, and includes inverted duplication of the bands 15q11-q13 (Cheng et al. 1994, Crolla et al. 1995), and therefore it is called an "inv dup(15)". In contrast, metacentric or submetacentric mar(15)s are found in normal individuals (Leana-Cox and Schwartz 1993). They are smaller than G-group chromosomes and the region of 15q11-13 are not involved (Cheng et al. 1994, Crolla et al. 1995) but it is still called an "inv dup(15)". Some small mar(15)s have been reported in PWS/AS patients. However, the syndrome could be attributed to uniparental disomy or a deletion of one of the "normal" chromosome rather than small mar(15)s per se (Robinson et al. 1993a, Spinner et al. 1995, Cheng et al. 1994).

Extra small ring chromosomes derived from 15 have only been reported in three patients (Callen et al. 1992a, Plattner et al. 1993b, Crolla et al. 1995). One case had mental retardation, delayed speech, and destructive behaviour. Cytogenetic study showed that the ring was approximately the size of a chromosome 21. In situ hybridization with the PWS/AS region cosmids SNRPN and GABRB3 were positive (Crolla et al. 1995), therefore this ring chromosome contained the imprinted region (15q11-q13). The phenotype in one case was not clear since the pregnancy was terminated (Callen et al. 1992a) , in the other case the answer whether the abnormal phenotype was caused by ring chromosome 15 was unknown since multiple rings derived from chromosomes 8, 14/22, 15 and X were also present (Plattner et al. 1993a).

In this study, microdissection and molecular microcloning have been used to characterize one extra small ring chromosome 15 in a patient with phenotypic abnormalities. The aim was to precisely determine the origin and genetic content of the ring and to compare the phenotype of this patient with the previously documented cases. In addition the PCR products of the microdissected DNA from this extra ring chromosome 15 was used as a FISH probe to the "inv dup(15)"s of two other patients. This allowed comparison between these two types of mar(15)s as to determine whether they were from the same origin and whether their genotypes could be related to their phenotypes.

4.2 Materials and Methods

4.2.1. Clinical details of patients

Patient C: This was a 10-year-old male and his parents were not consanguineous. He was noted to be a very active boy from the first one or two years of life. When he started school at 5 years of age, he had a high level of activity and problems in staying on task and problems with behaviour. His educational problems were significant, particularly in the area of language development. On examination as an 8-year-old, he had no dysmorphic features but he had generalized hypermobility of the joints. A psychological assessment indicated his cognitive functioning to be in the average range but he had a learning disability. The family history showed that his mother and a maternal uncle also found school difficult and had particular problems with language-based skills. His two young siblings, at the age of 1 and 4 years, were normal. His karyotype was 46,XY[28]/47,XY,+r[72]. His parents had normal karyotypes.

Patient D: This individual was a 36-year-old normal female. Karyotype was 47,XX,+mar and the size of this ESAC was smaller than a G-group chromosome and was bisatellited. C-banding and DA/DAPI staining were positive at one side of the

marker and this marker was considered to be derived from chromosome 15. Her mother and one of her brothers also possessed the same marker. His other six siblings and father had normal karyotypes.

Patient E: This patient was a male and his parents were nonconsanguinous. Pregnancy history was unremarkable but hypotonia was found at birth and epilepsy occurred from 5 months of age. Assessment at 31 years of age showed severe mental retardation, protruding tongue, mild scoliosis convex to right side, and minor dysmorphism included facial asymmetry, broad forehead, overhanging brow, strabismus, triangular face, and broad jaw tip. Karyotype showed 47,XY,+mar. The extra marker was bisatellited and the size of this ESAC was ≥ G-group. It was dicentric and considered to be derived from chromosome 15 since C-bands and DA/DAPI were positive at both ends of the marker. His brother had mild mental retardation and epilepsy. The karyotypes of his father and brother were unknown and his mother and three sisters were normal.

4.2.2. Preparation of metaphase chromosomes

Chromosome preparation of patients C, D and E was the same as described in Chapter 2. Metaphases in patient C were spread on clean coverslips (22 × 60mm) and stored at 37°C for 2-3 days. G-banding with trypsin-Giemsa (GTG) was performed on metaphase of patient C prior to microdissection.

4.2.3. Microdissection and amplification of chromosomal DNA

Microdissection of ring from patient C was performed with glass microneedles controlled by a Narashige micromanipulator attached to an inverted microscope as previously described (Meltzer et al. 1992). Microneedles were treated with UV light (Stratalinker, Stragagene) for 5 min prior to use. The dissected chromosome fragments adhered to the microneedle and they were transferred to a 20 μl collecting drop (containing proteinase-K 50 μg/ml) in a 0.5 ml microcentrifuge tube. A fresh

microneedle was used for each fragment dissected. For this library, 5 copies were dissected, the collection drops were incubated at 37°C for 1 hour, followed by 90°C for 10 min to inactivate the proteinase-K. The components of the PCR reaction were then added to a final volume of 50 μl in the same tube (1.5 μM universal primer -CCGACTCGAGNNNNNNATGTGG- (Telenius et al. 1992), 200 μM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.1 mg/ml gelatin, and 2.5 U Taq DNA polymerase (Perkin-Elmer/Cetus). The reaction was heated to 93°C for 4 min then cycled for 8 cycles at 94°C 1 min , at 30°C 1 min, and at 72°C 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 3 min at 72°C, with a 10-min final extension at 72°C. This work was kindly accomplished by Dr X.-Y. Guan of The University of Michigan Comprehensive Cancer Center, USA.

4.2.4. Molecular cloning

(1) Preparation of insert DNA: For cloning PCR amplified microdissected DNA, it was required to modify the preamplified PCR products. The components of the PCR reaction (50 μl) included 2 μM of primer (5-CUACUACUACUACCGACTCGAG -3), 200 μM each dNTP, 2.5 U Taq DNA, and 2 μl of preamplified PCR product in PCR buffer (Boehringer Mannheim). The reaction was then cycled by an initial 6 cycles at 94°C for 0.5 min, 48°C for 0.5 min, 72°C for 2 min, followed by 14 cycles at 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 2 min. For further amplification, 2 μl PCR product was added in 400 μl of PCR reaction with the same final concentration of reagents as above and amplified for 4 cycles at 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 2 min, extension at 72°C for 10 min. PCR products were purified by QIAquick PCR Purification Kit (QIAGEN) prior to cloning.

(2) Preparation of vector DNA: Two vectors, pGEM-3Zf(+) and pUC19 were independently linealized by digestion with X*ba*I. The digestion was performed by adding 1 μg of pGEM-3Zf(+) (or pUC19) to 20 μl of solution containing 2 μg BSA,

2 μl of 10× buffer, and 1 μl of X*ba*I (Biolabs) and incubated at 37°C for overnight. Both digested vectors (12.5 ng for each) were mixed and amplified by PCR using the primer V1 (5-UAGUAGUAGUAGGGAGCCCCGGGT-3) and V2 (5-UAGUAGUAGUAGGTCGACCTGCAG-3). PCR reaction solution (400 μl) included (final concentration) 250μM each dNTP, 1.1mM Mg^{2+}, 0.25 pMol/μl of primer V_1 and V_2, 0.25 ng mixture of pGEM-Zf(+) and pUC19, and 2 μl of Taq polymerase from Advantage™ KlenTaq polymerase mix (Clontech). The thermal cycling program was 24 cycles at 94°C for 0.25 min, 55°C for 0.5 min, and 72°C for 10 min. The PCR product was purified by QIAquick PCR Purification Kit (QIAGEN). This amplified vector DNA resulted in ends compatible with insert and suitable for ligation.

(3) Ligation and transformation: Fifty ng each of vector and inserts from PCR amplified microdissected DNA were added to 20 μl of ligation solution with 1 μl of BRL uracil N glycosylase enzyme (1U/μl) and incubated at 37°C for 30 min. Transformation was carried out by adding 5 μl of this solution to 100 μl competent cells (JM109). The details of making competent cell from bacteria and transformation procedures have been described in Chapter 2.

4.2.5. Analysis of microdissected products

Colony blot was performed to identify the clones containing high copy repeat DNA by hybridization of ^{32}P labelled genomic DNA to those clones. Colonies that were negative in colony blot potentially contained single-copy DNA and were amplified by PCR using identical oligoprimers from the vectors of pGEM-3Zf(+) and pUC19 (same primers can be used for either vector): forward, 5'-CACGACGTTGTAAAACGACGGCCAGT-3', and reverse, 5'-TGTGAGCGGATAACAATTTCACACAGG-3'. Insert sizes were estimated from agarose gels and those large than 300bp were selected for further study.

The PCR-amplified products from these selected clones were digested by *Eco*RI and *Pst*I to release the insert from the vector. These insert DNAs were labelled with ^{32}P and used to probe filters from Southern blots of digested DNAs from total human DNA, mouse DNA (A9) and mouse/human hybrids, CY126, CY9 and CY18 (Table 4-1). CY18 and A9 were used as controls.

Table 4-1 Chromosome constitution of mouse/human hybrids

Hybrid line*	Humans chromosomes present	References
CY126	5, 6, 7, 13, 15, 16, 18, 19, ±11, ±20, 21	(Shen et al. 1994)
CY9	4, 13, 15, 16, 21, 22	(Callen et al. 1990c, d)
CY18	complete 16	(Callen et al. 1986)

*Each hybrid contained total mouse DNA (A9).

Four clones were sequenced and oligoprimers were designed to check the origin of cloned, microdissected products by amplification of two hybrid DNAs, GM/NA10898 containing chromosome 13 and GM/NA11418 containing chromosome 15.

4.2.6. Screening of genomic PAC library

PCR-amplified products that were unique and mapped to chromosome 15 were digested by *Eco*RI and *Pst*I to obtain the insert. These inserts were mixed and labelled with ^{32}P by the Megaprime^{TM} DNA labelling systems to screen filters of a high density arrayed genomic PAC library (Genome systems, Inc) (see Chapter 2). Positive PACs were further purified by QIAGEN Plasmid Kit (QIAGEN) (see Chapter 2) and randomly selected for FISH study.

4.2.7. Fluorescence in situ hybridization (FISH)

FISH was applied to localize the origin of the ring chromosome using two types of probes. In reverse painting, the PCR product from the microdissected ring chromosome DNA was hybridized to the ring chromosome. To generate probes for reverse painting, initial PCR amplified microdissected DNA (2 µl) was labelled with spectrum-orange in a secondary PCR reaction identical to that described in section 4.2.3 (Amplification of chromosomal DNA) except for the addition of 20 µM of spectrum-orange-dUTP (BMB). The reaction was continued for 12 cycles of 1 min at 94°C, 1 min at 56°C, and 3 min at 72°C, with 10 min final extension at 72°C. The products of this reaction were purified with a Centricon 30 filter and used for FISH.

Isolated PACs, commercial cosmid PWS/AS region A and region B probes (Oncor, Gaithersburg, MD) and mixed clones containing high repeat DNA were also used for FISH on chromosome metaphase. The detailed procedure for FISH has been described in Chapter 2.

4.2.8. Combination of Distamycin A/DAPI with FISH

The metaphase spreads from patients D and E were prepared as described in Chapter 2. This combination staining was performed by inserting the FISH step between the conventional Distamycin A/DAPI staining. Distamycin A (0.05-0.1 mg/ml) was dissolved in McIlvaine's buffer (pH 7.0) and stored at -20°C in small aliquots. DAPI (0.8 µg/ml) was dissolved in McIlvaine's buffer and stored at 4°C. The DAPI concentration used here was different from that used for mounting slides following FISH.

The slides were stained with 1-2 drops of Distamycin A, coverslipped, and kept in the dark at room temperature for 30 min. The coverslip was washed off and the slide was briefly rinsed with distilled water and dried. FISH (see section 2.2 of Chapter 2) was performed with spectrum-orange labelled PCR-microdissected DNA derived from the ring chromosome of patient C. After overnight hybridization and

appropriate washing, 1-2 drops of DAPI were added to the slides which were coverslipped and kept in the dark at room temperature for 30 min. The coverslip was washed off, and the slide was briefly rinsed with distilled water and the slides were mounted in one drop of antifade solution (0.18 g DABCO, 1 ml of 20mM Tris pH 7.5 and 9 ml glycerol).

4.3 Results

4.3.1. Characterization of cloned, microdissected ring chromosome

PCR products from the microdissected ring chromosomes from patient C were cloned into pGEM-3Zf(+) and pUC19 vectors to produce a small library containing 260 clones. Colony blot probed with total human DNA showed that 23 clones were likely to contain highly or moderately repetitive DNA since there was a strong positive signal. The remaining clones were negative and potentially contained single-copy or low copy DNA, although some negative clones might contain only vector DNA (Fig. 4-1). Colony PCR was carried out on 208 colonies that were negative on colony blot, to recover the inserts from those clones (Fig. 4-2). Fifty-two clones with inserts larger than 300bp were digested by *Eco*RI and *Pst*I for further analysis (Fig. 4-3). Probing DNA from somatic cell hybrids (CY126, CY9 and CY18) demonstrated that four clones, W5, W59, W110 and W172, mapped to chromosome 15 since there was hybridization to the somatic cell hybrids CY126 and CY9 but not to CY18 (Fig. 4-4). These four clones represented four different sizes of inserts. Inserts from most of the remaining clones had the same size as in these 4 clones. Few other clones did not hybridize to somatic cell hybrids and total human DNA at all.

The clones W5, W59, W110 and W172 were sequenced (Fig. 4-5A, B) using dye primer cycle sequencing ready reaction kit (see Chapter 2) and oligoprimers were synthesized from clone 59 (forward: 5'-GTAGGTCTGGTGATAATGAATTCC-3',

reverse: 5'-GAGATGAGGAGAATGGAACCAAC-3') (Fig. 4-5A, B) and clone 172 (forward: 5-GGACCATGTTTAAACTTCTGAGT-3, reverse: 5'-CAGAGTTGAATGATGCTCTTGG-3'). PCR amplified DNA from hybrid, GM/NA11418 containing human chromosome 15, GM/NS10898 containing human chromosome 13 and total human DNA using the primers from clone W59 and W172 showed that a band was on GM/NA11418 and total human DNA but not on GM/NS10898 (Fig. 4-6). The band for W59 was 489 bp and the band for W172 was 500 bp. These results suggested that clone W59 and W172 contained insert sequence derived from chromosome 15.

4.3.2. Fluorescence in situ hybridization

Reverse painting using the spectrum-orange labelled microdissected PCR-amplified DNA from the small ring chromosome 15 from patient C to metaphases of the same patient showed that the signal was on the ring and at the region from the centromere to q11 of chromosome 15 (Fig. 4-7A).

To confirm the reverse painting result, and to refine the origin of this ring chromosome, PACs were identified from the filters of a high density arrayed genomic PAC library using a mixture of four inserts from clones W5, W59, W110 and W172. Thirteen PACs were strong positive (data not shown) from which eight were randomly selected (117C2, 146P9, 150O2, 17A7, 170N20, 286K14, 173B17 and 186K18) for further study by FISH. FISH to chromosome metaphase of patient C showed that all eight PACs hybridized to band q11 of chromosome 15 and to the ring (one example is presented in Fig. 4-7B). FISH with cosmids from the PWS/AS region A and B showed no signal on the ring but at the q11-13 region of the normal chromosome 15 homologues (Fig. 4-7C). Taken together, these results suggested that the ring of chromosome 15 from patient C contained a contiguous region from the centromere to 15q11.

Eight clones (W36, W70, W84, W108, W120, W124, W148 and W224) containing high repeat DNA were mixed and hybridized to chromosome metaphases. The results showed that FISH signals appeared on the ring chromosome and the centromere of chromosome 15 (Fig. 4-7D). Such mixed clones potentially can be used as a probe for clinical diagnosis.

4.3.3. Analysis of "inv dup(15)"s

Banding study for the marker from the patient D indicated that marker was a small bi-satellited "inv dup(15)". A combination of DA/DAPI and FISH with a probe from labelled microdissected products from patient C was carried out on metaphases of patient D. This showed a single focal hybridization and overlapped with a single bright DAPI signal on the marker. FISH signal also appeared around the centromere area of the normal homologous chromosome 15 (Fig. 4-8A).

The marker from the patient E was a large bi-satellited "inv dup(15)" from banding studies, and C-banding disclosed a second inactive centromere. DA/DAPI staining combined with FISH showed a similar pattern: both sides of the marker had DAPI positive region. FISH with microdissected product from patient C showed two distally located foci of hybridization and a small but clearly defined region between two FISH signals and furthermore those two hybridization signals overlapped with both DAPI signals (Fig. 4-8B).

4.4 Discussion

With microdissection and molecular cloning, a small ring chromosome in patient C was demonstrated to consist of a contiguous region from centromere to q11 of chromosome 15 but did not contained the PWS/AS region (15q11-13).

Combination of the DA/DAPI staining and FISH with the microdissected product on the marker of patient D indicated that the marker was bi-satellited,

monocentric and originated from the centromeric heterochromatin of chromosome 15 and again did not contain the PWS/AS region. Together with the normal phenotype in patient D and the finding that the marker was familial, this marker was considered to have no euchromatin involved. In contrast, the marker in patient E was found to be bi-satellited, dicentric and highly likely to include symmetric inverted duplication of PWS/AS region since FISH study showed clearly defined euchromatic region between the two FISH signals. Therefore patient E contained at least three copies of PWS region. The origins of the three mar(15)s are summarized in Figure 4-9.

It is common to analyze small extra ring chromosomes by FISH with specific-centromere probes (Callen et al. 1990b, 1991, 1992a) or with PCR-amplified DNA from microdissected ESACs as a probe (Thangavelu et al. 1994, Viersbach et al. 1994). With centromere probes, the chromosome origin of the ring can be identified but the content of euchromatin can not be determined. Reverse painting is able to provide more information about the composition of the ring but this approach is not necessarily sufficiently sensitive to detect small non-contiguous regions of euchromatin. In this study, eight PACs that were isolated with cloned microdissected DNA showed a location at 15q11 and all of them were hybridized to the ring chromosome. Together with reverse painting of microdissected products, the origin of this extra small ring from patient C were consistent with contiguous region from the centromere to 15q11.

Similarity exists between the clinical feature of patient C and that of a previously reported patient with an extra ring chromosome 15 (case 6, in Crolla et al. 1995). Both of them were very active, had behaviour problem and delayed language development. However, our patient had hypermobility of the joints while the patient of Crolla et al. had mental retardation. Also from same report (Crolla et al. 1995), three patients, all with the "inv dup(15)", had language problems, lax joint, mental retardation and two patients had seizures. It should be noted that all these mar(15)s

contained the PWS/AS region (15q11→q13) while the ring chromosome in our patient only contained the segment from the centromere to 15q11. This suggests that the extra euchromatin in 15q11 may be associated with some phenotypic abnormalities such as hyperactivity, hypermobility of the joints, behaviour problem and delayed language development.

Several investigators have attempted to characterize 15q arm material present in "inv dup(15)"s and to determine the copy number of probes mapping to the PWS/AS region by dosage analysis (Nicholls et al. 1989, Robinson et al. 1993b, Shibuya et al. 1991, Crolla et al. 1995, Leana-Cox et al. 1994). On the other hand, these "inv dup(15)"s can be further classified according to cytogenetic distinction (Maraschio et al. 1988) or different breakpoints of the markers which detected by FISH with the classical satellite probe (D15Z1) and the single-copy chromosome 15q11-q13-specific probes (Cheng et al. 1994, Mignon et al. 1996). In these classification schemes, for the carriers who had normal phenotypes, their "inv dup(15)"s were categorized as type I containing entirely heterochromatin. For the abnormal phenotypic carriers, their "inv dup(15)s" were further classified into two to four types and all these types were symmetric duplications. One type classified by Mignon et al. 1996, an "inv dup(15)" in an abnormal patient had asymmetric duplication.

The mechanism of origin of small r(15) and "inv dup(15)" may be different. The formation of extra small ring chromosome has been discussed in Chapters 1 and 3. The "inv dup(15)" may involve in non-sister chromatid translocation, U-type exchange and following by miotic non-disjunction and inactivation of one of the centromeres (Schreck et al. 1977, Wisniewski et al. 1979, Van Dyke et al. 1977) (Fig. 4-10).

4.5 Summary

Three mar(15)s were shown by FISH to have derived from and composed entirely of chromosome 15 material. Using a combination of microdissection, molecular cloning and FISH, a small extra ring in a patient with problems of behaviour, language development and hypermobility of the joints chromosome 15 has been revealed to contain the segment from the centromere to 15q11. Analysis of metaphase spreads with "inv dup(15)s" from a normal and an abnormal individuals using probes from the microdissected DNA generated from above ring chromosome, clearly differentiated these two "inv dup(15)s" which was either monocentric, only alpha centromere heterochromatin or dicentric, with duplication of proximal 15q material. The results suggested that the mar(15)s contained different genetic materials and this results in diverse clinical features.

Fig. 4-1. Partial colony blot with total human DNA from patient C showed that colonies 36, 107, 108, 118, 120, 124, 131, 138 and 148 were positive and the remaining colonies were negative. The positive clones indicate that they contained highly or moderately repetitive DNA while negative ones potentially contained single-copy DNA or vector DNA only.

1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28
29 30 31 32 33 34 35 36 37
38 39 40 41 42 43 44 45
46 47 48 49 50 51 52

104 105 106 107
108 109 110 111 112 113
114 115 116 117 118 119 120 121
122 123 124 125 126 127 128 129
130 131 132 133 134 135 136 137
138 139 140 141 142 143 144 145
146 147 148 149 150 151 152 153

Fig. 4-2. Inserts recovered by PCR from 4 individual microclones (lanes 2-5 from left). The size of inserts were between 400bp to 800bp which included approximately 200bp of vector sequence.

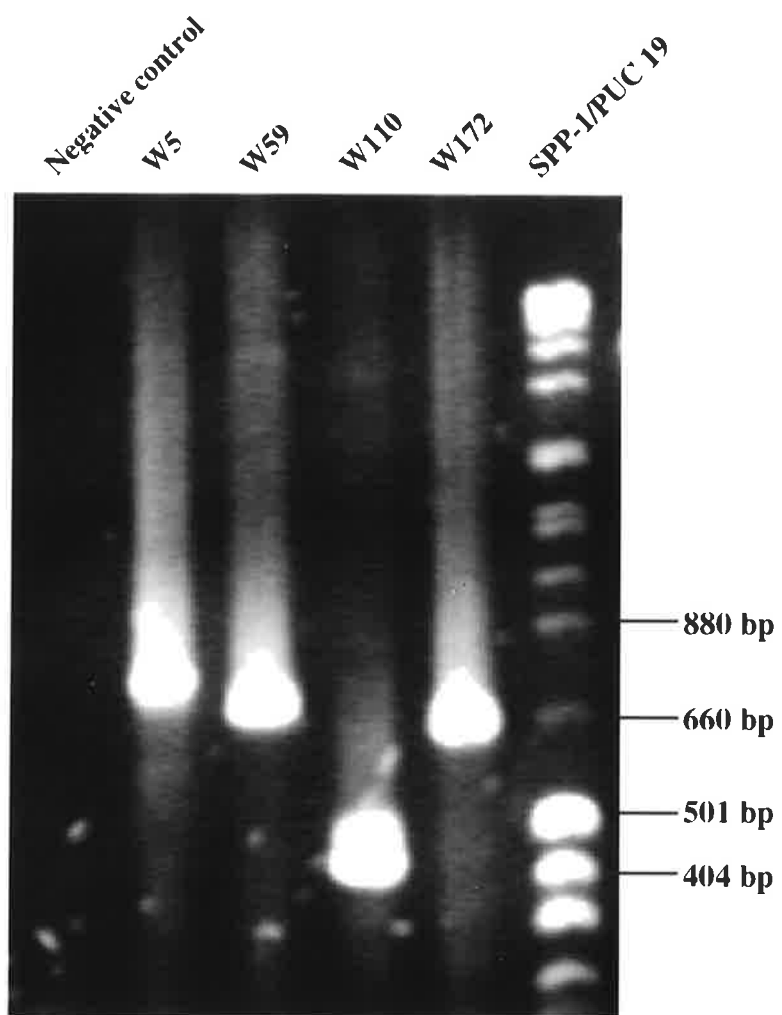

Negative control
W5
W59
W110
W172
SPP-1/PUC 19
880 bp
660 bp
501 bp
404 bp

Fig. 4-3. Four clones (W5, W59, W110 and W172) were digested with *Eco*RI and *Pst*I. The size of inserts were between 300 - 660bp. W59 showed two bands, one was 147bp and the other was 501bp, indicating that W59 contained a restriction site of either *Eco*RI or *Pst*I. The vector band is around 3000bp. The size pGEM-3Zf(+) is 3199bp and pUC19 is 2686bp.

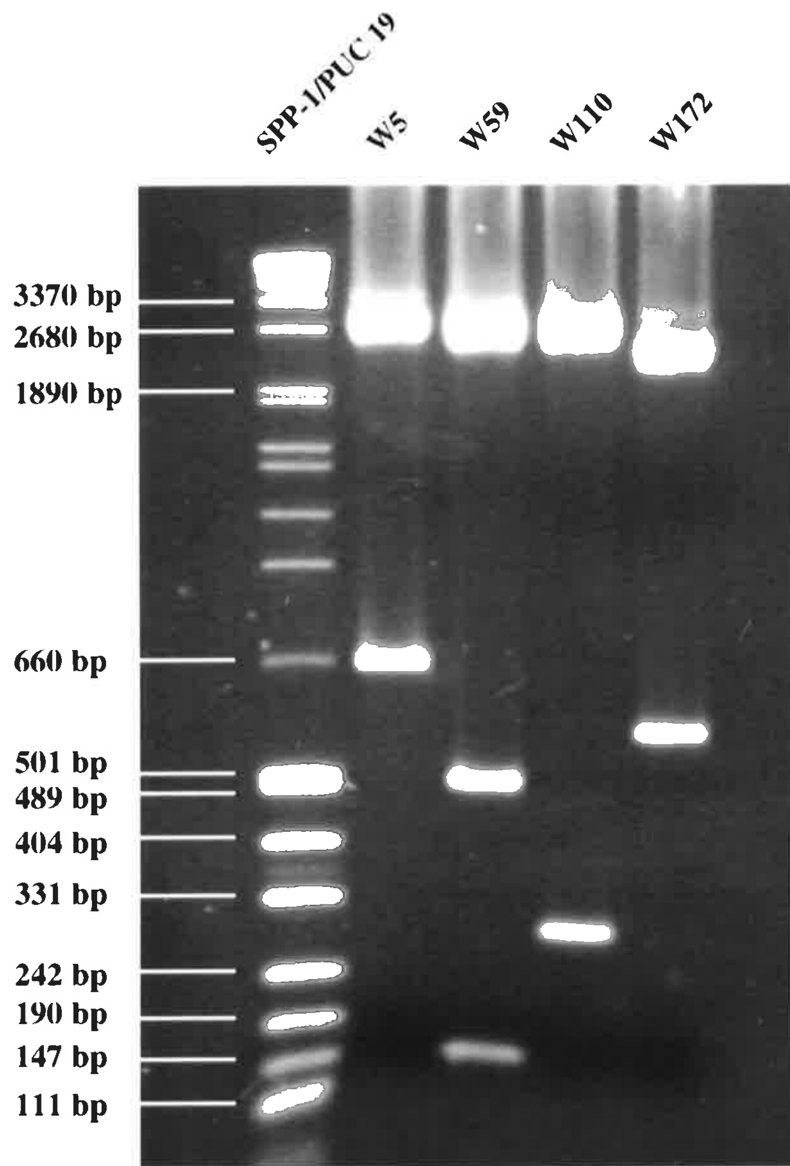
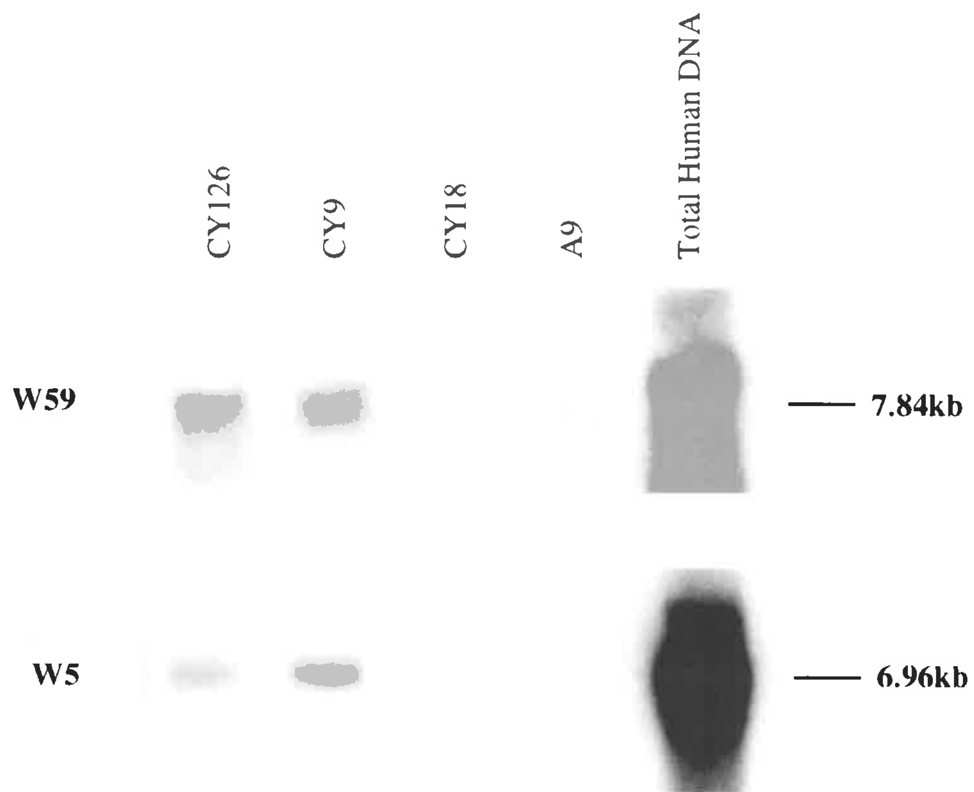

SPP-1/PUC 19
W5
W59
W110
W172
3370 bp
2680 bp
1890 bp
660 bp
501 bp
489 bp
404 bp
331 bp
242 bp
190 bp
147 bp
111 bp

Fig. 4-4. Southern blots using clones W5 and W59 as probes against *Hind*III-digested rodent/human hybrids CY126, CY9 and total human DNA. CY18, A9 were as controls.


CY126
CY9
CY18
A9
Total Human DNA
W59
7.84kb
W5
6.96kb

W5 sequence (611 bp):

GATTTTAATGACCAAGGAAATTTGCAGTGCAGATATCCTGACTCAGACAGCATAA
GTCAGCGACAGCTGGCTTTAGCCTCTGTCTCTGGAAGCACTTCTACACTTGTGCAG
CAGTCATGTTTCCAACAGTCATAGAGCTACATGGGGATTGTATGTGAGGACGTTT
CCAGTGAGTTGTGAGAGTCTCCAATGAGTAATTTTAATTTAAGGAGTCCGCAGTG
ACCTAGGCCAAAGCATTCTTGGAACCACATCTCCCCTCGAGTCGGCCACATGAACG
CCTCGAGTCGGACCACATTTAATCCTCGAGTCGGACCACATTTAATCCTCGAGTCG
GCCACATCCGGACCTCGAGTCGGACCACATGCCCCCCTCGAGTCGGACCCACATAC
ACATCTCGAGTCGGACATGTGCTCCTCCACATCCCCCGCTCGAGTCGGAGACATCC
CAACCACATGCTCGTCTCGAGTCGGACCACATCAGGGTCACCACATCCCACATTCT
AGTCGGCCACATTACAACCTCGAGTCGGCCACATGCCCCCCTCGAGTCGGACCACA
TCAGGGTCACCACATCCCACATTCTAGTCGGCCACATTACAACCTCGAGTCGGC

W59 sequence (552 bp):

GGTGCCCATGTGGTCTTTTCTTTCCATATTTAGTGCTTCCTTCAGGAGCTCTTGTAA
GGTAGGTCTGGTGATAATGAATTCCCTCAGCATTTGCTTGTCTGAAAAGGATCTTG
TTTCTCCTTCACTTATGATGCTTAATTTTGCTGGACATGAAATTCCGGGTTGAAAT
TTCTTTTCTTTAAGATGTTGAATATCTTTTCTGGCTTGTATAGTTTCAGTTGAGAG
GTCTGCTAAGTCTGATGGAATTTCCTTTGCAGGTGATGTTGCCTTTCTCCCTAGCT
GCCTTTAATACTTTTTCTTTCATTTTGACCGCAGAGAATCTGATGATTATGTGTCTT
GGGGATGATCTTCTCATGGCATATCTTACTGAGGTTCTCTGGATTTCCTGAAGTTG
AGTGTTGGCCTGTCTGGCTAGGTTGGGGACATTCTCATGAATGATATTCTGAAAT
GTGTTTTCCAAGTTGGTTCCATTCTCCTCATCTCTTTCAGGTACATTAATCACGTCA
TAGATTTAGTCGTTTATATAATCCCACATCCCCCAC

W110 sequence (181 bp):

GACGTAGAAGAAGATTTTAGTAAAAAGAAACTTTAATAATTAAAGAAATGGAAA
ACAGAATCTAGAAGGGACTATAACAGAATTAGGTAGTCTTAAGACAATATTGCCA
TGAAACCTGTGCCTTCAGTTATGTAAATTTGGTCCTATCGTATCCAAATAT
AGCAACTGTC TTCTAAGATG C

W172 sequence (499 bp):

GGGACAATGTGGGCCACATGACCCAAGGGGGACCATGTTTAAACTTCTGAGTTTT
CACCGAGGTTAACATGCATTTGTTGAAAGAGAAACCCCTTTTCCCCTACTCCCCCA
GCTGCAAATGCCTTCAGGGATTATATCATGTTGGAACATTTGGTTACAGTGTTTCC
TAAACTTTGGGGGTAAAAATTGTTCAAGTAGGTAAAAATGGAGCACACACAAAG
AAAAAAGGAGTCCAGAAATAACAAATAAAGAAAGGGCCTCCATAAAATCATTTG
AACTTATGATTAATTCATTAGTCATTAAAATAAGTTTAGTGTACAAAGAATCATC
CCTCCAACCACCCTTTATTCCTTCACCAGGTTTAAGTTACATTTTTAAACTTGCAAA
CAAAAGATTTGTCATTAACTTAGACATCAAAATCCCTTGTCTCCAAGAGCAATCAT
TCAACTCTGTCCCTCTCATTATTACAATAATATGTTCACTTTATTCCACATCCATAA

Fig. 4-5A. Complete sequence of clones W5, W59, W110 and W172. The bases underlined indicated the sequence of the oligoprimers synthesized.

Fig. 4-5B. Partial sequence of the clone W59. The insert start from the base 85 and before this (arrow) was the sequences of adaptor and partial vector. The bases from 131 to 154 were synthesised forward oligoprimer (underlined in red).

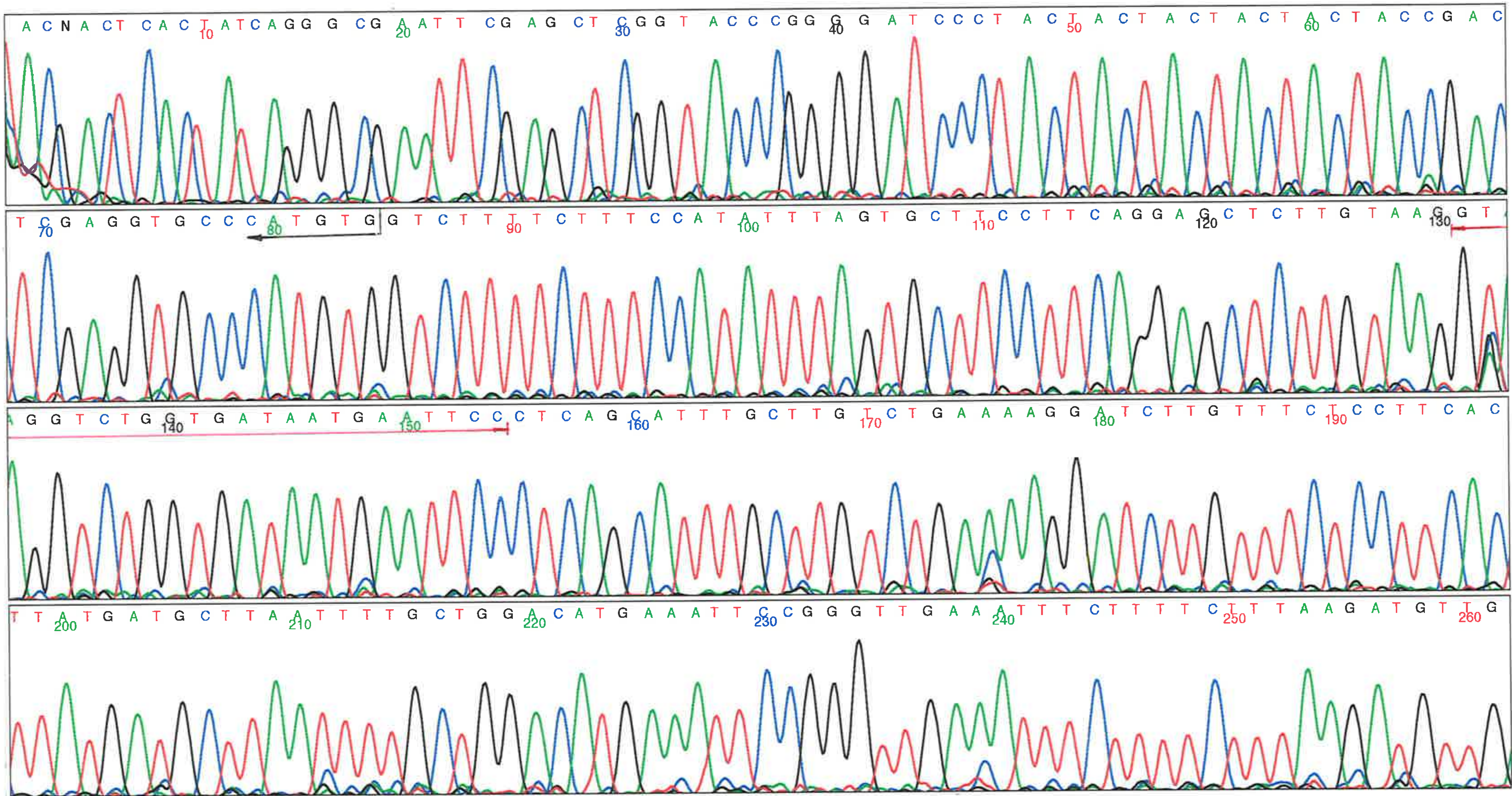
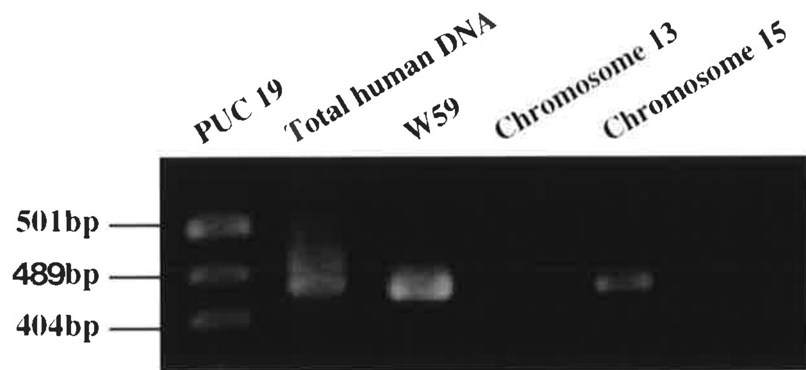


Fig. 4-6. (Top panel) Oligoprimers produced from clone W59 amplified total human DNA, chromosome 15 and W59, but not chromosome 13.

(Bottom panel) Oligoprimers produced from clone W172 amplified total human DNA, chromosome 15 and W172, but not amplified chromosome 13.


PUC 19
Total human DNA
W59
Chromosome 13
Chromosome 15
501bp
489bp
404bp

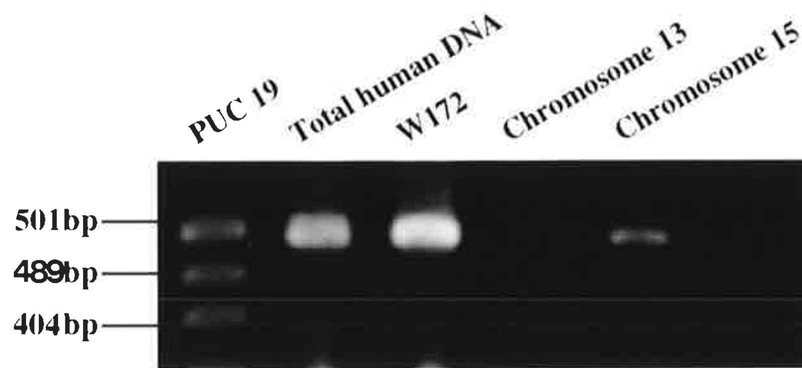

PUC 19
Total human DNA
W172
Chromosome 13
Chromosome 15
501bp
489bp
404bp

Fig. 4-7. (A) Metaphase after *in situ* hybridization with microdissected DNA labelled spectrum-orange. Signals appeared around the centromeres of chromosome 15s (thin arrows) and the ring (thick arrow) in patient C. **(B)** PAC17A7 located at q11 of chromosome 15 was hybridized to chromosome 15 (arrowheads) and also to the ring of patient C (arrow) . **(C)** FISH with PWS's probe showed no signal on the ring of patient C (arrow) but on the normal chromosome 15 homologous (arrowheads). **(D)** FISH with mixed DNA from 8 repetitive DNAs hybridized to the ring of patient C (arrow) and centromere of chromosome 15 (arrowheads).

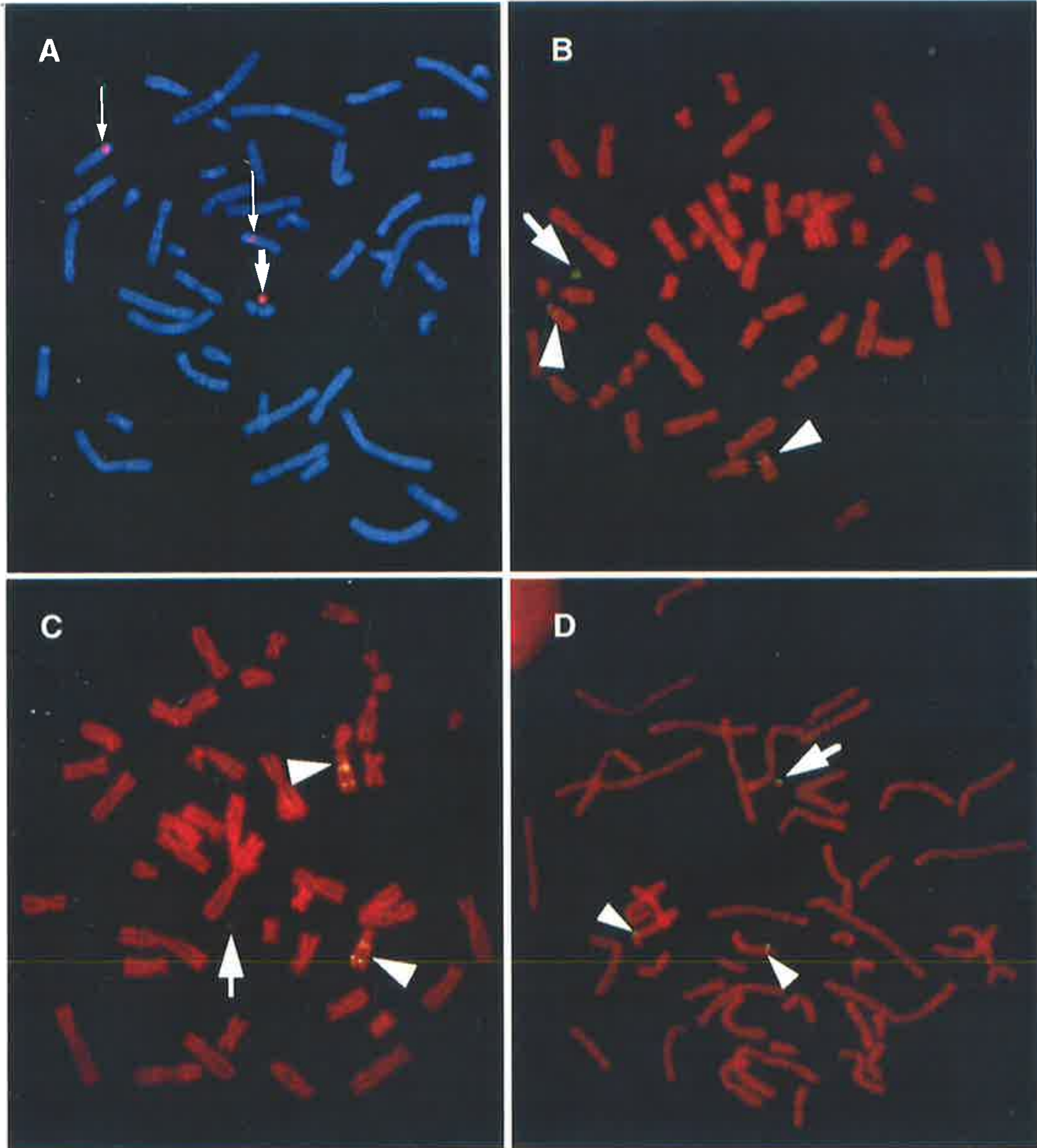
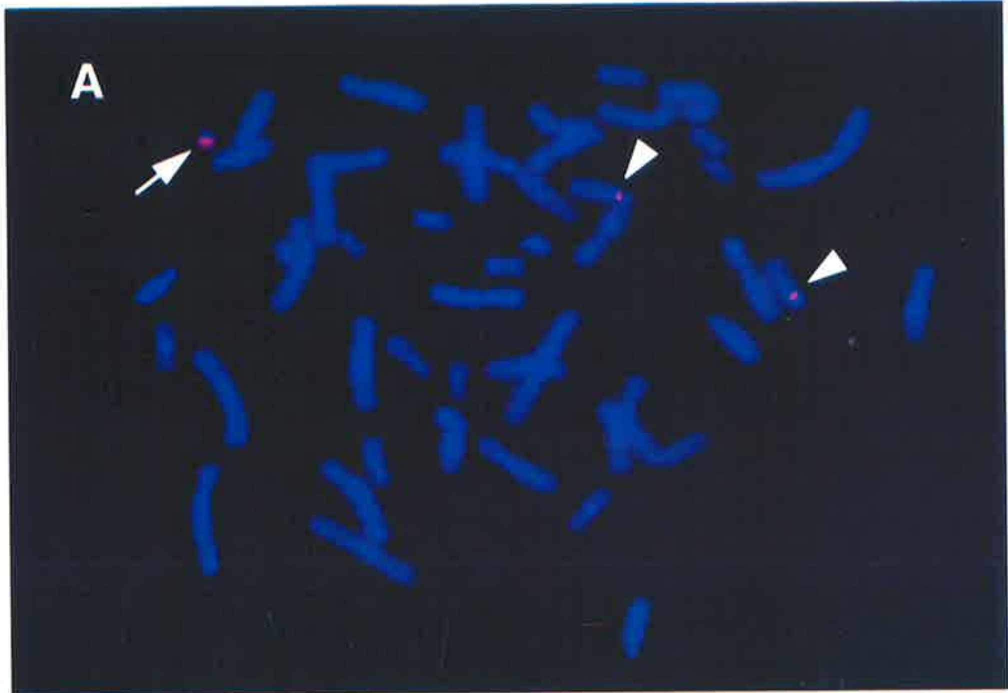
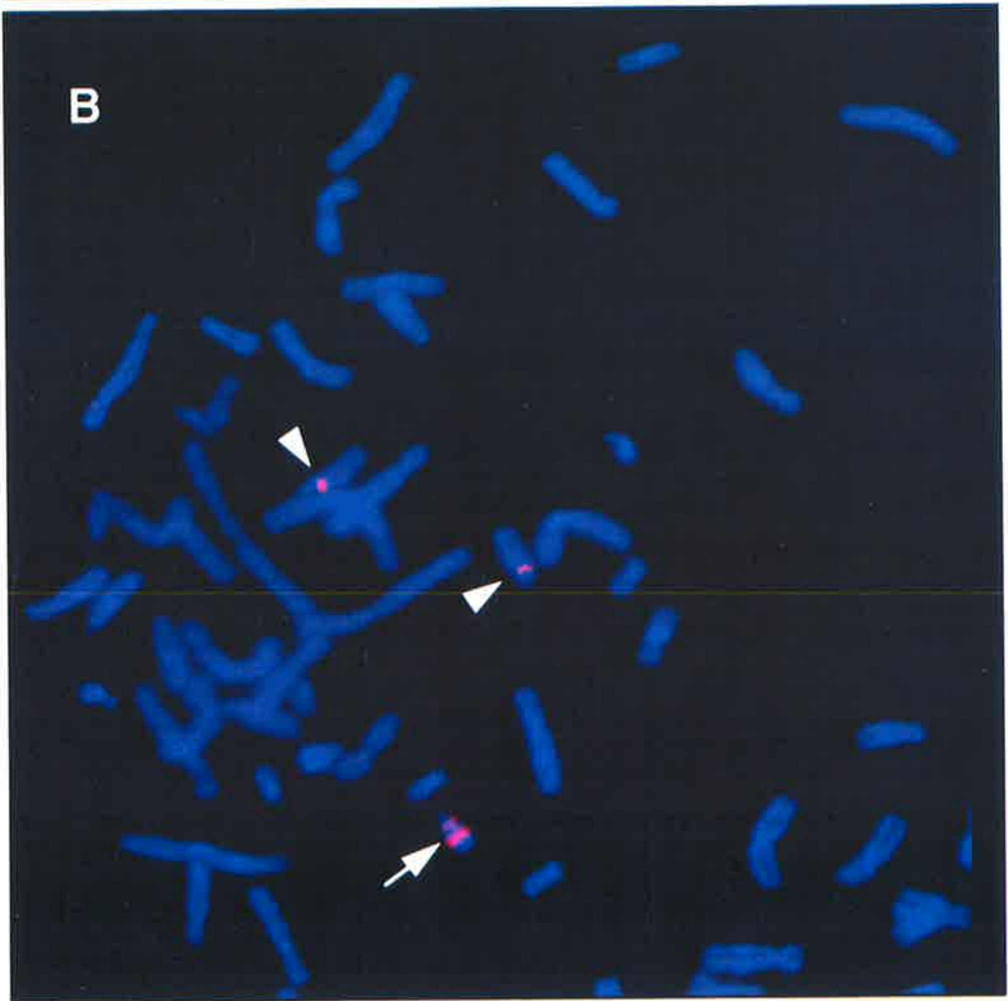

A
B
C
D

Fig. 4-8. Combination of DA/DAPI and FISH with microdissected product from patient C on inv dup(15)s in patient D and E. **(A)** One FISH signal overlapped on DA/DAPI signal showed in patient D (arrow). **(B)** Two FISH signals which were covered on DA/DAPI signal showed in patient E (arrow).


A


B

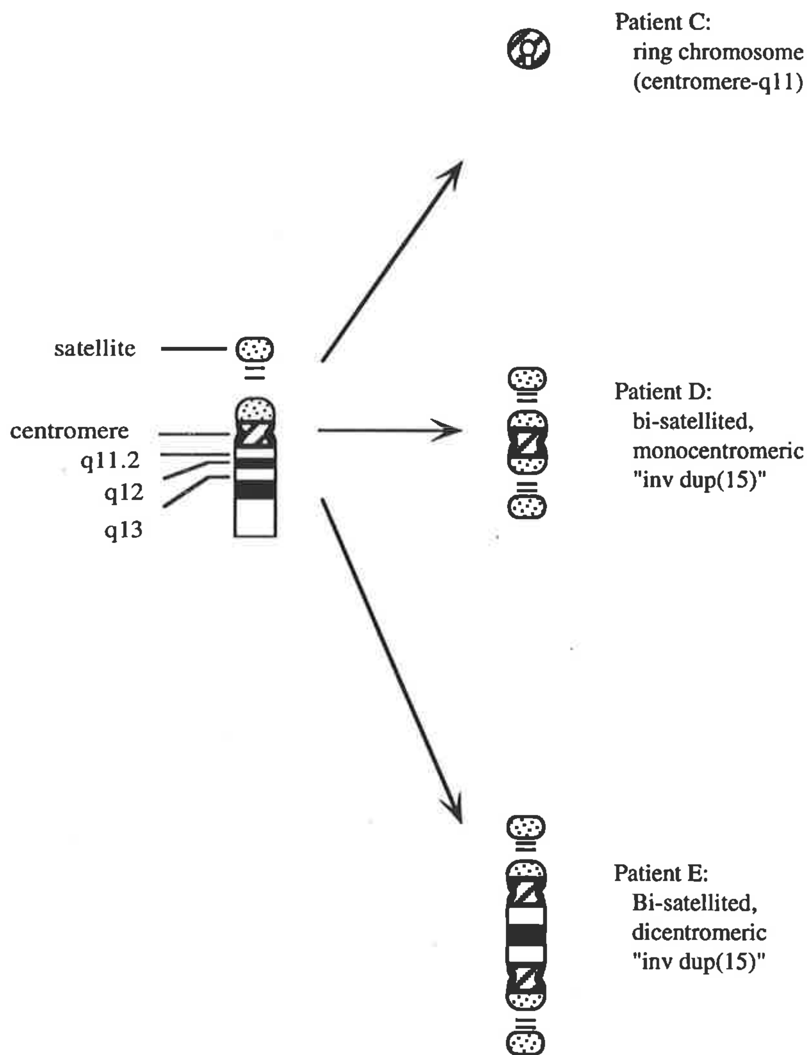


Fig. 4-9: Diagram of partial chromosome 15 (left side) and three mar(15)s (right side)

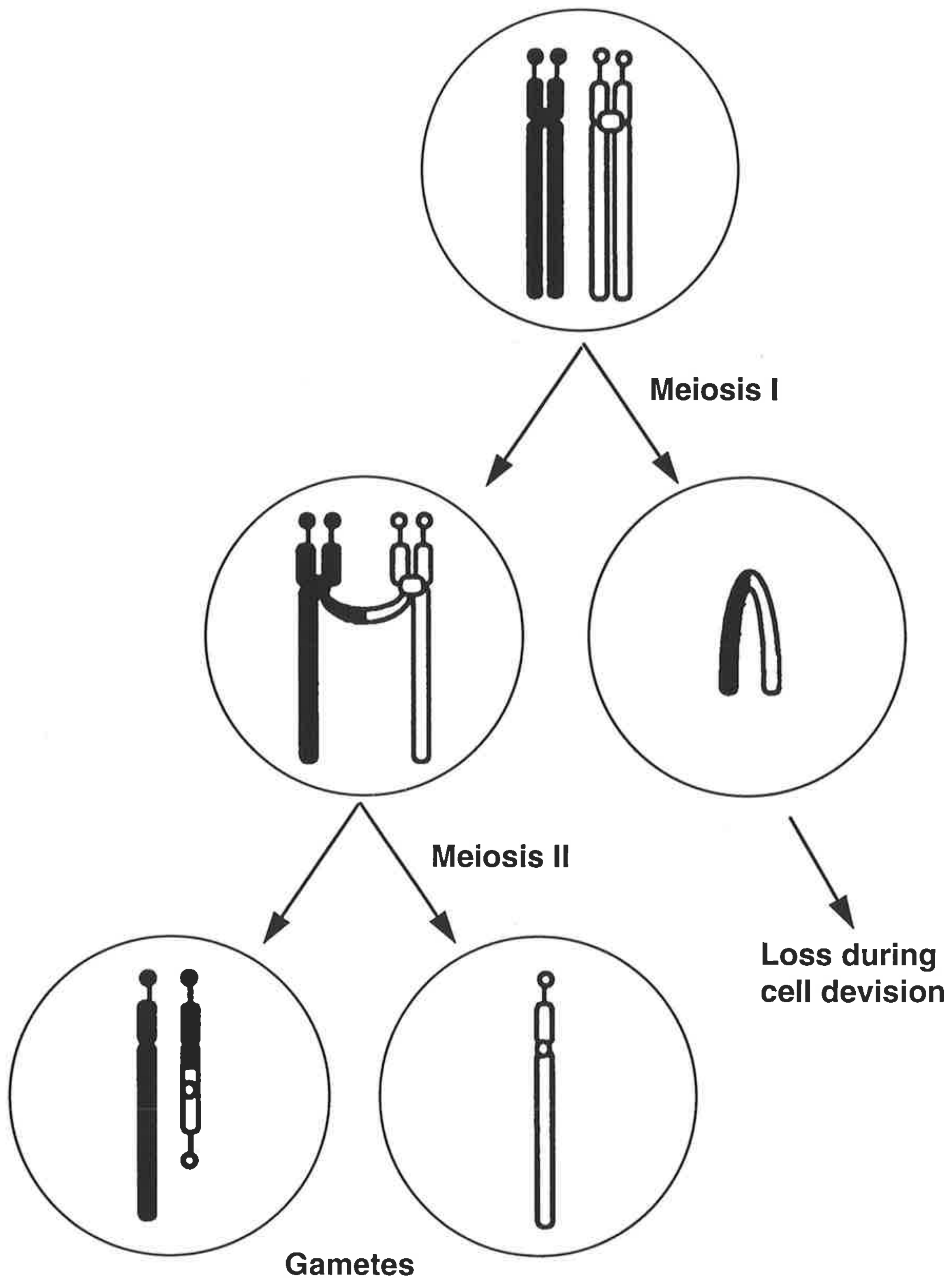


Fig. 4-10. Schematic illustration of a possible mechanism for the formation of an isodicentric chromosome, including non-sister chromatid U-type exchange and non-disjunction during meiosis. Adapted from Blennow 1994a.

CHAPTER 5

IDENTIFICATION OF SMALL EXTRA RING CHROMOSOME 1, 4 AND 8 BY REVERSE PAINTING

5.1 Introduction

One subclass of ESACs is small extra ring chromosomes which consist of approximately 10% of all ESACs (Blennow et al. 1994b). Whether the rings cause phenotypic abnormality depends on their derivation and whether euchromatin is involved. Some rings are associated with normal phenotype (Callen et al. 1991, Michalski et al. 1993) while others are associated with mental and physical abnormality (Lanphear et al. 1995, Chen et al. 1995b). Since only a limited number of rings have been reported, the relationship between the patient's phenotypes and the chromosome aberration has not been defined. Detailed characterization of the origin of the rings and the comparison of multiple patients with rings of the same origin may contribute to the correlation between the phenotype and genotype.

In this study, the origins of four extra small ring chromosomes in both normal and abnormal individuals were identified by microdissection of rings, DOP-PCR amplification of microdissected products and hybridization back to the ring chromosomes. The information obtained with this combination of techniques was compared with hybridization using centromere derived probes.

5.2 Materials and Methods

5.2.1 Patients

Patient F: He was the first child of healthy caucasian parents of maternal age 30 years and paternal age 43 years. Assessed as a 7-year-old, he had severe mental retardation associated with significant bilateral sensorineural hearing loss and significant visual impairment. He tended to be awake at all hours of the night and has chronic constipation. He had short stature with a head circumference of 50 cm at that age (2-50%). The surface of the skull was irregular with plagiocephaly (right forehead forward). He had a flat round face, widely set eyes with nystagmus, puffy eyelids, a broad fleshy nose with prominent nares and thick alas nasi, cleft palate, full lip, anteverted cup shaped ears with thick helices, and malocclusion with abnormal teeth. He also had narrow, slopping shoulders, a long thorax with chest wall asymmetry (left anterior chest forward), widely spaced nipples and tiny accessory nipples, overlapping finger posture on both hands, mild fingernail hypoplasia, micrognathia. His two younger siblings and another two siblings from his father's previous marriage were all normal. Chromosomal analysis of peripheral lymphocyte culture showed a karyotype of 46,XY[42]/47,XY,+r[58].

Patient G: A 18-year-old male had been admitted to the hospital three times since 1995 due to suffering from schizophreniform psychosis (paranoid features). Psychiatric problem included bizarre and aggressive behaviour, social withdrawal auditory hallucinations, thought insertion and ideas of reference. Patient had multiple drugs use history. No abnormality was found during physical examination, CT head scan, EEG, complete blood examination and biochemical analysis. The parents were normal both physically and mentally and had a normal karyotype. His brother and a step-sister on the father's side were normal but a nephew of his father committed suicide due to major depression and another nephew was mentally retarded with

Down syndrome. Venous blood lymphocyte cultures showed 46,XY[18]/47,XY,+r[82].

Patient H: She was an aboriginal girl delivered by emergency caesarian section at 32 weeks gestation since the mother had worsening pre-eclampsia. Birth weight was 1760 gram and there was frequent gastroenteritis and failure to thrive. Assessed at a 2-year-old, she had no dysmorphic features. Peripheral lymphocyte cultures showed 46,XX[10]/47,XX,+r[90]. Karyotypes of the parents were normal.

Patient I: She was referred for a genetic consultation at the second month after birth due to heart murmur and large tongue found at the neonatal stage. Physical examination showed telecanthus, high arched palate and plagiocephaly. Subsequently her heart was examined several times and the heart murmur disappeared. Peripheral lymphocyte cultures showed 46,XX[85]/47,XX,+r[15]. The cytogenetic analysis for her parents was normal.

5.2.2 Microdissection and degenerate oligonucleotide-primed-polymerase chain reaction DOP-PCR)

The chromosomal preparation for patient F and G was processed as described in Chapter 2. The ring chromosomes were microdissected with fine glass needles controlled by micromanipulator under an inverted microscope (Nikon Diascopic Dic Nomarki) The microdissection was carried out by Dr A. Houben from The University of Adelaide, Australia.

For patient F, 25 pieces of ring chromosomes were obtained from metaphases and 5 pieces of nuclei from interphase cells. They were separately collected in 1 μl solution containing proteinase K (Boehringer, 0.5 mg/ml) in 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.1 % (w/v) SDS and overlaid with paraffin oil. Genomic DNA from interphase cell was used as DNA quality control since the suspension had been fixed by methanol: acetic acid and kept in -20°C for several months before this study.

The DNA with proteinase solution was incubated at 50°C for 30 min and subsequently amplified at a final volume of 100 μl solution of DOP-PCR master (Boehringer Mannheim) using the degenerate oligonucleotide primer MW-6 (Telenius et al. 1992) with some modification. The PCR was performed on a FTS-960 Microplate Fast Thermal Sequencer (Corbett Research): 5 min at 95°C, followed by five cycles of 1 min at 94°C, 1.5 min at 30°C, 25 sec each at 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 3 min extension at 72°C, subsequently with 35 cycles of 1 min at 94°C, 1 min at 62°C and 3 min at 72°C. The final extension was lengthened to 7 min. The PCR product was checked by electrophoresis and purified using a QIAquick-spin column (QIAGEN) prior to nick translation.

Microdissection and DOP-PCR amplification in patient G were similar to patient F but the slide containing metaphases was stained with Leishman prior to microdissection and a total of 20 pieces of ring chromosome from metaphases were collected.

For patients H and I, the microdissection and spectrum-orange directly labelling by PCR were the same as described at Chapter 4. This work was preformed by Dr X.-Y. Guan of The University of Michigan Comprehensive Cancer Center, USA.

5.2.3 Fluorescence in situ hybridization (FISH)

The probes from DOP-PCR products of the microdissected rings and centromere probes from most chromosomes were used for FISH to identity the origins of these rings. The details of FISH have been described in Chapter 2.

5.3 Results

Patient F: Several banding studies were performed for this patient and with G-banding, the ring was present in 21 out of 50 spreads (Fig. 5-1A). C-banding showed that most rings had one centromere and an occasional ring had two

centromeres. Ag-NOR and DA/DAPI was both negative in the ring. Ring chromosome (25 pieces) and 5 pieces of interphase cell were microdissected and amplified by DOP-PCR. Electrophoresis showed that the PCR products from the rings and from the interphase cells were a smear and the size ranged from 400 to 880bp (Fig. 5-2). DNAs were amplified indicating that their quality was still good. Reverse painting with the DOP-PCR product of the microdissected DNA showed that the hybridization signal covered the majority of the area of the ring and localized at the chromosome 8 from p11→centromere (Fig. 5-3A).

Patient G: Metaphases were studied by conventional staining and FISH. G-banding indicated that the size of the ring was smaller than a G-group chromosome (Fig. 5-1B). Ag-NOR and DA/DAPI staining were negative on the ring. FISH was performed with classical alphoid centromere probes, including those for chromosomes 1, 2, 3, 5, 11, 17, 18 and 19 and those showed no signal on the ring. FISH with the centromere probes from chromosome 4, 9 and 14/22 showed some faint signals on the ring. Therefore, microdissection was performed and reverse painting with the microdissected products indicated that the hybridization signal was located at the centromeres of chromosome 8 homologues and on the ring (Fig. 5-3B).

Patient H: Cytogenetic investigation including G-banding, Ag-NOR and DA/DAPI revealed an extra small ring (Fig. 5-1C) which was Ag-NOR and DA/DAPI negative. Initial FISH study with alphoid centromere probe of chromosomes 1, 9, 11, 13/21, 16, 17, 18 and X were all negative. Reverse painting with spectrum-orange labelled microdissected ring DNA indicated that the hybridization signal covered the whole ring and just at centromeres of the chromosome 4s, without signals elsewhere (Fig. 5-3C).

Patient I: Cytogenetic studies showed that the marker appeared as a small ring chromosome and did not contain any satellites (Fig. 5-1D). DA/DAPI staining was positive and therefore suggested that the ring was probably derived from either

chromosome 1, 9 or 16. FISH with centromere probes of chromosome 1, 9 and 16 showed that the ring was derived from chromosome 1. Reverse painting with spectrum-orange labelled PCR product from the microdissected DNA of the ring showed the signal was on the region of chromosome 1 from centromere to 1q12 and on the ring. FISH using the alphoid centromere probe for chromosome 1, D1Z1 together with the microdissected products showed that both signals were on the centromere area but the signal obtained by using the microdissected products also covered the pericentromeric heterochromatic region of the long arm (1q12) (Fig. 5-3D).

5.4 Discussion

With microdissection, PCR amplification, and reverse hybridization, the origin of four ring chromosomes have been identified. They were derived from chromosomes 1 (patient I), 4 (patient H), and 8 (patients F and G), respectively. These rings were only derived from the vicinity of the centromere in patients G and H while the ring in patient F also included 8p11 and in patient I included the heterochromatic region of chromosome 1q12.

Although the origin of small ring chromosomes can be identified by FISH with specific centromere probes, multiple probes are normally needed to screen a number of different chromosomes. To reduce this time-consuming process, a method using multiple stringency conditions has been applied to identify such ring chromosomes (Plattner et al. 1993a). Ring chromosomes were initially screened with probes used at low stringency of post hybridization washes allowing the detection of similar alphoid satellite families and finally they were identified by high stringency. However, with this procedure, at least one week was needed. This can be reduced to 3-4 days when microdissection of the marker and reverse hybridization with PCR amplified microdissected DNA was applied. Therefore reverse painting is more

effective and straightforward for the rapid identification of the origin of ring chromosomes.

Moreover, reverse painting is able to provide more information about the composition of the ring. For example, reverse painting showed that a ring chromosome 4 in Chapter 3 consisted of centromere and 4q31. The ring chromosome from patient F was found to include 8p11, and from patient I was found to include the heterochromatic region of chromosome 1q12 using reverse painting. Although DA/DAPI staining can detect such heterochromatic regions, it could not distinguish the origin of chromosomes 1 from that of chromosomes 9 and 16. Heterochromatic regions are known to be polymorphic to contain only repetitive DNA sequences. Thus, this ring chromosome in patient I might not exert any phenotypic effect.

A total of three ring chromosome 4s have been characterized in this thesis. Two cases were studied by the molecular cloning techniques as described in Chapter 3. Some degrees of correlation between genotype and phenotype were presented such that different composition of the ring resulted in different clinical features (Table 5-1).

Table 5-1 Comparison of patient phenotypes and the contents of three ring chromosome 4s

Patients	Phenotype	Contents of the ring
A*	Moderate mental retardation, minor facial anomalies	4p13/14 :: cen :: 4q31
B*	Severe mental retardation, delayed motor development, no development of language skills, insulin-dependent diabetes mellitus	cen → 4q12
H#	normal	centromere

*Patients A and B were presented in chapter 3

#Patient H was described in this chapter.

Ring chromosomes in patients F and G were both derived from chromosome 8 and the hybridization signal was spread from 8p11→centromere (patients F) or was located at the centromere area (patients G) with reverse painting. The phenotypes were quite different in both patients (Table 5-2). Compared with three other reported cases (Table 5-2), their phenotypic abnormalities were dissimilar, except for some non-specific features such as their development delay and mental retardation. The phenotype of patient F may associated with the euchromatin in 8p11 or one plausible explanation is that the ring chromosome in these two patients contained small fragments of euchromatin and this fragment is beyond the detection with the reverse painting method.

5.5 Summary

Four patients with *de novo*, different small extra ring chromosomes were characterizated with microdissection and fluorescence *in situ* hybridization (FISH). Analysis with FISH probes produced from the ring ESACs indicated that two rings from different patients were both derived from chromosome 8, one contained 8p11→centromere and the other mainly included the centromere. There was no apparent phenotypic similarity between these two patients. Characterization of two other ring chromosomes showed that one was from chromosome 1, the region of centromere→1q12 and the other ring was from chromosome 4, involving the centromere area. Not surprising of the three individuals with ring chromosome 4s in this study (patient H) had a normal phenotype while the two other patients had abnormal phenotypes (patient A and B in Chapter 3) since their ring chromosomes also involved euchromatin (one was from 4p13/14 and 4q31, while the other included 4q12). Microdissection in combination with FISH has been proven to be a valuable technique in determining the chromosomal origin of ring ESACs.

Table 5-2 Comparison of the clinical features in extra small r(8)s

Phenotype	Karyotype	References
Mental retardation, language development delay, autistic behaviour, difficulty with fine and gross motor coordination, mildly dysmorphic features included epicanthic folds, hypoplastic and widely spaced nipples.	46,XY[5]/ 47,XY,+r[95]	Plattner et al. (1993b)
Hypotonia, dysmorphic features included coarse face, hypertelorism, bulbous nose, low-set ears with a prominent helical root, accessory nipple, narrow shoulders, and bilateral pes equinovarus.	46,XX[60]/ 47,XY,+r[40]	Blennow et al. (1993)
Development delay, seizure, hypotonia, minor anomalies included a round face, slightly up slanted deep set eyes with small epicanthal folds, flat nasal bridge, downturned corners of the mouth.	47,XX,+r	Melnyk and Dewald (1994)
Global delay, poor language comprehension and social skills, dysmorphic features included broad nasal bridge, triangular face, large low-set posteriorly rotated ears, divergent squint, hyperextensible elbows, mild clawing of toes 2-5.	46,XY[50]/ 47,XY,+r[50]	Daniel et al. (1994)
Mental retardation, sensorineural hearing loss, dysmorphic features included nystagmus, puffy eyelids, a broad fleshy nose with prominent nares and thick alas nasi, cleft palate, anteverted cup shaped ears with thick helices, and malocclusion with abnormal teeth, narrow, slopping shoulders, chest wall asymmetry, tiny accessory nipples, overlapping finger posture, mild fingernail hypoplasia, micrognathia.	46,XY[42]/ 47,XY,+r[58]	Present study (Patient F)
Schizophrenia	46,XY[18]/ 47,XY,+r[82]	Present study (Patient G)

Fig 5-1. Metaphase from patient F **(1A)**, G **(1B)**, H **(1C)** and I **(1D)** showing ring ESACs (double arrowheads) detected by Leishman **(1A)** or Giemsa **(1B-1D)** straining.

A

B

C

D

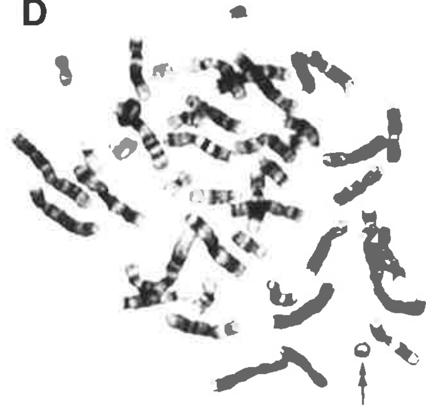


Fig 5-2. PCR products from microdissection of ring ESAC of patient F resulting in a smear with some bands ranging from 400 to 900bp (lane 3). Lane 1, PCR reaction with no DNA; Lane 2, PCR product of DNA from DOP-PCR Master kit (Boehringer mannheim) as positive control; Lane 3, PCR product from microdissected ring ESAC. Lane 4, PCR product of genomic DNA.

1 2 3 4

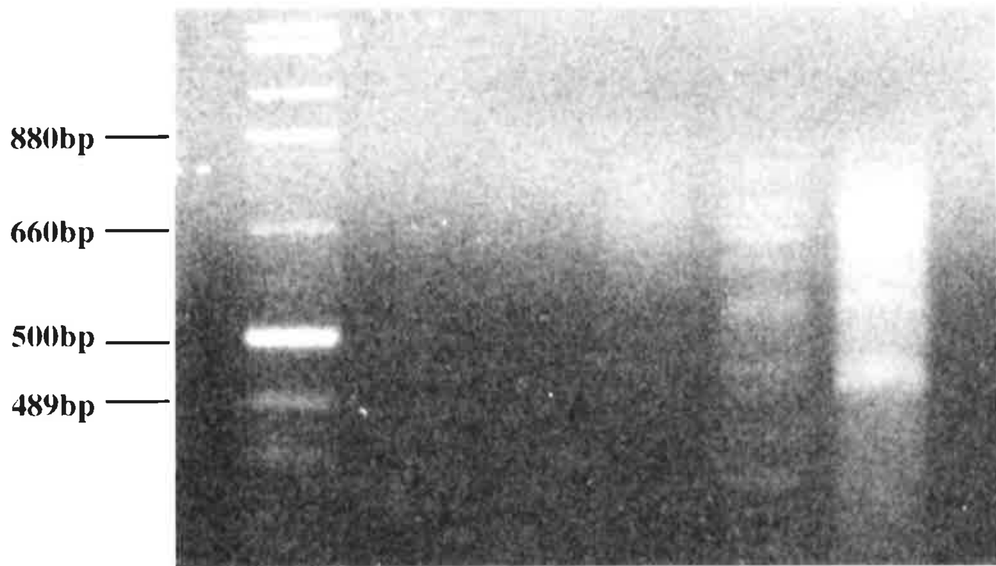
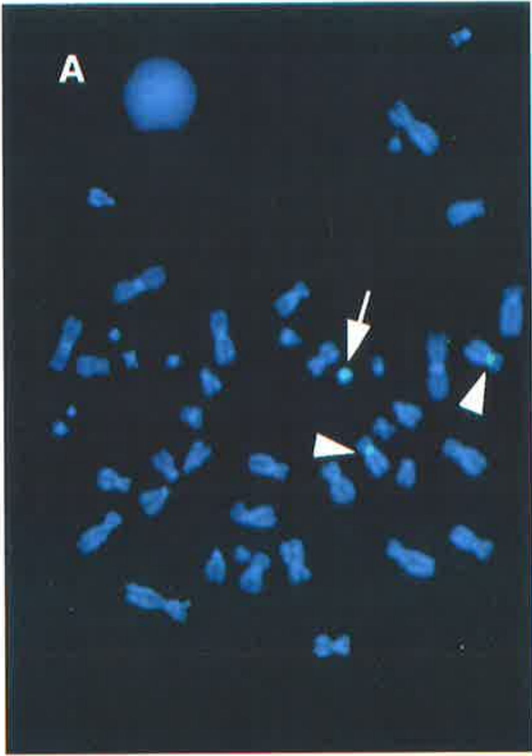


Fig 5-3. FISH analysis with the probes developed from the ring ESACs in patients F **(3A)**, G**(3B)**, H**(3C)** and I**(3D)**. These probes were hybridized to the centromere of chromosome 8 **(3A)**, 8 **(3B)** and 4 **(3C)**, respectively. FISH analysis with the combination of a centromere probe, D1Z1 and microdissected ring in patient I **(3D)**. Hybridization with D1Z1 was shown in white on the ring and the centromere of chromosome 1 while hybridization with probe created from ring ESAC was shown in red which span from centromere to 1q12 area **(3D)**.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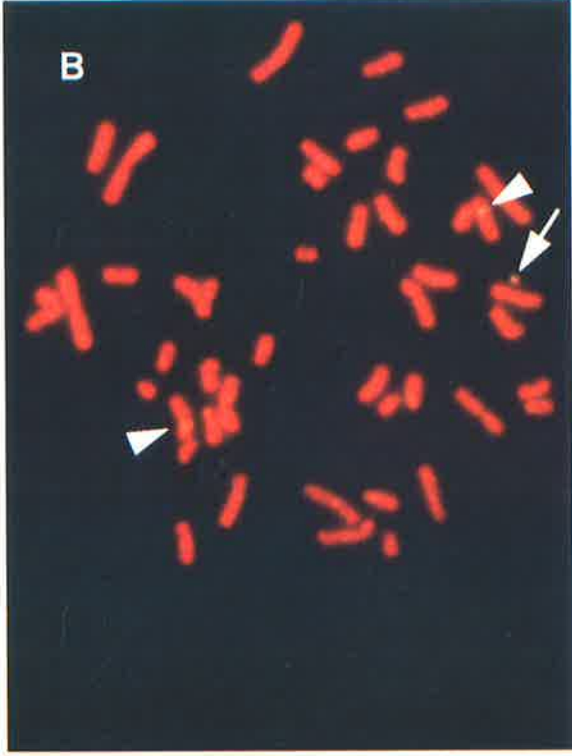
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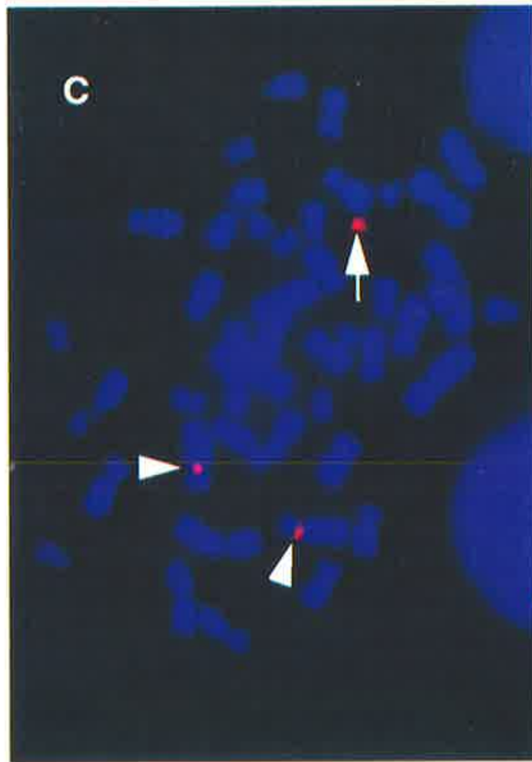
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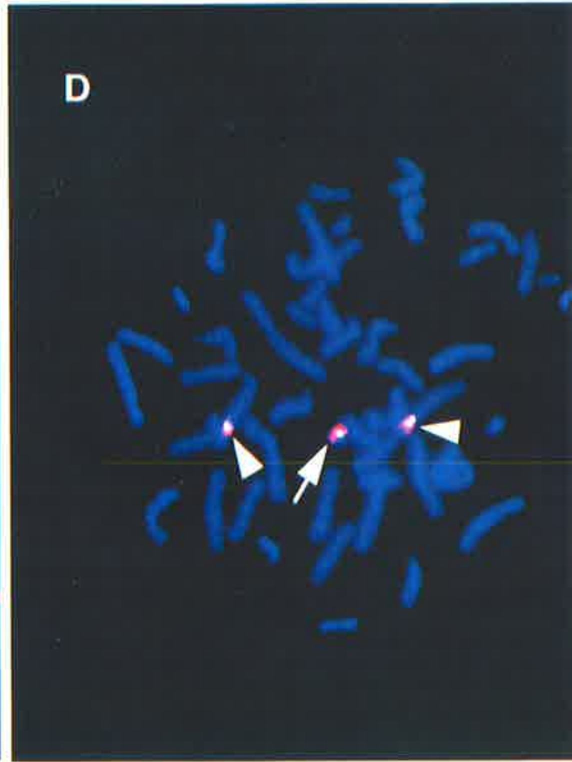
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CHAPTER 6

CHARACTERIZATION OF RING CHROMOSOME 1 AND 20 WITH MICRODISSECTION AND MOLECULAR CLONING

6.1 Introduction

Patients with phenotypic abnormalities resulting from an extra small ring chromosome have now been described (see Table 1-3 in Chapter 1). Different patient phenotypes and the origin of the ring from any chromosome makes it difficult for a clinical counselor to estimate the potential risk for developmental abnormality when such a ring is ascertained in the clinic. Therefore, the precise definition of the original euchromatin of the ring by a prospective study and retrospective studies may yield important information that is useful in assessing potential risks.

Throughout the literature to date, 6 cases of ring ESACs have been reported that were derived from chromosome 1 (Callen et al. 1990b, 1991, Michalski et al. 1993, Chen et al. 1995a, Lanphear et al. 1995, Plattner et al. 1993b). Some of the reported accessory marker chromosome 1s did not describe the structure of the marker and karyotypes were not presented and these have not been included. The clinical features of these patients extended from normal (Callen et al. 1990b, Michalski et al. 1993, Plattner et al. 1993b) to abnormal (Callen et al. 1991, Chen et al. 1995a, Lanphear et al. 1995). Only three patients have been described with accessory ring chromosome that originate from chromosome 20 and in all three cases, the patients showed abnormal phenotypes (Batista et al. 1995, Blennow et al. 1993, Callen et al. 1991). The origins of these rings were mainly identified using FISH with specific centromere probes. In addition, FISH with probes from flow-sorted DNA and microdissected DNA have also been employed to characterize the ESACs (Blennow et al. 1994b, Blennow et al. 1992, Thangavelu et al. 1994, Viersbach et al. 1994).

In Chapter 3, a combination of microdissection and molecular cloning has been successfully used to characterize an extra small ring chromosome 4. In this study, we attempted to apply these techniques to the identification of two further cases of extra small ring chromosomes to precisely determine their origins and genetic contents. Both ring ESACs have been previously reported using FISH with centromere probes (Callen et al. 1991) and were derived from chromosome 1 and chromosome 20, respectively.

6.2 Materials and Methods

6.2.1 Patient description

Patient J: This patient was a male born after normal pregnancy, labor and delivery. Chromosome studies were requested at 2.5 years of age because of delayed speech development and dysmorphic features. At 4 years of age the Reynell developmental language scale showed receptive language abilities to be at a level of 2 years and 11 months. Bat ears were corrected at 5 years of age. Assessed at 8 years 3 months of age, he was functioning satisfactorily in a language-disorder unit of a normal school. Height was 1.35m (90th percentile), weight was 25kg (50th percentile), and head circumference was 50.3cm (10th percentile). He had a lean build with narrow shoulders, bifrontal narrowing, a long face, ear-lobule creases, slightly up-slanting palpebral fissures, a long nose with broad nasal bridge, featureless philtrum, upturned corners to the mouth, mild micrognathia, bilateral clinodactyly of the little fingers, and inverted nipples. Karyotype from father was normal and the mother's karyotype was not available.

Patient K: The patient was a male with unremarkable labor and delivery after normal pregnancy. Birth weight was 2,530 g (<10 percentile). At 14 months of age he was considered to be dysmorphic and there was concern about his development.

When psychological assessment by the Wechsler scale showed that IQ was at normal range at 7 years 10 months of age, his height was 1.12 m (<3rd percentile), weight was 18.7 kg (<3rd percentile), and head circumference was 52.5 cm (50th percentile). He had scaphocephaly, a high-pitched voice, low anterior hairline, abnormally folded low-set ears, synphrys with bushy eyebrows, a featureless philtrum, high palate, open bite and dental crowding, micrognathia, narrow shoulders, hyperextensible elbows and fingers, clinodactyly of fingers 2, 4, and 5 toward the third finger, transverse palmar creases, and partial soft-tissue syndactyly of the fingers 2-5. The right lower limb was 1 cm shorter than the left, resulting in a mild compensatory scoliosis. The family history and karyotype from parents were normal.

6.2.2 Preparation of metaphase chromosomes

Lymphocyte cultures and chromosomal preparation were similar to that described in Chapter 2 but the cells were harvested in only 100% methanol, followed by a quick fix in 3:1 [v/v] methanol:glacial acetic acid before spreading. Chromosomes were stained for 30 seconds in 20-25% Giemsa and stored in sealed Petri dishes at 4°C prior to microdissection.

6.2.3 Microdissection, PCR and FISH

Micropitettes were made from 20 μl microcap capillary tubes, using a pipette-puller (Dacid Kopf Instruments) and a microforge (Narishige). All micromanipulations were conducted under paraffin oil (Merck parrafin Flüssig) in specially constructed glass microdissection dishes, which had been washed with chromic acid and treated with EDTA and mercaptoethanol (200 μl 0.5M EDTA and 100 μl 2-mercaptoethanol in 100 ml distilled water) prior to use. Manipulations were performed on an inverted microscope (Zeiss) with the aid of a manual micromanipulator (Leitz).

From each patient, five pieces of ring chromosome were collected into microdroplets under oil and their DNA was released by Proteinase K treatment and phenol extraction. The microdissected product was digested with *Sau*3A, ligated to adaptors (5' GATCAGAAGCTTGAATTCGAGCAG 3'), and amplified by PCR with primer (5' TCTTCGAACTTAAGCTCGTC 3') using the Perkin-Elmer Cetus Amplitaq kit. Amplifications were conducted in a Corbett Research FTS-1 Fast Thermal Sequencer, using one cycle at 94°C for 3 min, followed by 37°C for 1 min (after which *Taq* polymerase [Perkin Elmer Cetus] was added); 40 cycles at 94°C for 1 min, followed by 55°C for 1 min and then 72°C for 3 min; and one cycle at 55°C for 1 min, followed by 72°C for 10 min. Ten percent of the sample was size-fractionated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The work of microdissection and PCR were kindly completed by Dr R.J. Hill in CSIRO Division of Biomolecular Engineering, Laboratory for Molecular Biology, NSW, Australia.

FISH with PCR amplified microdissected DNA was carried out for both patients as described in Chapter 2.

6.2.4 Molecular cloning, isolation of unique DNA and mapping

PCR products were purified with the Pre-A-Gene (Bio-Rad) and cloned into the pGEM®-T vector (Promega). The procedure of cloning has been detailed in Chapter 2. To isolate clones containing single-copy DNA, all colonies were transferred to Hybond-N^{+} nylon membrane (Amersham, UK) and probed with ^{32}P labelled human genomic DNA (see Chapter 2). These negative colonies might contain single-copy DNA and were amplified by PCR using the vector oligoprimers: forward, 5'-CACGACGTTGTAAAACGACGGCCAGT-3' and reverse, 5'-TAATACGACTCACTATAGGG-3'. For each clone, the size of insert was estimated from agarose gels and those with inserts greater than 400bp were selected for further study. The PCR amplified products from these selected clones were labelled with ^{32}P

and used to probe filters of Southern blots of restricted DNAs from total human, total mouse, and rodent/human hybrids which contained either chromosome 1 or chromosome 20 (Table 6-1).

Table 6-1 Chromosome constitution of mouse/human hybrids

Hybrid line*	Portion Autosome Present	Other Humans Chromosome Present	References
CY151	1q1→pter	Unknown	Callen et al. (1995)
CY152	1pter→p11	Many	Callen et al. (1992b)
CY182	14q32→qter	1, 3, 5, 6, 15, 17 +others	Shen et al. (1994)
CY130		3, 6, 15, 20, 22	Callen et al. (1990c)
CY153	11pter→p11.2	5, 20	Callen et al. (1990c)
CY170	4pter→q35	5	Callen et al. (1990c,d)
CY18	complete 16	absent	Callen et al. (1986)

*Each hybrid contained total mouse DNA (A9) and portion of human chromosome 16 DNA.

Hybrids CY151, CY152 and CY182 were selected for mapping study of patient J. To confirm that CY151, CY152 and CY182 still contained chromosome 1, the hybrids were checked by PCR with polymorphic markers D1S164, D1S185 and D1S249. For mapping study of patient K, the hybrids CY130, CY153 and CY170 were selected since they contained completed human chromosome 20. CY170 and CY18 were chosen as a control.

6.2.5 Sequence, synthesis of oligo and PCR amplification

Clones isolated from patient J were sequenced as described in Chapter 2. From the sequence of one of these clones, P210, oligoprimers were designed to check the origin of cloned, microdissected products by amplification of hybrids DNA from NIGMS human/rodent somatic cell hybrid mapping panel #2: Chromosome 1 (GM/NA7299), Chromosome 2 (GM/NA10826B), Chromosome 3 (GM/NA10253),

Chromosome 4 (GM/NA10115), Chromosome 5 (GM/NA10114), Chromosome 6 (GM/NA10629), Chromosome 7 (GM/NA10791), Chromosome 8 (GM/NA10156B), Chromosome 9 (GM/NA10611), Chromosome 10 (GM/NA10926B), Chromosome 11 (GM/NA10927A), Chromosome 12 (10868), Chromosome 13 (GM/NA10898), Chromosome 14 (GM/NA10479), Chromosome 15 (GM/NA11418), Chromosome 16 (GM/NA10567), Chromosome 17 (GM/NA10498), Chromosome 18 (GM/NA11010), Chromosome 19 (GM/NA10449), Chromosome 20 (GM/NA10478), Chromosome 21 (GM/NA10323), Chromosome 22 (GM/NA10888), Chromosome X (GM/NA06318B), Chromosome Y (GM/NA06317).

6.3 Results

The karyotype of patient J was 46, XY/47, XY,+r with the ring present in 70% of the metaphases (Fig. 6-1A). Distamycin A/DAPI banding and Ag-NORs staining for the ring was negative. FISH with centromere probes indicated the ring was originated from chromosome 1 (Fig. 6-1B). The amplified, microdissected products from patient J were hybridized to metaphase chromosomes and the hybridization was only observed at the centromere of the two normal chromosome 1s and on part of the ring (Fig. 6-1C). Therefore this ring was further confirmed to originate from chromosome 1.

The microdissected products from patient J were cloned into the TA cloning vector (Promega). A total of 67 colonies were obtained and colony blot showed that 4 clones were positive and were considered to contain highly or moderately repetitive DNA. The remaining negative colonies might contain single-copy DNA, or low-copy repetitive DNA or no insert. Colony PCR was performed to recover the inserts of those clones which were negative in the colony blot. Forty-five clones that contained inserts larger than 400bp (Fig. 6-2A) and selected for further mapping studies.

The hybrid lines CY151, CY152 and CY182 were checked with three polymorphic (AC)n microsatellite markers of human chromosome 1, that D1S164 located at 1p35, D1S185 located at centromere and D1S249 located 1q31-32. CY151 was positive for D1S164 and D1S185 and was confirmed to have 1q1→pter, CY182 was positive for all three markers and was confirmed to contain the completed sequence of human chromosome 1 while CY152 was found not to contain these markers (Fig. 6-2B), therefore CY152 was not selected for mapping studies.

In mapping of the forty-five clones, only four clones (P72, P204, P206 and P210) were hybridized to the hybrids and total human DNA; the remaining clones, unfortunately, did not hybridized to any hybrid or total human DNA. The clones P72, P204, P206 and P210 were used to probe Southern blots of DNA from these somatic cell hybrids and showed that all four clones hybridized to both CY182 and total human DNA but did not hybridize to CY151 (Fig. 6-3A). This indicates that the inserts of those four clones may derived from the long arm of chromosome 1.

Analysis of the sequences of these four clones (Fig. 6-4A, B) indicated that the clones P72 and P204 were identical and contained an insert of 89bp. The clone P206 contained an insert of 190bp and P210 contained 427bp. Oligoprimers were synthesized from P210; forward 5'-ACCCGATATCATGTACCTCT-3' and reverse 5'-CCCTCAGCTCCTGTATTCTTCA-3' (Fig. 6-4A, B). These oligoprimers were used to amplify DNAs from 24 human/rodent somatic cell hybrids (NIGMS), each of them contained single human chromosome, respectively. Clone P210 and genomic DNA were used as positive control. The PCR results showed a band of approximately 495bp from the hybrid, GM/NA10629 which contained only human chromosome 6, the plasmid P210 from which the sequence was derived and total human DNA but not from the hybrid, GM/NA7299 containing human chromosome 1 (Fig. 6-3B). Primers were not synthesized from clone P72/P204 since the insert was less than 90bp. Primers were generated from clone P206; forward 5'-GGACAATACCTATTGGGACAA-3' and reverse 5'-

CGCTGTTTCCGAAAACCGAT-3'. PCR amplification showed a band of approximately 170bp from the origining clone P206 and some human genomic DNA (positive for three out of ten different individuals) but no band was observed on any lane of mouse/human hybrids with single human chromosomes. These results were supposed to associate with polymorphism. Based on these PCR results from clone P210, it was concluded that the library constructed from the microdissected products of the ring chromosome 1 was contaminated. Therefore this study was not pursued further.

The karyotype of patient K was 47,XY,+r[72]/48,XY,+r,+r[28] (Fig. 6-5A). All rings were negative for DA/DAPI and Ag-NORs staining. In situ hybridization with centromere probes showed that the ring was derived from chromosome 20 (Fig. 6-5B). When analyzed on an agarose gel the PCR amplified, microdissected ring chromosome 20 appeared as a smear with several bands in the size range of 200-500bp (data not shown). FISH to metaphase spreads with the biotin-labelled microdissected DNA showed no signal on any chromosome or to the ring (data not shown). After molecular cloning, a total of 98 colonies were obtained and colony blot showed 30 clones were positive, indicating these clones contained highly repeat copy DNA.

Colony PCR was performed on the negative colonies and 33 clones with insert sizes larger than 400bp (Fig. 6-6A) were selected for further mapping study. Probing Southern blots of DNA from the somatic cell hybrids demonstrated that 27 clones did not hybridize to any hybrid or total human DNA. These negative results might be due to the loss of inserts during processing or false positive of colony PCR.

Only 6 clones (C32, C79, C82, C85, C156 and C170) were mapped to hybrids and to total human DNA. The clones C32, C79 and C82 hybridized to the hybrids CY130, CY153 and total human DNA (Fig. 6-6B), indicating these three clones are likely to map to chromosome 20. However, clones C85 and C156 hybridized to

CY153, CY170 and total human DNA but not to CY130 (Fig. 6-6B), suggesting these two clones contained DNA from either chromosomes 4 or 5. Clone C170 hybridized to CY130 and total human DNA but not to CY153, indicating the insert of this clone was from human chromosomes other than chromosome 5 and chromosome 20. These results suggested that the microdissected library was contaminated and any further work was abandoned.

6.4 Discussion

Microdissection and molecular cloning are the most sensitive methods to identify the detailed origin of small ring ESACs, as was successfully used in the characterization of a ring chromosome 4 (see Chapter 3). The investigation of a ring chromosome 1 in patient J and a ring chromosome 20 in patient K was attempted using a similar microdissection and molecular cloning strategy. The rings from both patients were microdissected and DNAs were amplified and cloned in a TA vector. For patient J, isolated clones with single-copy DNA were sequenced since mapping studies could not confirm that the insert originated from chromosome 1. All four clones hybridized to CY182 which contained chromosome 1, 3, 5, 6, 15, 17 but not to the hybrid CY151(1q1→pter) in which the other human chromosome contained in this hybrid have not been characterized. After sequencing and oligoprimer synthesis, PCR with the oligoprimers confirmed that the microdissection library was contaminated with DNA from chromosome 6, although FISH with the microdissected DNA showed only hybridization to the ring and the centromere of chromosome 1 but not to chromosome 6. This result was possibly caused by preferential amplification of the abundant repeat DNA from the microdissected ring chromosome 1 rather than contaminated single-copy DNA from chromosome 6. The FISH resolution was presumably not sensitive enough to detect the contaminating single-copy DNA of chromosome 6 in the presence of the amplified repeat DNAs from chromosome 1.

Similar studies in patient K indicated that this microdissection library likely contained DNA from chromosome 20 but was also contaminated with DNA, of either chromosome 4 or chromosome 5, since 3 out of 6 isolated clones mapped to other chromosomes than chromosome 20. Reverse painting showed an absence of signal on the ring or any other chromosome. This was supposedly due to either very low proportion of DNA from the marker since only five pieces of ring chromosomes were microdissected or DNA degeneration since the sample had been kept in freezer for several months before FISH study.

The problems of contamination may be related to several factors. Firstly, very small numbers of microdissected ring chromosome have been used in this study. In both libraries, only 5 pieces of each ring were dissected by an oil chamber method. Secondly, these 5 pieces of ring chromosome under paraffin oil were transferred to microdrops for s series of DNA preparation, including treatment by proteinase K, extraction by phenol, digestion by *Sau*3A and ligation with adaptors. Thirdly, microdissected product had 40 cycles of amplification following the enzyme digestion and ligation, and this is likely to result in preferential amplification of certain types of DNA such as repeat DNA. All these procedure are technically demanding and therefore the chance of contamination was high.

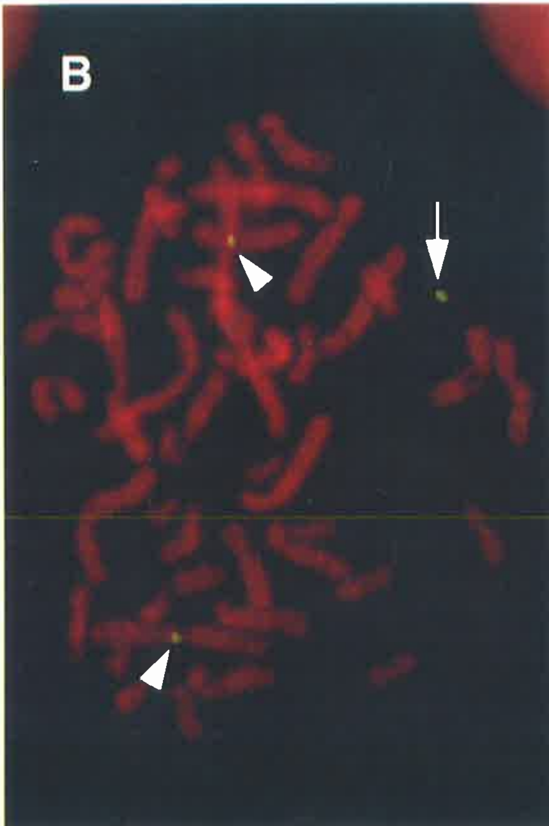
It is important to avoid possible contamination at the microdissection and amplification step since subsequent analysis by molecular cloning and mapping techniques are time-consuming and contamination can be discovered only after prolonged analysis. To prevent contamination with other chromosome during dissection, Hagag and Viola (1993) have suggested that three aspects which should be observed: (1) increasing the precision of the dissection including a thin dissection needle of less than 0.5 μm in tip diameter, (2) using a fresh microneedle for each ring chromosome dissected and (3) choosing a ring chromosome set apart from other chromosomes.

More recently developed techniques involve microdissection performed directly on the slide without oil and more than 5 pieces were dissected as was performed by Dr Bohlander in Chapter 3 and Dr Guan in Chapter 4. Furthermore, microdissected products were directly transferred to a PCR tube for preparation and amplification. These procedures greatly reduce the chances of contamination. The two ends of the microdissected products were linked to universal primers by PCR (see Chapters 3 and 4). The PCR amplification was normally performed by first undertaking several cycles at low annealing temperatures (30-37°C) to allow the primer to anneal at random sites along the DNA, and then by no more than 30 cycles at 55-56°C annealing temperature to allow symmetrical amplification of the microdissected DNA (see Chapters 3 and 4).

The characterization of seven rings from Chapters 3 to 5 showed no probe generated from microdissected products hybridized to chromosomes other than the original chromosome of the ring. Despite this fact and the above discussion about likely contamination, there is a remote possibility that the unexpected results are genuine. Multiple ring ESACs and that rings derived from the various chromosomes have been reported (Callen et al. 1991, Mascarello et al. 1987, Pezzolo et al. 1993a, Plattner et al. 1993b, Tozzi et al. 1988). Is it possible that the ring ESAC can involve non-homologous chromosomes? ESACs can be derived from the 3:1 segregants of reciprocal translocations (Brondum-Nielsen 1991, Stamberg and Thomas 1986, Winsor and Van Allen 1989). The ring chromosomes could be derived from complex interchromosomal rearrangements. Nevertheless, this possibility could be resolved by further studies in these two cases.

Fig. 6-1 A metaphase from patient J after Giemsa staining **(A)**. FISH on metaphase from patient J with D1Z1 **(B)** and with microdissected DNA **(C)**, both probes gave signals on the ring chromosome (arrows) and two normal chromosome 1 (arrowheads).

A


B

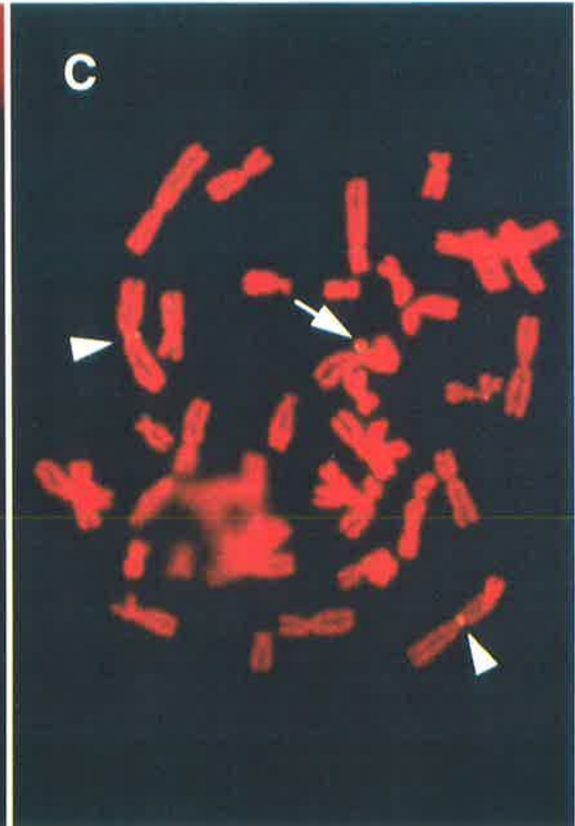
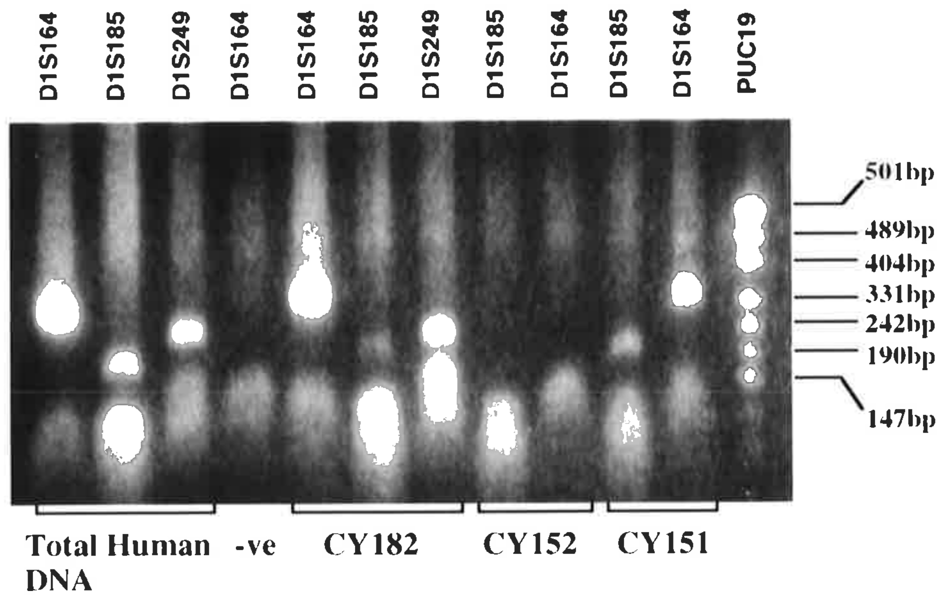

C

Fig. 6-2. Top panel, Inserts recovered by PCR from 4 individual microclones (lanes 3-6 from left) in patient J. The size of inserts were between 400bp to 660bp which included approximately 200bp vector sequence. **Bottom panel,** Chromosome 1 sequence in CY182, CY152 and CY151 were checked with three polymorphic (AC)n microsatellite markers of human chromosome 1, D1S164, D1S185, D1S249. D1S185 and D1S164 on CY152 lanes showed no band, indicating CY152 did not contain chromosome 1 DNA.

SPP-1
PUC19
P72
P204
P206
P210
1090bp
880bp
660bp
501bp
489bp
404bp


D1S164
D1S185
D1S249
D1S164
D1S164
D1S185
D1S249
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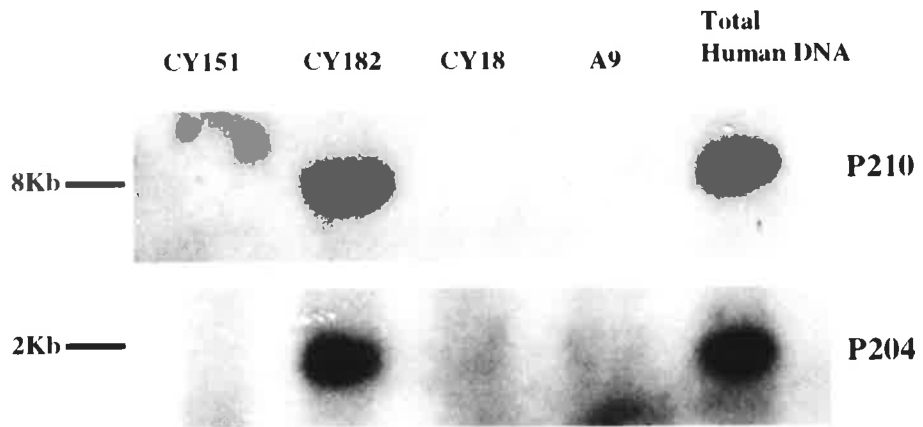
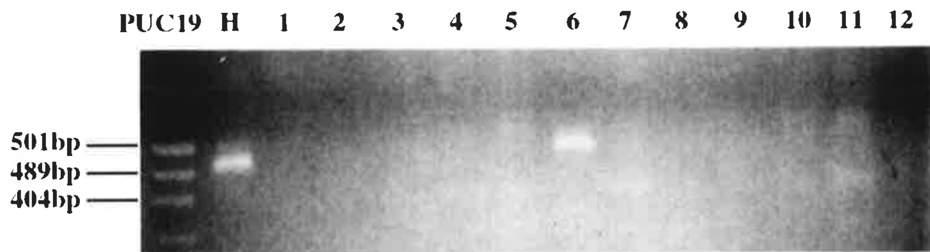
D1S164
PUC19
501bp
489bp
404bp
331bp
242bp
190bp
147bp
Total Human DNA
-ve
CY182
CY152
CY151

Fig 6-3. Top panel, Southern blots using clones P204 and P210 from patient J as probes against Hind III-digested rodent/human hybrids CY151 and CY182. CY151 contained the region from pter-1q1, while CY182 contained the entire human chromosome 1. Total human DNA was positive control, CY18 and A9 were negative controls. **Bottom panel,** PCR amplified each chromosome with oligos from P210. The lane of Chromosome 6 and total human DNA were positive but lane of chromosome 1 was negative.


CY151
CY182
CY18
A9
Total
Human DNA
8Kb
P210
2Kb
P204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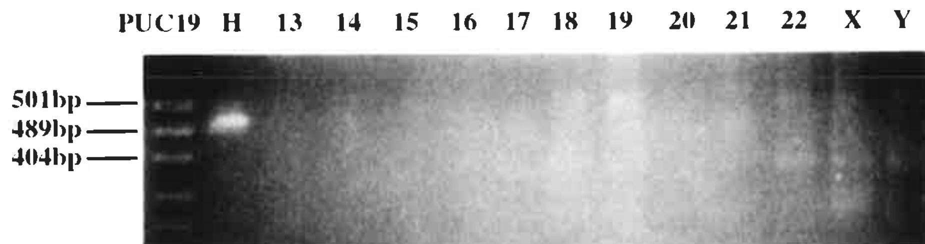
PUC19
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22
X
Y
501bp
489bp
404bp

P72 and P204 (89 bp): 5-ATA AAA ATG GAC TCC AAA GAG CTA ACT TTG TAC CAT TCA TAG CAG TCT TGA AGG TAA AAA CAA ATC ATT TAT TTG TGT TTA CTA AGG TG -3.

P206 (190 bp): 5-GCT CGA AGA AAC GTG ATT TAG TGC GGA CAA TAC CTA TTG GGA CAA AGC AGG AGT CCG CTC CCC GAC TAG GAA AAA AAA GCT GGC GCC GTT TCC GTT CGT CAG AGC GTT CCC AGG AAG GCC GCC ACA TCC TCG CGC TCT TCC GCC GTC AGT TCG CGG CGG CGC ANA GGG GGA TCG GTT TTC GGA AAC AGC G-3'.

P210 (436 bp): 5-GAG TCT GGN AAC CCG ATA TCA TGT ACC TCT AAA TGT GAA GTA TGC GGT AGA GCT AAT GTT TTG TCA GAG TTT AAA GAA GGA AGC AAT AAT ATG GGC AAA GTT GTT GAG CAA GGC TTC AGG GAG GAA GTA GAA CCT GAA CAG GCT TTT AAA AAC ACT GGC ACC ATT TGG ATT GGT GAG GAG GCG GTA GAA GGG TGT CCT CTC TGG GTG GGA CAG CAC AAA CAA AGG TGT GGG ACC AAA ACT CAA CAA GGC ACA TTT GGG CAA CAC TTG GTT GAG CAA TTC CAT GGG AGT GCA AGA CTC ATG GGC GAA ATC ATT GGA AGC CAC ATG GTG GAA GGC TTT AAA TGT CAA TGT GTT CTT CAT GCC ATG GAA ATT CCA CAA GGC TTG GCA GTT TGC AGG AAA GAC CAG AAT TGA AGA ATA CAG GAG CTG AGG G-3

Fig. 6-4A. Complete sequence of clones P72 (P204), P206 and P210. The bases underlined indicated the sequence of the oligoprimers synthesized.

Fig. 6-4B. Partial sequence of clone P210. The insert started from the base 103 and before this was the sequences of adaptor and partial vector (indicated by the black arrow). The bases from 127 to 148 were synthesised reverse oligoprimer (underlined in red).

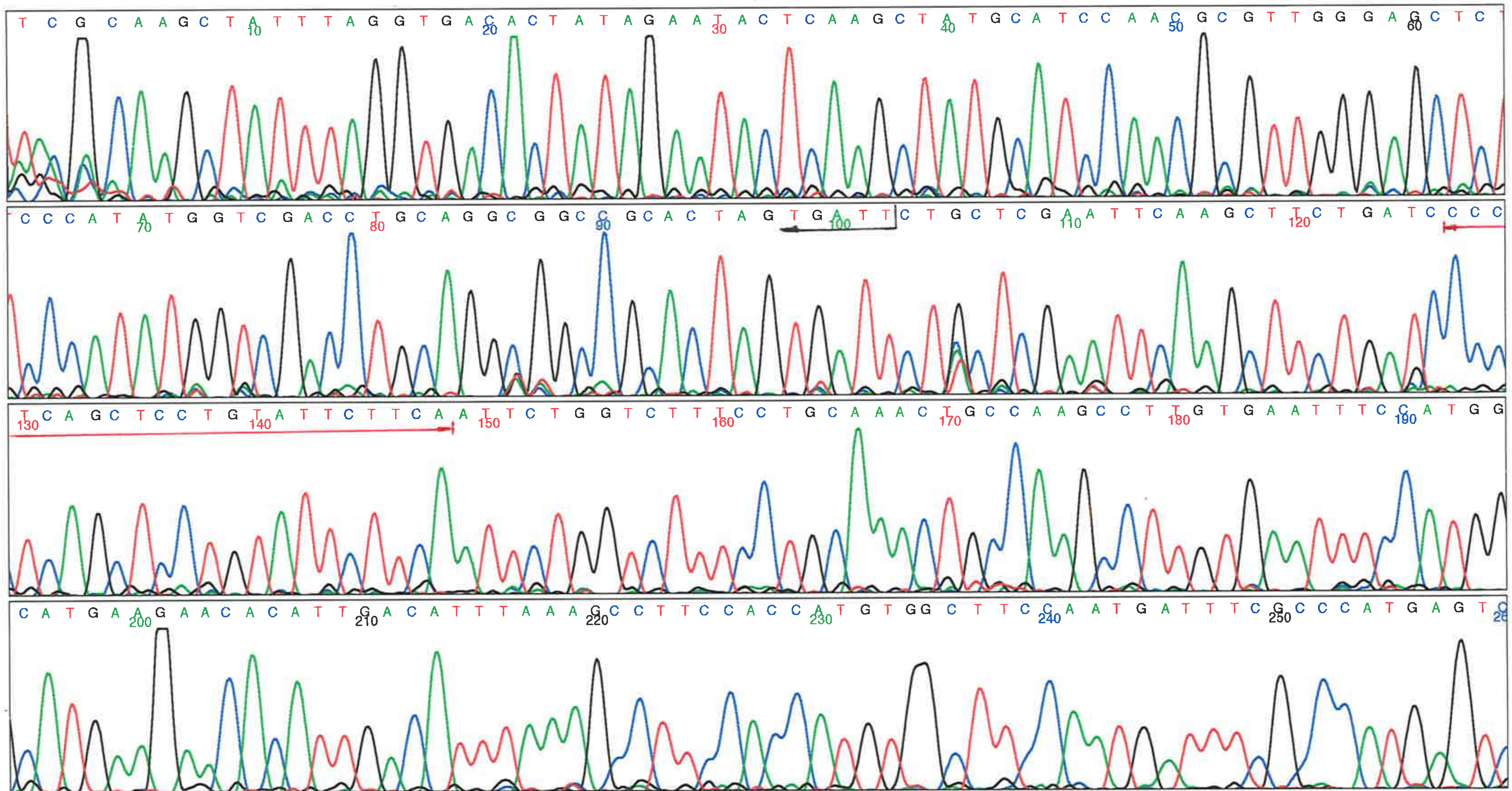
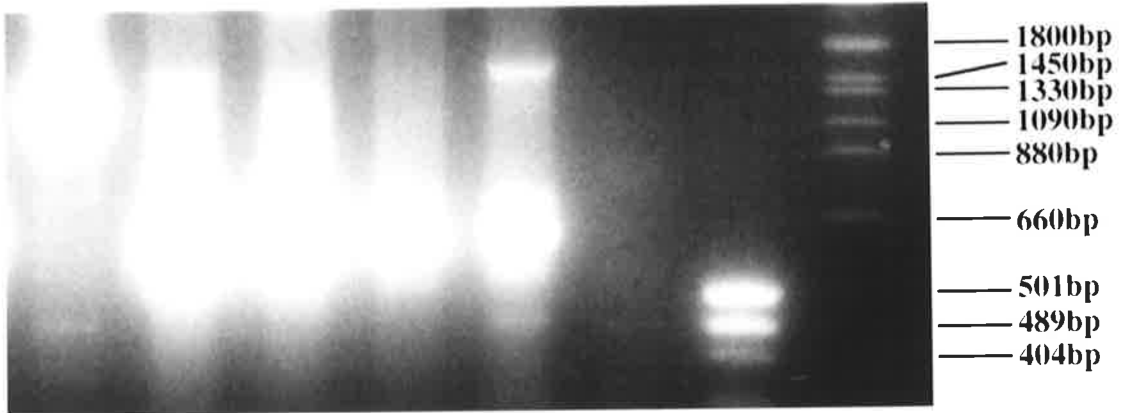

T C G C A A G C T A T T T A G G T G A C A C T A T A G A A T A C T C A A G C T A T G C A T C C A A C G C G T T G G G A G C T C
C C C A T A T G G T C G A C C T G C A G G C G G C C G C A C T A G T G A T T C T G C T C G A A T T C A A G C T T C T G A T C C C C
T C A G C T C C T G T A T T C T T C A A T T C T G G T C T T T C C T G C A A A C T G C C A A G C C T T G T G A A T T T C C A T G G
C A T G A A G A A C A C A T T G A C A T T T A A A G C C T T C C A C C A T G T G G C T T C C A A T G A T T T C G C C C A T G A G T C

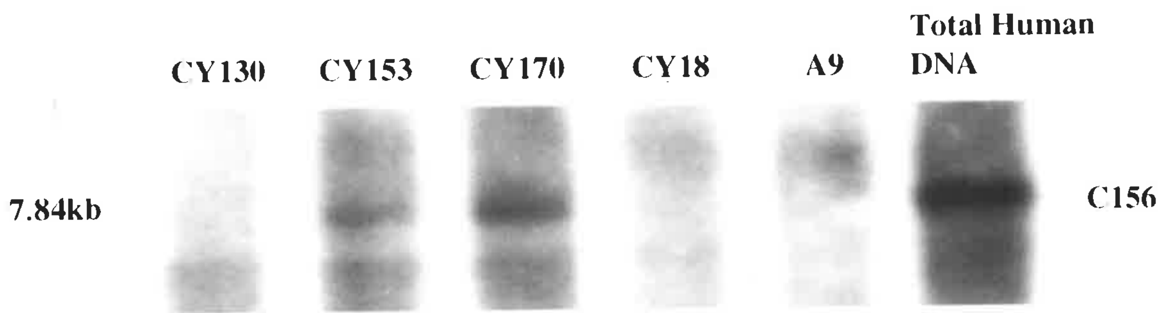
Fig. 6-5. A metaphase from patient K after Giemsa staining **(A)** and *in situ* hybridization with D20Z1 showed the signal on the ring chromosome (**B**, arrow) and on homologous chromosome 20s (**B**, arrowheads).

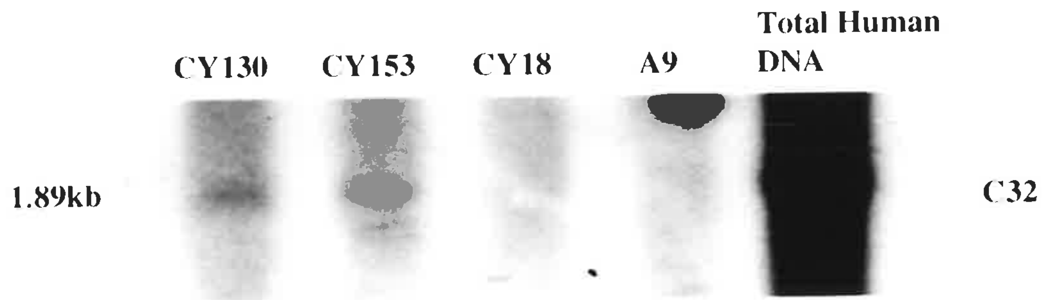
A

B

Fig. 6-6. Top panel, Inserts recovered by PCR from 5 individual microclones (lanes 1-5 from left) in patient K. The sizes of inserts were between 500bp to 1300bp including approximately 200bp of vector sequence. **Bottom panel,** Southern blots using clones C32 and C156 as probes against Hind III-digested rodent/human hybrids CY130 and CY153. Both hybrids contained the entire chromosome 20 but CY153 also contained chromosome 5. CY170, which contained chromosome 5, was used as a control. Total human DNA was the positive control, CY18 and A9 were negative controls.


C32
C79
C82
C156
C170
-ve
PUC19
SPP-1
1800bp
1450bp
1330bp
1090bp
880bp
660bp
501bp
489bp
404bp


CY130
CY153
CY170
CY18
A9
Total Human DNA
7.84kb
C156


CY130
CY153
CY18
A9
Total Human DNA
1.89kb
C32

CHAPTER 7

HIGH RESOLUTION CHARACTERIZATION OF AN INTERSTITIAL DELETION OF LESS THAN 1.9 MB AT 4p16.3 ASSOCIATED WITH WOLF-HIRSCHHORN SYNDROME

7.1 Introduction

Wolf-Hirschhorn Syndrome (WHS) was independently described by Wolf and Hirschhorn in 1965 (Wolf et al. 1965, Hirschhorn et al. 1965) and has an incidence of 1/50000 live births (Johnson et al. 1976, Goodman and Gorlin 1983). It is characterized by severe growth and mental retardation, seizures, and distinct facial features described as "Greek warrier helmet " (Fig. 7-1C). It is due to deletions of the distal short arm of chromosome 4.

The deletions in the short arm of chromosome 4 can range from one-half of the short arm (Lurie et al. 1980, Wilson et al. 1981) to cytogenetically undetectable (Preus et al. 1985). These undetectable cases can be either small deletions or cryptic translocations which can be detected by fluorescence in situ hybridization (FISH) (Altherr et al. 1991, El-Rifai et al. 1995) or polymerase chain reaction (PCR) (Altherr et al. 1992). The critical deletion region of WHS within 4p16.3 was estimated to be 2 Mb between the markers D4S142 and D4S43 (Gandelman et al. 1992, Estabrooks et al. 1992). Subsequently, Reid et al. (1996) excluded the loci D4S111 and D4S115 from the critical deletion region and Somer et al. (1995) reported a patient with typical WHS who was not deleted for D4S96, therefore the critical region was further reduced to 1.2 Mb between the loci D4S96 and D4S43. The present study characterized a subtle interstitial deletion of 4p16.3 in a girl possessing mild WHS

manifestations. FISH analysis maps this deletion to the critical WHS region. The interstitial deletion in this patient is estimated to be approximately 1.9 Mb in size.

7.2 Materials and Methods

7.2.1 Clinical report

KS was the first child of Caucasian parents, maternal age 18 years and paternal age 25 years. Birth was at 32 weeks by emergency Caesarean section because of antepartum haemorrhage. Apgar scores were 4 at 1 minute and 7 at 5 minutes. Birth weight was 1480 g (25th centile) and head circumference (OFC) was 28cm (10th centile). She required ventilation for hyaline membrane disease and phototherapy for jaundice.

She fed poorly, had gastro-oesophageal reflux and failed to thrive, resulting in gavage feeding between 12 and 15 months. Development was noted to be delayed in the first months of life and there were minor facial anomalies. Chromosomes were initially reported to be normal. Ultrasound study showed mildly dilated ventricles of the brain. There were recurrent respiratory infections during infancy. Immunoglobulin A deficiency could be demonstrated until 2 years of age. Generalised seizures occurred with fever at 3 and 5 years of age.

The patient was reviewed at 5.5 years. There was moderately developmental delay. Height, weight and head circumference were well below, but tracking parallel, to the 3rd centile. Mental retardation was mild. She had minor anomalies of ear shape, upslanting palpebral fissures, telecanthus, broad nasal bridge, relatively short philtrum, small mouth and small chin (Fig. 7-1A, B). There was minor clinodactyly of the right little finger and broad halluces. There was an exaggerated lumbar lordosis. Chromosomal analysis was repeated because the craniofacial changes suggested a diagnosis of Wolf-Hirschhorn syndrome.

7.2.2 Cytogenetic Analysis and FISH Study

High resolution cytogenetic studies was performed on the patient and her mother using trypsin-Giemsa (GTG) banding. The patient's father was not available for study.

Molecular cytogenetic study of the patient was performed by FISH with markers located at 4p16.3, including D4S142 (2R88), D4F26 (pC847.351), D4S90 (CD2), D4S133 (cDp16), D4S96 (pC678), D4S168 (8C10E4), D4S113 (A62.5), D4S98 (pC385.12), D4S166 (L6), D4S43 (C9A), L25G12, 79F5, L65C1, D4S182 (247F6), D4S127 (195C9), and D4S180 (21F12). The relative order and distance between probes is given in Figure 7-3. A cosmid DNA , 77G3, located at 4q25 was used as a control probe for chromosome 4. The FISH procedures were used as described in Chapter 2. A fluorescein isothiocyanate-labelled avidin/antibody step was used to amplify the hybridization signal. Cosmid DNA biotin labelled by nick translation were hybridized to metaphase spreads from the patient and her mother. Counterstaining of the propidium iodide stained chromosomes with DAPI allowed identification of chromosomes and location of the signals to specific chromosome bands. Images of metaphase preparations were recorded on colour slides.

7.3 Results

High resolution studies suggested a possible deletion of 4p16.3 although this could be observed in some prometaphase spreads (Fig. 7-1D). This deletion was confirmed by FISH using the DNA probes, D4S168 (8C10E4), D4S113 (A62.5), D4S98 (pC385.12), D4S166 (L6), D4S43 (C9A), L25G12, 79F5, L65C1. A hybridization signal from the 4p16.3 region was only detected on one chromosome 4, and was missing from its homolog (Fig. 7-2A). FISH with probes, D4S142 (2R88), D4F26 (pC847.351), D4S90 (CD2), D4S133 (cDp16), D4S96 (pC678), D4S182 (247F6), D4S127 (195C9), and D4S180 (21F12) showed a hybridization signal on

both chromosomes 4 (Fig. 7-2B). Therefore, there was an interstitial deletion of 4p16.3. The proximal breakpoint was between L65C1 and D4S182 (247F6), which are approximately 3.2-3.4 Mb from the telomere, and the distal breakpoint between D4S96 (pC678) and D4S168 (8C10E4), which are approximately 1.5-2.06 Mb from the telomere. Since the deletion spans from D4S96/D4S168 to L65C1/D4S182, this patient has an interstitial deletion up to 1.9 Mb in size.

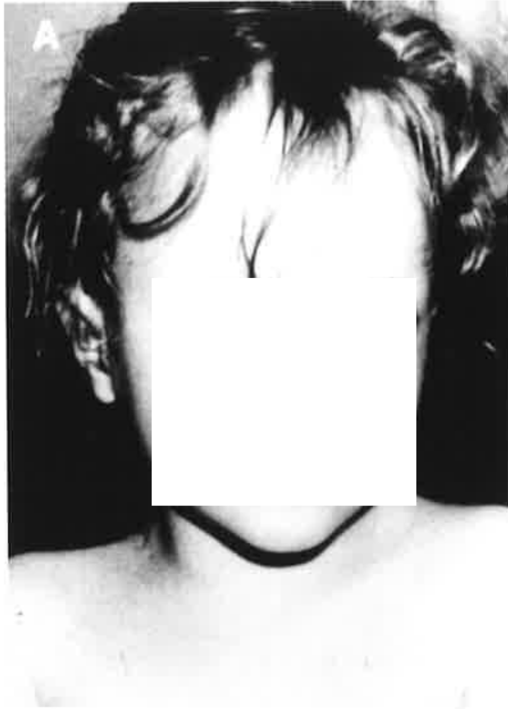
Lymphocyte cultures from the patient's mother showed a normal karyotype, and FISH studies with the probes D4S43 (C9A) and D4S168 (8C10E4), which are located in the critical region of Wolf-Hirschhorn syndrome at 4p16.3, were normal.

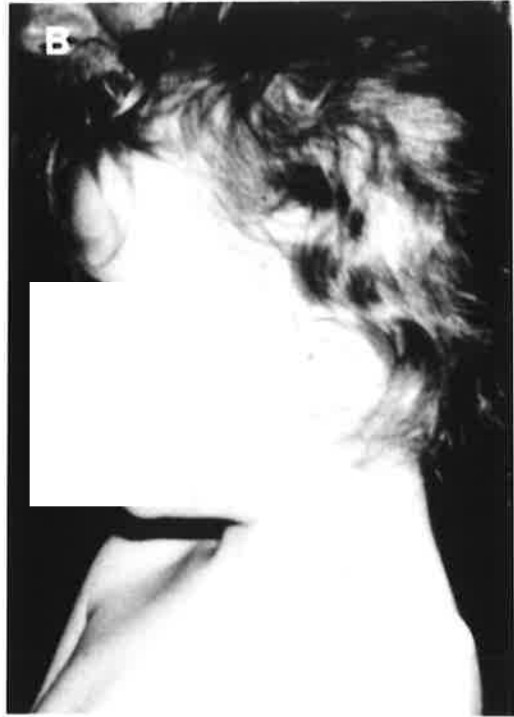
7.4 Discussion

Molecular analysis of various WHS patients localized the critical region to an approximate 2-Mb interval between D4S43 and D4S142 (Gandelman et al. 1992, Estabrooks et.al. 1992). Subsequently, molecular characterizations of several patients have reduced the size of this critical region. Reid et al. (1996) excluded the loci D4S111 and D4S115 while Somer et al. (1995) excluded D4S96. In addition, Wright et al. (1996) described a WHS patient with a distal breakpoint between D4S168 and FGFR3. Therefore, the critical region of WHS is now between D4S168/FGFR3 and D4S166/D4S43 which spans an interval of 450-700 kb (Fig. 7-3). The patient, KS, in this report is consistent with this critical region for WHS and confirms that the loci D4S111, D4S115, and D4S96 are outside the critical region. The probe D4S96 should be used with caution for the FISH diagnosis of WHS since it is outside the critical region and therefore could lead to false negative results.

A closely related syndrome is Pitt-Roger-Dank syndrome (PRD). Sixteen cases with Pitt-Roger-Dank syndrome (PRD) have been reported (Donnai 1986, 1996, Oorthuys and Bleeker-Wagemakers1989, Lindeman-Kusse et al. 1996, Zollino et al. 1996, De Die-Smulders and Engelen 1996) since it was initially described by Pitt et

Fig. 7-1. A, B. Photographs of patient at 5.5 age. **C.** Greek worrier helmet, which was described as typical WHS facial features. The picture was adapted from DeGrouchy and Turleau (1985). **D.** The deletion of chromosome 4 is indicated in the G-banded partial metaphase by the arrows.


A


B

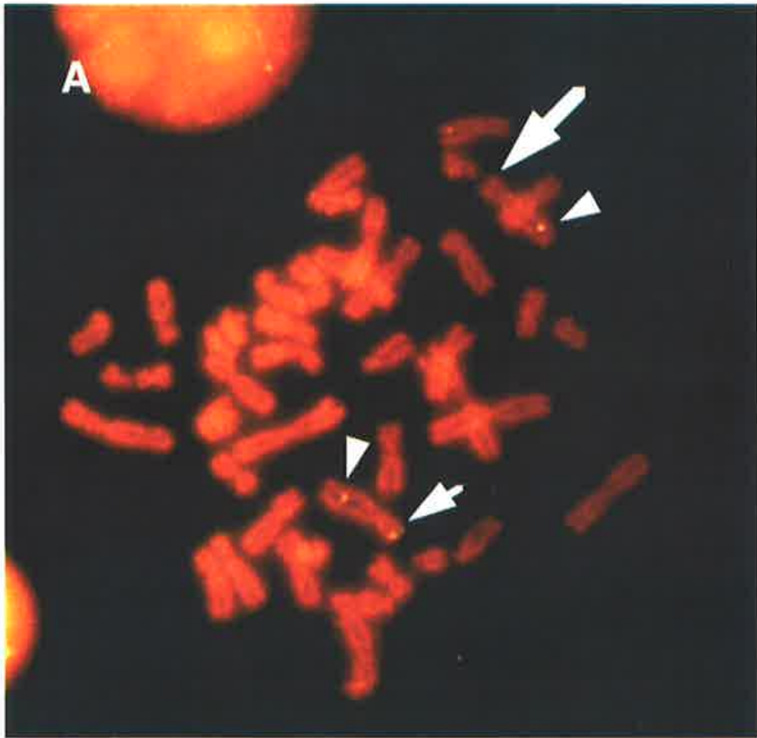
C

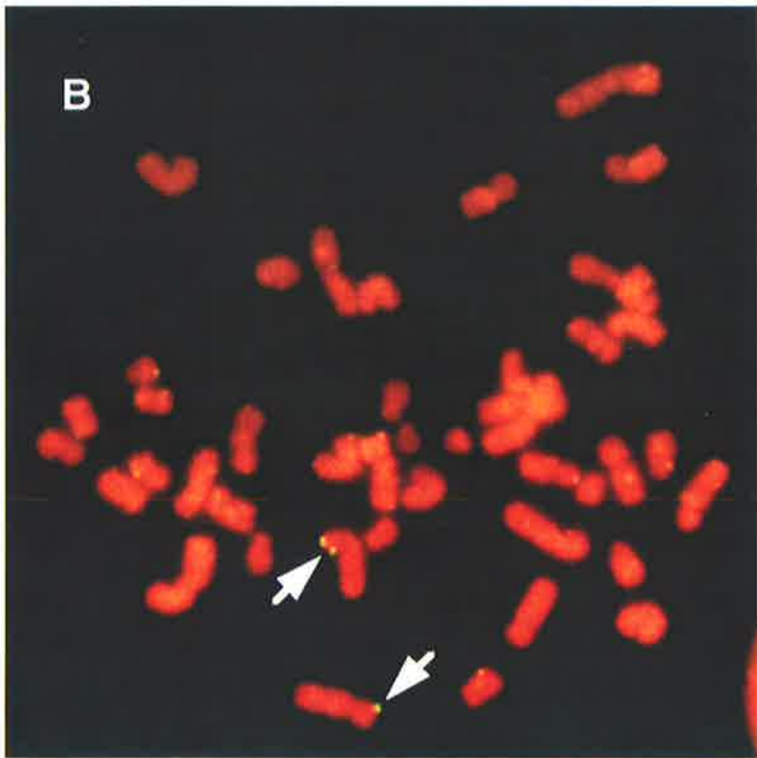
D

Fig. 7-2. Partial metaphases after *in situ* hybridization with 4p probes.

A. Probe D4S166, signal can be seen on the normal chromosome 4 (small arrow) but not on the other partially deleted chromosome 4 (large arrow). The chromosome 4 was additionally indicated by the 77G3 which is located on 4q31 (arrowheads).

B. Probe D4S127 was present (arrows) on both chromosome 4s and is therefore not deleted. The chromosomes were identified by Distamycin A/DAPI banding (not presented).


A


B

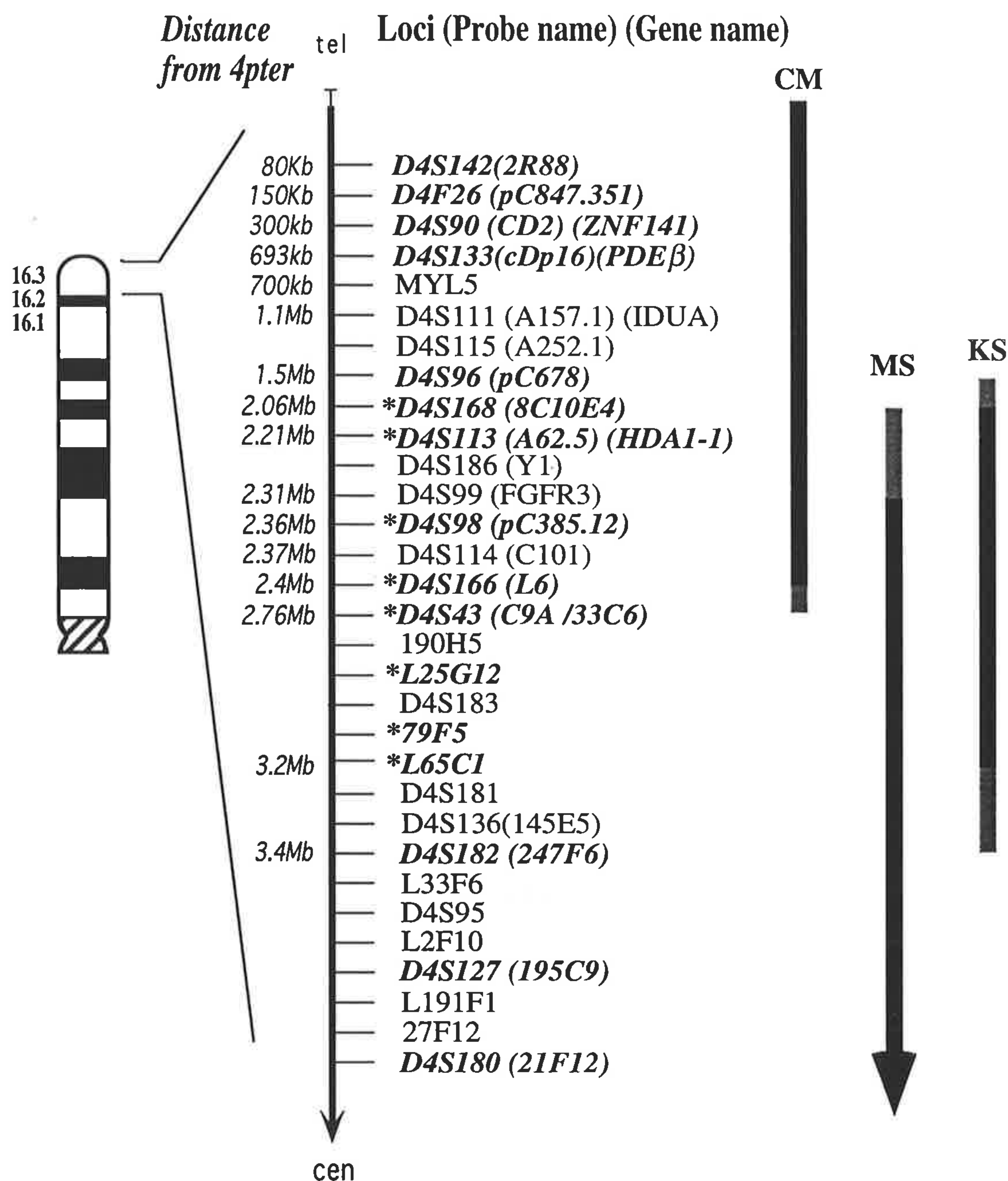


Fig. 7-3. Markers are listed in order from the most distal to the proximal according to Whaley et al. 1988, 1991; Snell et al. 1992; McCombie et al. 1992, Collins et al. 1992b, and Baxendale et al. 1993. The probes with italic were tested in KS patient and the probes with prefix asterisk were deleted in KS patient. Corresponding to each patient's name is a darkly-shaded region representing the deleted loci, and a lightly-shaded region representing the boundary breakpoint region. Patient CM was quoted from Gandelman et al. 1992, patient MS was quoted from Wright et al. 1996, and results for patient KS are reported in this paper.

CHAPTER 8

GENERAL DISCUSSION

A large number of experimental studies have demonstrated that dosage changes of chromosomes cause various abnormalities in humans (see Chapter 1). The results of previous work provided a framework and an impetus for the present investigation of extra structurally abnormal chromosomes (ESACs) and Wolf-Hirschhorn syndrome. The present systematic investigation has used, and further developed, a combination of techniques and leads to the characterization of several ring ESACs: two cases of ring 4 (Chapter 3), one case of ring 15 and two cases of inv dup(15) (Chapter 4), one case of ring 1, one case of ring 4, and two cases of ring 8 (Chapter 5), a further case of ring 1 and the other of ring 20 (Chapter 6).

8.1 Methodology for characterization of ring ESACs

Two main strategies have been used throughout this study. The first was microdissection of the marker chromosomes followed by amplification of the microdissected DNA and direct labelling of these amplified products to allow FISH (reverse painting). The second was microdissection of the marker chromosomes followed by molecular cloning of the microdissected products (see later for details). Compared with either conventional banding or FISH with specific-centromere probes, reverse painting proved effective and straightforward for the identification of the origin of ring ESACs (Chapter 5) while molecular cloning provides accurate information about the exact structure of the rings (Chapter 3). In the following section, the advantages and disadvantages of the two strategies will be discussed.

8.1.1 Microdissection and FISH

The conventional cytogenetic banding procedures are G-banding, C-banding, staining for the satellites associated with NORs, and DA/DAPI staining. Although the origin of the rings from chromosome 15 can be determined when both of Ag-NOR and DA/DAPI are positive, for most rings, their origin can not be identified in this way. G-banding provides little information with these small rings other than their sizes and DA/DAPI only reveals rings that originate from chromosomes 1, 9, 15 and 16. Furthermore, Ag-NOR is often negative on the ring chromosomes since the ring chromosomes rarely have satellites.

This situation has been improved since the introduction of FISH with specific-centromere probes to detect the origin of the ring chromosomes. In these studies, multiple chromosome probes are usually needed, and this is a time-consuming and expensive process. In spite of this, several reports demonstrated that the origin of some ring ESACs may not be revealed by FISH with centromere probes when the ring is negative for all centromere probes, including the probe RR216 which was positive for all centromeres (Callen et al. 1992a). For example, one reported case had two ring chromosomes in the same metaphase in which one ring originated from chromosome 3 but the origin of the other ring could not be determined (Callen et al. 1991). In another study, one ESAC was negative for all centromere DNA and was finally identified to originate from chromosome 9 with a flow-sorting method (Raimondi et al. 1991). Furthermore, a marker chromosome 10 failed to be detected by probes of alpha-satellite, satellite III and CENP-B protein but was identified to be associated with some centromeric proteins using CREST antiserum (Voullaire et al. 1993). These results, together with the very small size of the ring chromosomes, suggest that in such cases alphoid repeat sequences are absent or rare, well below the sensitivity of FISH detection (Callen et al. 1992a), and that repeat sequences may not be essential for full centromere activity (Voullaire et al. 1993).

The limitation of centromere probes can be overcome by use of probes generated from flow-sorting or microdissection, since the probe DNA is directly generated from the marker chromosome and is able to hybridize back to ring and corresponding chromosomes. Microdissection has advantages over flow-sorting for the following reasons. (1) For DOP-PCR amplification, only 5-50 microdissected chromosome fragments are required (Meltzer et al. 1992, Deng et al. 1992, Guan et al. 1993) while 300-500 specific chromosomes have to be sorted from a cell suspension (Blennow et al. 1992, Carter et al. 1992); (2) Microdissection has no limitation in collecting tiny ESACs that are smaller than one third of chromosome 21, whereas no distinctive peaks appears on the flow karyotype with such small chromosomes (Carter et al. 1992); (3) Microdissection can isolate specific DNA from the ESACs, compared with flow-sorting which contains non-specific DNA from chromosomal debris sorted along with the ESACs (Ferretti et al. 1987).

With reverse painting, the entire constituents of the microdissected DNA, both repetitive and single-copy, are amplified. Although, demonstrated in Chapter 3, the presence of these repeats may limit the sensitivity of FISH in detecting small regions of euchromatin.

8.1.2 Microdissection and molecular cloning

A further development in identifying the origins of ring ESACs was the combination of microdissection with molecular cloning (Chapter 3 and 4) which is able to distinguish clones containing repetitive DNA from clones obtaining single-copy DNA by probing total human DNA to clones of the microdissected DNA library (colony blot). The single-copy DNAs were used to screen cosmids/phage libraries to generate probes for subsequent FISH hybridization to chromosome metaphases. This allows the content of the ring chromosome to be precisely determined. This has been applied in Chapter 3. After the segment of centromere and 4q31 was detected by microdissection-FISH, an additional segment of 4p13/14 was detected with

microdissection and molecular cloning. Furthermore, those cloned repetitive DNA can be used as probe for clinical diagnosis (see Chapter 4) and the primers generated from sequencing repetitive DNA can be used for PRimed IN Situ labelling (PRINs).

The combination of microdissection and molecular cloning can provide information that cannot be achieved with any other method. However, As demonstrated in Chapter 6, this is dependent on a high quality microdissection library. When contamination is present in the initial microdissection, this cannot be discovered until the last step, i.e. hybridization, after the time-consuming procedure of microdissection and cloning. Contamination must be avoided at all cost.

The combination of microdissection and molecular cloning allowed the discovery of a new type of ring formation. It has been suggested that extra small ring chromosomes arise from one break at the centromere, a second break in close proximity on either the long or short arm of the chromosome and subsequent rejoining of the broken ends (Callen et al. 1991). This type of ring chromosome will contain a continuous segment of a chromosome. However, the ring chromosome 4 presented in Chapter 3 was derived from three discontinuous regions, and the ring formation could not be explained by the above mechanism. Here we proposed that the ring may be formed by an initial large ring without any material lost (Dutrillaux et al. 1978, Cote et al. 1981, Zuffardi et al. 1980, Pezzolo et al. 1993b), followed by interlocking, breakage and reunion of the large ring during cell division, resulting in the generation of small ring with stable form (see Fig. 3-7).

8.2 Correlation between ring ESACs and patients phenotypes

Extra small ring ESACs constitute approximately 10% of all ESACs (Blennow et al. 1994b). When a ring chromosome is ascertained in prenatal diagnosis, the potential risk for mental retardation or abnormality can not be defined precisely. Among a total of 60 cases of single ring chromosome reported to date (Callen et al.

1990b, 1991, 1992a, Pezzolo et al. 1993a, Wiktor et al. 1993, Michalski et al. 1993, Plattner et al. 1993b, Voullaire et al. 1993, Daniel et al. 1994, Melnyk and Dewald 1994, Crolla et al. 1995, Chen et al. 1995a, Lanphear et al. 1995, Blennow et al. 1993, 1995, Blennow and Tillberg 1996, Begleiter 1996, Brondum-Nielsen and Mikkelsen 1995, James et al. 1995, Rosenberg et al. 1995, Morrison et al. 1997), nine cases were reported or re-investigated in this thesis (see Table 1-4 in Chapter 1). Thirteen cases showed normal phenotype and 44 cases had abnormal phenotype, including developmental delay, dysmorphic features and mental retardation. Three cases were not assessed due to the choice of termination of pregnancy (Callen et al. 1991, Blennow et al. 1993, Brondum-Nielsen and Mikkelsen 1995). Within these cases, the ring chromosomes have been shown to originate from all chromosomes except chromosomes 5 and 11. When rings originated from chromosomes 13, 21, 14 or 22, they could not be further verified since the same centromere sequences are shared by each pair of chromosome. Obviously this could be resolved by the use of single-copy DNA generated from microdissection and molecular cloning. There is variation in the phenotype of the patients with marker chromosome of the same origin, making the correlation between the origin and phenotype difficult. For example, in six cases of small accessory ring chromosome 1s, three carriers had normal feature while the other three had different degrees of abnormalities, including dysmorphic features with or without mental retardation and delayed development. This may be due to the variation in the euchromatin involved. As demonstrated in Chapter 3, both rings originated from chromosome 4 but they consist of different segments of euchromatin; one consisted only of the proximal part of the q arm, while the other was formed by a more complex rearrangement involving discontinuous regions. Some abnormal phenotypes may be caused by uniparental disomy of the two normal homologous rather than trisomy of ring chromosome per se. Therefore, detailed investigation of the origin of each ring chromosome using microdissection and molecular cloning is warranted.

8.3 Relationship between ring ESAC and chromosome trisomy

It is possible that ring ESACs originate from a trisomic line after a partial deletion of one of the trisomic chromosome. In two cases of ring chromosome 9 (Raimondi et al. 1991) and ring chromosome 20 (Batista et al. 1995), these ring chromosomes co-existed with a mosaic trisomy cell line and both ring chromosomes originated from the same chromosome as the trisomy. A high proportion of ring ESACs cell line presented in both cases suggested that ring ESAC are probably more stable while trisomy cells are less likely to survive in fetal tissues. The stability of a ring ESAC probably depends on the integrity of the centromeric sequences. In most cases these components are expected to be intact and functionally normal since ring ESACs can be maintained in successive cell generations.

8.4 Future directions

8.4.1 cDNA selection

Some *de novo* ring ESACs appear to contain euchromatin and are associated with developmental delay and/or phenotypic malformation. It is presumed that the phenotypes are due to the abnormal dosage of relatively few genes. Genes in the ring ESACs can be isolated by direct selection of transcribed sequence using the microdissected DNA of the ring (Wei et al. 1995). The steps one: (1) to eliminate repetitive sequences from the microdissected DNA by hybridization of the biotinlated, microdissected DNA to Cot1 human DNA; (2) to then hybridize the isolated unique DNA of the microdissected sequences to cDNA libraries such as fetal brain and human placenta; (3) to clone isolated cDNA.

8.4.2. Cloning of breakpoint

This thesis has established at least two different mechanisms for the formation of ring ESACs. One is the rejoining of a small chromosomal segment generated from one break at the centromere and a second break at either short arm or long arm adjacent to the centromere. This would result in small ring chromosomes containing contiguous segment, either from the short arm to the centromere or from the centromere to the long arm. The other involves non-contiguous segments from a chromosome. This type of small ring chromosome may be formed by an initial large ring followed by interlocking, breakage and reunion during cell division. To help elucidate the mechanisms of formation, the breakpoints of the ring ESAC could be cloned and sequenced.

This could be achieved as follows: (1) to generate sequence-tagged sites (STS) from cloned microdissected sequences of the ring ESAC; (2) to isolate either BACs or PACs by PCR with STS; (3) construct cosmid library of YAC by cloning of digested YAC; (4) to establish cosmids contig by STS; (5) to hybridize cosmid to ring ESAC by FISH and finally (6) to sequence the cosmid containing end fragment.

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American Journal of Human Genetics, v. 57(5), pp. 1137-1142

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Fang, Y.-Y., Bain, S., Haan, E.A., Eyre, H.J., MacDonald, M., Wright, T.J., Altherr, M.R., Riess, O., Sutherland, G. & Callen, D.F. (1997) High resolution characterization of an interstitial deletion of less than 1.9 Mb at 4p16.3 associated with Wolf-Hirschhorn Syndrome.
American Journal of Medical Genetics, v. 71(4), pp. 453-457

NOTE:
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http://dx.doi.org/10.1002/(SICI)1096-8628(19970905)71:4<453::AID-AJMG15>3.0.CO;2-F