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CHARACTERISATION OF A CHROMOSOMAL TRANSLOCATION IN AN OVARIAN CARCINOMA CELL LINE USING FLUORESCENCE 'IN SITU' HYBRIDISATION

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of Master of Science (Haematology)

Declaration:

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science (Haematology) in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university.



Brett Friedman

27th day of June , 1996

Ethics Committee Clearance Number M960407

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TABLE OF CONTENTS

Declaration i

Acknowledgements ii

Table of Contents iii

List of Figures viii

List of Tables xi

ABSTRACT xii

SECTION 1.0 INTRODUCTION 1

1.1 **THE BIOLOGY OF OVARIAN CARCINOMA**..... 2

1.1.1 Chromosomal Translocations..... 3

1.1.2 Loss of Heterozygosity in Ovarian Carcinoma..... 6

1.1.3 The Role of TSG's in Ovarian Carcinoma..... 7

1.1.4 Candidate Genes on Chromosome 11p..... 8

1.2 **FLUORESCENCE in situ HYBRIDISATION (FISH)**..... 11

1.2.1 Background..... 11

1.2.2 Probes..... 13

1.2.3 Whole Chromosome Probes..... 13

1.2.4 Repeat Sequence Probes..... 13

1.2.5 Gene-Specific Probes..... 13

1.2.6 Chromosomal in situ Suppression Hybridisation.... 14

1.2.7 Applications of FISH..... 14

1.2.8 Metaphase Analysis..... 15

1.2.9 Interphase Analysis..... 16

Aims of This Study 17

SECTION 2.0 MATERIALS AND METHODS 18

2.1 **CELL CULTURE AND CYTOGENETIC TECHNIQUES**..... 19

2.1.1 Tissue Culture of Cell Lines..... 19

2.1.2 Tissue Culture of Fresh Tumour Material..... 19

2.1.2.1 Collection and Transport of Specimens..... 20

2.1.2.2 Cell Dissociation..... 20

2.1.2.3 Culture Media..... 21

2.1.2.4 Incubation 21

2.1.3 Cell Culture of Ascitic Fluid 21

2.1.4 Metaphase Arrest 22

2.1.5 Cell Cycle Synchrony 22

2.1.6 Culture of Human Peripheral Blood Cells 23

2.1.7 Harvesting of Cell Cultures 23

2.1.8 Slide making 24

2.1.9 G-T-G-Banding Technique 25

2.2 **PLASMID PROBES** 25

2.2.1 Collection of Plasmid Probes 25

2.2.1.1 Chromosomally Enriched Plasmid Probes 25

2.2.1.2 Centromeric Alphoid Repeat Probes 26

2.2.1.3 Single Copy Gene Probes 26

2.2.2 **PLASMID PROBE PREPARATION** 26

2.2.2.1 Bacterial Transformation 26

2.2.2.2 Plate Inoculation and Plasmid DNA Amplification . 27

2.2.2.3 Plasmid Extraction 28

2.3 **YEAST ARTIFICIAL CHROMOSOME (YAC) PROBES** 30
2.3.1 Collection of YAC Probes 30
2.3.1.1 YAC Amplification and DNA Extraction 30
2.4 **ESTIMATION OF DNA YIELDS** 33
2.4.1 Agarose Gel Electrophoresis 33
2.4.2 Spectrophotometry 33
2.5 **PROBE LABELLING** 34
2.5.1 Purification of Labelled DNA 35

2.6 **FLUORESCENCE IN SITU HYBRIDISATION (FISH)** 36
2.6.1 Slide Preparation 36
2.6.2 Probe Preparation 38
2.6.3 Hybridisation 39
2.6.4 Immunodetection 40
2.6.4.1 Immunodetection of Biotin-labelled Probes 40
2.6.4.2 Immunodetection of Digoxigenin-labelled Probes .. 41
2.6.4.3 Immunodetection for Dual FISH 42
2.7 **FLUORESCENCE MICROSCOPY** 44
2.8 **GENE DOSAGE STUDIES** 44
2.8.1 DNA Extraction 44
2.8.1.1 Phenol/Chloroform Extraction 44
2.8.2 Slot Blot Assay 45
2.8.2.1 DNA Preparation 45
2.8.2.2 Blot Preparation 45

2.8.2.3 Blotting Procedure 45

2.8.2.4 Probe Labelling 45

2.8.2.5 Prehybridisation of the Blots 46

2.8.2.6 Hybridisation 46

2.8.2.7 Post-hybridisation Washes 46

2.8.2.8 Autoradiography 46

SECTION 3.0 RESULTS 47

3.1 Cytogenetic Analysis of cell Line UWOV2(Sf) 48

3.2 Cytogenetic Analysis of Parent Line UWOV2 48

3.3 Cytogenetic Analysis of Fresh Tumour Material .. 51

3.4 Cytogenetic Analysis of Ascitic Fluid Cultures . 51

3.5 FISH Studies on Cell Line UWOV2(Sf) 51

3.6 FISH Studies on Cell Line UWOV2 57

3.7 Mapping of the Breakpoint Region in Cell Line UWOV2(Sf) Using FISH Studies 66

3.8 Mapping of the Breakpoint Region in Cell Line UWOV2 Using FISH Studies 66

3.9 FISH Analysis of Fresh Tumour Material 71

3.10 Gene Dosage Studies 71

SECTION 4.0 DISCUSSION 73

4.1 Cytogenetic Analysis of the 11p Abnormality in Cell Lines UWOV2 and UWOV2(Sf) 74

4.2 FISH Analysis of Cell Lines UWOV2 and UWOV2(Sf) 74

4.3 Mapping Studies on Chromosome 11p 78

4.4 Analysis of Fresh Tumour Material 80

SECTION 5.0 CONCLUSION 83

SECTION 6.0 APPENDIX 85

APPENDIX A (Buffers,Stains and Reagents) 86

APPENDIX B (Growth Media) 88

SECTION 7.0 REFERENCES 89

LIST OF FIGURES

Figure 1: A map of the short arm of chromosome 11 (11p) indicating the location of candidate genes in the region 11p13 and 11p15.

Figure 2: G-T-G-banded karyotype of a metaphase cell from cell line UWOV2(Sf) showing the clonal structural abnormalities.

Figure 3: Partial G-T-G-banded karyotype from cell line UWOV2 showing the chromosome 11 abnormality der(11)t(3;11) and a cytogenetically normal chromosome 3.

Figure 4a: G-T-G-banded karyotype of a metaphase cell from specimen T5 showing the abnormality del(6)(q21q27) and a single giant marker chromosome.

Figure 4b: G-T-G-banded karyotype of a metaphase cell from specimen T5 showing the abnormality del(6)(q21q27). Two marker chromosomes are present.

Figure 5a: Cells from ascitic fluid culture AF-1 showing a fibroblastoid-like morphology.

Figure 5b: Cells from ascitic fluid culture AF-2 showing a fibroblastoid-like morphology.

Figure 5c: Cells from ascitic fluid culture AF-3 showing a fibroblastiod-like morphology.

Figure 5d: Cells from ascitic fluid culture AF-5 showing a fibroblastoid-like morphology. Multinucleated cells are present.

Figure 6: Biotinylated whole chromosome 11 library probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing the 11p+ abnormality and a normal chromosome 11.

Figure 7: Biotinylated chromosome 3 library probe and biotinylated chromosome 11 centromeric probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing positive hybridization of chromosome 3 material to the distal portion of the 11p+ abnormality. Several other structural abnormalities involving chromosome 3 are present.

Figure 8: Dual FISH with biotinylated chromosome 8 library probe and DIG-labelled chromosome 3 library probe on a metaphase cell from cell line UWOV2(Sf) showing the t(3;8) and positive hybridization of the chromosome 8 probe and feint hybridization of the chromosome 3 probe to the distal portion of the 11p+ abnormality.

Figure 9: Dual FISH with a chromosome 8 library probe (FITC signal) and a chromosome 11 library probe (Rhodamine signal) to a metaphase cell of cell line UWOV2(Sf) showing chromosome 8 material on the distal portion of the 11p+ abnormality.

Figure 10: Biotin-labelled chromosome 14 library probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing trisomy 14 and 14q+.

Figure 11: Biotin-labelled chromosome 8 centromeric probe hybridized to a metaphase cell of cell line UWOV2 showing three cytogenetically normal copies of chromosome 8.

Figure 12: Biotin-labelled chromosome 11 centromeric probe and pSBβ gene probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing positive hybridization of pSBβ to the normal chromosome 11.

Figure 13: Biotin-labelled YAC probe 847a12 hybridized to a normal metaphase cell showing FITC signals at the distal portion of chromosome 11p15.

Figure 14: Biotin-labelled YAC probe 954f4 hybridized to a normal metaphase cell showing FITC signals at the distal portion of chromosome 11p15.

Figure 15: Biotin-labelled YAC probe 845a3 hybridized to a normal metaphase cell showing FITC signals at the region 11p15.1 (arrows) (DAPI counterstain, triple filter x1000).

Figures 16a & b: Metaphase cells of cell line UWOV2(Sf) showing hybridization of biotin-labelled YAC probe 845a3 to the chromosome 11p+ abnormality and to the normal chromosome 11.

Figure 17: Metaphase cell of cell line UWOV2(Sf) showing hybridization of the biotin-labelled YAC probe 966e8 to the normal chromosome 11 but not to the chromosome 11p+ abnormality.

Figure 18(a & b): Dual FISH on interphase cells of cell line UWOV2(Sf) with YAC probe 845a3 (FITC signal) and 966e8 (Rhodamine signal) (arrows).

Figure 19: A hypodiploid metaphase cell of specimen T5 showing dual FISH with YAC probes 845a3 (FITC signal) and 966e8 (Rhodamine signal).

Figure 20: A hypodiploid metaphase cell of specimen AF-5 showing dual FISH with YAC probes 845a3 (FITC signal) and 966e8 (Rhodamine signal).

Figure 21: Autoradiograph of slot blot assays with probes IGF-II **(a)** and β-actin **(b)**. Lane 1 indicates cell line UWOV2(Sf) DNA, Lane 2 indicates control DNA and Lane 3 indicates cell line UWOV2 DNA.

Figure 22: A physical integration map of a portion of the region 11p14.3-p15.1 indicating the possible position of the t(3;11) breakpoint region on chromosome 11 in cell lines UWOV2 and UWOV2(Sf) relative to the two YAC probes 845a3 and 966e8.

Figure 23: A map of the distal portion of chromosome 11p15 showing the position of the YAC probes used in this study relative to various DNA markers. The region of the t(3;11) breakpoint is indicated.

Figure 24: A map of the short arm of chromosome 3 indicating the various regions implicated in ovarian carcinoma. The position of the t(3;11) breakpoint is indicated.

LIST OF TABLES

Table I: The Human Genome Mapping regional assignments of CEPH YAC probes to the chromosomal region 11p15.5-14.3.

Table II: Results showing the hybridisation signal between the chromosomal libraries and the marker chromosome 11 as determined by Fluorescence in situ hybridisation studies with cell line UWOV2(Sf).

ABSTRACT

The region 11p13-p15 on the short arm of chromosome 11 (11p) has been implicated in the initiation or progression of several human malignancies including the embryonic rhabdomyosarcoma Wilms' tumour, bladder, renal cell and ovarian carcinoma. In this study, Fluorescence In Situ hybridisation (FISH) was used to identify the nature of a chromosome 11p+ abnormality present in two ovarian carcinoma cell lines after conventional cytogenetic techniques had failed to elucidate the chromosomal origin of the abnormality. Using whole chromosome library probes, the abnormality in cell line UWOV2 was found to be composed entirely of chromosome 3 material representing the translocation t(3;11)(p12-14;p15). In the protein-free subline UWOV2(Sf), the abnormality was found to consist of the complex translocation t(3;8;11)(p12-14;q22-24;p15). It is possible that the involvement of chromosome 8 in this translocation was a cell culture phenomenon. Other structural and numerical abnormalities elucidated with FISH in cell line UWOV2(Sf) included 1q+, +5, +7, 7q-, 8q+, +12, +14, 14q+, -15, 16q- and -18.

Using FISH together with the gene probe pSBβ and the CEPH YAC probes 892g9, 785e5, 847a12, 954f4, 966e8 and 845a3, the breakpoint region on chromosome 11 in the two cell lines was narrowed down and mapped to the region 11p14.3-p15.1 lying between probes 966e8 (D11S902) and 845a3 (D11S899). This represents a physical distance of approximately 1 Mb. The breakpoint in the two cell lines appeared to involve the same region on 11p15.1.

In a separate study, three epithelial ovarian tumour specimens and four ascitic fluid specimens were obtained. Tumour specimens T2 and T4 and ascitic fluid specimens AF-1, AF-2 and AF-3 were all cytogenetically uninformative. Cytogenetic analysis of specimen T5 revealed a single clonal abnormality involving a deletion in the region 6q21. Ascitic fluid specimen AF-5 yielded cytogenetically normal metaphases. Both specimens were hypodiploid and revealed a cytogenetically normal chromosome 11. Using FISH and CEPH YAC probes 966e8 and 845a3, no abnormalities were detected in the region 11p14.3-p15.1 in these two specimens but one cannot rule out the possibility of submicroscopic abnormalities lying within the region between these probes. From this study we speculate that chromosome 6 abnormalities may be important in the initiation of these tumours.

From the results obtained with cell lines UWOV2 and UWOV2(Sf) we speculate that the chromosome 3 abnormalities were an early event in the evolution of these tumours while the chromosome 11 abnormality was a later event. Little is known about the region 11p14.3-p15.1 and very few disease loci have been assigned to this region, however, we may speculate that this region harbours a tumour suppressor gene or an oncogene whose disruption or activation is critical to the pathophysiology of ovarian carcinoma and other genitourinary cancers.

SECTION 1.0

INTRODUCTION

1.1 THE BIOLOGY OF OVARIAN CARCINOMA

Ovarian carcinomas encompass a wide range of tumour types the commonest being the epithelial tumours. The human ovary is a complex organ with two major normal physiological functions; the production of steroid hormones and the timely release of the ova, that is, ovulation. Embryologically, the ovarian surface epithelium is related to the epithelial lining of the fallopian tubes, endometrium and endocervix. The ovarian surface epithelial cells are a modified peritoneal mesothelium which originates from the same coelomic epithelium which invaginates laterally to the gonads during embryonic development forming the paramesonephric or müllerian duct system of the embryo. This process also results in the formation of the fallopian tubes, endometrium, uterus and part of the vagina. These epithelia and the ovarian surface epithelium are thus closely related.

During the fourth or fifth month of foetal development, the ovarian epithelial cells undergo intense mitotic activity possibly as a result of hormonal stimulation (Gondos 1975). The presumptive granulosa cells of the ovary form as a result of downgrowths of this surface epithelium thus the coelomic epithelium has the capacity to differentiate along several lines. The striking number of phenotypes found associated with ovarian carcinomas may be explained by this differentiation potential displayed by the coelomic epithelium.

The majority of ovarian tumours are thought to arise from the ovarian surface epithelial cells. These common epithelial tumours display a range of histological features which enable them to be classified according to their histological similarities with the epithelial components characteristic of different müllerian structures. The most common of these epithelial tumours are the serous, mucinous and endometroid types and consist of biologically and morphologically benign neoplasms called cystadenomas as well as malignant cystadenocarcinomas.

Several hypotheses have been proposed to explain the development of ovarian carcinomas. These models are purely speculative and although animal systems have been looked at, little work on human systems has been undertaken. The most popular hypothesis is based on evidence that during the course of reproductive life, the ovarian germinal epithelium may become entrapped within the ovarian stroma due to continuous ovulation, resulting in the formation of epithelial inclusion cysts. This process places the ovarian surface epithelial cells in close proximity to the growth factor and steroid hormone producing components of the ovary. Subsequently, this process may induce oncogenic factors which cause the benign epithelium to undergo malignant transformation (Cramer *et al.*1983).

Another hypothesis is that the surface epithelium is stimulated into growth and repair during ovulation. Frequent ovulation would thus imply frequent cycles of cell division by these cells which increases the risk of mutation during DNA replication (Fathalla, 1971).

It is unknown whether or not these carcinomas arise *de novo* or through clonal expansion from pre-existing cystadenomas, that is, if the benign epithelium leads directly to invasive carcinoma or passes through stages of benign/borderline carcinoma. Puls *et al.* 1992, identified areas of benign epithelium adjacent to borderline or malignant epithelium in the majority of mucinous and serous ovarian cystadenocarcinomas which suggested that certain benign or borderline tumours may be precursors to invasive carcinomas. Bourne *et al.*1991, showed that malignant transformation of benign epithelial ovarian tumours was increased in women with a genetic predisposition while Dubeau *et al.*1993 also showed areas of benign growth adjacent to malignant growth.

Although the relationship between benign and malignant carcinomas has been investigated, the genetic evolution of these tumours has remained elusive. By the time these tumours are detected, they usually exhibit complex structural and numerical abnormalities and thus it is difficult to distinguish between early and late genetic events.

1.1.1 Chromosomal Translocations

Cancer cells usually harbour chromosomal abnormalities the complexity of which increases with an increase in tumourigenicity. Such abnormalities may be inherited that is, carried within the germline of an individual or they may be acquired sporadically resulting in a somatic mutation. They may also be present in different forms as a result of certain genetic events such as translocations, deletions, amplifications and numerical abnormalities.

Chromosomal translocation involves the transposition of a chromosome segment to a new location within the genome. Translocations are usually specific for the type of malignancy such as leukaemias, lymphomas, sarcomas and mesenchymal neoplasms. They occur at nonrandom sites and may involve genes and oncogenes relevant to the pathophysiology of the disease. The translocation event may lead to the activation of a gene or cellular oncogene resulting in the overexpression of that gene product. Two examples of this are described here. The t(11;14) and t(14;18) in lymphomas where the bcl-1 and bcl-2 genes juxtapose respectively with the immunoglobulin heavy chain promoter sequences results in the overexpression of these two genes respectively. (Reed, 1994). The second group of translocations result in the production of chimaeric proteins. Here, the recombination event fuses two genes, one of which is

always a transcription factor. In the t(1;19) seen in acute lymphoblastic leukaemia, the E2A transcription factor gene on chromosome 19 fuses with the PBX homeobox gene on chromosome 1 (Cleary, 1991). The resulting chimaeric protein acts as a novel transcription factor interfering with proliferation and survival of lymphoid hematopoietic precursors. A translocation event may also result in the inactivation of an anti-oncogene or tumour suppressor gene resulting in tumourigenicity.

The translocation event may also lead to the activation of certain cellular proto-oncogenes. Proto-oncogenes are known to code for many components of the signalling pathways which carry growth stimulatory messages from the local environment to the nucleus. These components include growth factors, cell surface receptors, cytoplasmic signal mediators and transcription factors within the nucleus. The activation of certain protooncogenes such as *c-erbB-2*, *c-fms*, *c-myc* and *K-ras* occurs relatively frequently in ovarian tumours but appears not to be related to prognosis.

The two most widely studied oncogenes in ovarian cancer are the *K-ras* and HER-2/*neu* oncogenes. Human ovarian cancers have demonstrated over-expression of the HER-2/*neu* gene which codes for a growth factor receptor similar to the epidermal growth factor (EGF) receptor. Over expression of this gene was found to be caused by gene amplification in this locus and was associated with poor disease prognosis (Berchuck *et al.*1990).

Mutations in the *ras* family of proto-oncogenes have been identified in many human adenocarcinomas including lung, colon and pancreas. Point mutations in the ras proto-oncogene result in a mutant ras protein which is constitutively activated sending growth stimulatory messages. In ovarian carcinomas, *K-ras* mutations have been detected in borderline and invasive mucinous tumours. These mutations may represent an early event in the progression of malignancy (Toneriello *et al.*1993; Mok *et al.*1993) and may indicate an event in the transition between borderline and invasive carcinomas.

The translocation event may also result in the inactivation of anti-oncogenes or tumour suppressor genes (TSGs). With the development of chromosomal banding techniques, the normal and abnormal appearance of chromosomes could be discerned in a process known as 'karyotyping'. This process facilitated the identification of regions of chromosomes that were deleted, duplicated or translocated. The link between such chromosomal abnormalities and the presence of a malignant phenotype lent support to the hypothesis that cancer was a disorder of the chromosomes. Since the 1960's, there has been increasing evidence to suggest that fusing normal cells with malignant cells resulted in some fused cells reverting to a less malignant phenotype (Harris *et al.*1969). Later, with a technique known as microcell transfer, it was possible to demonstrate that the

introduction of single chromosomes to tumour *in vitro*, resulted in the reversion of the malignant phenotype which suggested that these single chromosomes harboured differentiation inducing genes or tumour suppressing genes (Saxon *et al.*1986).

The discovery and cloning of the gene responsible for the paediatric eye tumour, retinoblastoma (RB) accelerated research into the discovery of genes responsible for suppression of the malignant phenotype. Retinoblastoma occurs in two forms: sporadic, where the tumour is unilateral and the condition is not inheritable; and familial, where usually bilateral tumours are seen and the condition can be traced through families. Knudson, (1971), proposed that two mutations were necessary for tumour formation that is, both alleles of the same unidentified gene were mutated. In the inherited form of the disease, children inherited a mutant allele and the second mutation occurred somatically whereas in sporadic cases, both mutations occurred somatically. This 'two-hit hypothesis' explained why only cells with two mutant alleles developed into cancers while cells with one normal gene could suppress tumourigenesis. These studies led to the idea that tumour suppressor genes (TSG's) act recessively at the cellular level as opposed to oncogenes which act dominant to the wild type.

The techniques used in isolating the *RB* TSG serves as a model by which other TSGs may be assessed including those thought to be associated with ovarian carcinoma. Cloning of the *RB* gene depended on clinical and epidemiological data, cytogenetics, somatic cell hybrids, linkage analysis studies and loss of heterozygosity (LOH) studies.

The presence of minor interindividual sequence variations or polymorphisms in the human genome formed the basis for initial protocols in disease tracking. Probes recognising length polymorphisms (RFLP s) could be used to track RFPLs coinherited within a family. Somatic cell hybrids and the presence of hypervariable regions in the genome allowed the discovery of more probes which were linked to disease loci. Using this technique, the region within which the gene of question lay was considerably narrowed down and the tight linkage between anonymous, accurately localised markers and specific diseases could be shown.

The generation of DNA polymorphisms had another invaluable application. They could be used to study the differences between DNA extracted from tumours and that extracted from normal tissue where both tumour and constitutional DNA are studied under the same experimental conditions. The DNA is digested with restriction endonucleases to reveal the polymorphisms. The digested DNA is then resolved by electrophoresis on an agarose gel and transferred to a filter. The filter is then hybridised to radiolabelled probes of known localisation. The resulting autoradiographic pattern reveals the presence or absence of

targeted alleles. If only one allele is present in the tumour sample while two copies are found in the normal tissue this is referred to as reduction to homozygosity, or loss of heterozygosity (LOH). In this way submicroscopic deletions, non-disjunction with reduplication or mitotic recombination can be distinguished.

Chromosomal recombination normally occurs during meiosis where homologous chromosomes exchange sequences, but during mitotic recombination, if a chromosome contains a putative tumour suppressor gene (TSG), the chromosomes will be identical below the crossover point resulting in LOH and therefore any crossover centromeric to the TSG will result in homozygosity at that locus. Non-disjunction may also occur during mitosis and is characterized by paired chromosomes failing to separate at cell division. The resulting cell will have both chromosomes derived from the same parent and LOH will occur over the whole chromosome. Under the above models, a second mutation in the TSG or a deletion of the wild-type gene is not required.

In all cancers, mitotic recombination and non-disjunction appear to be the most common mechanisms of LOH. Terminal and interstitial deletions, gene conversions and translocations are less common, however if tumours present with these abnormalities, they may precisely localise the position of a TSG rendering cloning of the gene more manageable. Cloning of a putative TSG using the above method may prove to be an arduous and laborious task (Foulkes, W.D.; Trowsdale, J., 1995).

1.1.2 Loss of Heterozygosity in Ovarian Carcinoma

Numerous studies on LOH in ovarian carcinoma have been presented and have been largely based on the protocol described by Cavanee *et al.*(1983). For the most part, these studies have detailed LOH on those chromosomal arms known to harbour TSGs but little work has been done on the TSGs themselves. The conclusion that the LOH on a particular arm can be accounted for by a known TSG is thus purely speculative. Foulkes *et al.*(1993) described a provisional allelotype for ovarian carcinoma based on a compilation of published data on LOH in ovarian carcinoma. Chromosome arms 6q, 11p, 3p, 13q, 16q, 17q and 17p are regions of the genome that exhibit LOH and may contain TSGs important in the pathogenesis of ovarian carcinomas. Chromosome 17p and 17q show high rates of allelic imbalance and recently the breast-ovarian cancer gene (BRCA1) was assigned to the region 17q12-21 by linkage analysis (Easton *et al.*1993). This gene however, rarely contributes to ovarian tumourigenesis in common sporadic, non-familial cases (Merajver *et al.*1995;Trowsdale *et al.*1995) and thus another mechanism must exist in these cases.

Abnormalities involving the short arm of chromosome 11 (11p) have been extensively described in epithelial ovarian carcinomas. Deletions and unbalanced translocations involving

the region 11p13-p15 have been implicated in the development of the malignant phenotype (Pejovic *et al.*1992; Jenkins *et al.*1993; Thompson *et al.*1994). The chromosomal origins of these abnormalities could not be determined cytogenetically due to their complexity. Loss of heterozygosity studies have revealed certain loci spanning the region 11p15.1-p15.5 to be deleted. Gallion *et al.*1992, demonstrated LOH at the *HRAS1* locus (11p15.5) in the majority of invasive tumours but not in borderline tumours which confirms previous studies by Zheng *et al.*1991 and Lee *et al.*1989 who noted that *HRAS1* losses were most common in high grade or metastatic tumours suggesting that 11p loss may be involved with later steps in ovarian tumourigenesis. Kiechle-Schwarz *et al.*1993 using DNA recombinant technology together with Southern blotting and polymorphic probes to the regions *HRAS1* (11p15.5), *INS* (11p15.5), *PTH* (11p15.2-p15.1) and *CALCA* (11p15.2-15.1) found LOH in informative cases of 47.5% reduction to homozygosity. The study suggested the presence of a TSG in the region of the *HRAS1* locus that was important in tumour progression. Later studies showed similar findings (Weitzel *et al.*1994).

Allelic loss on chromosome 11p thus correlated with a clinically more aggressive disease possibly reflecting a progression from benign to malignant or low grade to high grade state. Although the above loci studied have been informative, one cannot rule out the possibility of there being other regions on 11p harbouring a TSG(s) which may contribute to the mechanism of the disease.

1.1.3 The Role of TSGs in Ovarian Carcinoma

Little is known about the role of many of the known TSGs in ovarian carcinoma and no TSG specific to this cancer has been isolated. The reasons for this are several. Firstly, despite the knowledge that heritable forms of ovarian cancer did exist, it has only recently been acknowledged that ovarian cancer formed part of a group of familial cancer syndromes. Secondly, the pathogenesis of ovarian carcinoma is not fully understood and it is not known if tumourigenesis occurs in a stepwise progression from benign adenoma to malignant carcinoma or if another mechanism exists. Thirdly, by the time that ovarian carcinomas are detected, they usually present with karyotypes exhibiting complex structural and numerical changes and it has not been possible to find chromosomal areas consistently involved in all tumours.

The tumour suppressor gene *P53* on chromosome 17p has been studied in some detail in ovarian carcinomas. Growth suppression by this gene may be two-fold, firstly, p53 binds to and in turn suppresses various transcription factors including those that bind to TATA elements. Secondly, it transcriptionally activates the expression of a number of genes which encode proteins that can suppress cell division (Levine *et al.*1991). p53 Mutations in

ovarian cancer may be a later event in tumour progression as mutations have not been detected in borderline cases (Kupryjanczyk *et al.*1995). Abnormalities observed, reveal a predominance of transitional mutations as well as transversions and microdeletions (Kohler *et al.*1993).

Mutations at the *RB* gene locus on chromosome 13q have also been investigated as a candidate TSG for ovarian cancer, however, inactivation of the *RB* gene leading to abnormal RB protein expression is a rare event in ovarian cancer (Dodson *et al.*1994) and thus its role in ovarian tumourigenesis remains to be elucidated.

1.1.4 Candidate Genes on Chromosome 11p

The pathophysiology of several human malignancies is thought to be linked to the inactivation or loss of a putative TSG on chromosome 11p.This region is known to harbour TSGs as revealed by microcell fusion studies conducted by Weismann *et al.*1987. The region 11p15 is specifically of interest as similar studies have shown that it has tumour suppressor activity (Dowdy *et al.*1991).

Chromosomal abnormalities involving 11p13-p15 loci have been demonstrated in: breast carcinoma (Winqvist *et al.*1993) who implicated the region between the *TH* and *HBB* genes at 11p15.5; prostate cancer where two prostatic carcinoma metastasis suppressor genes have been localised to the region 11p12-p13 (Isaacs *et al.*1992) and hepatocellular carcinoma (Wang *et al.*1988) (figure 1).

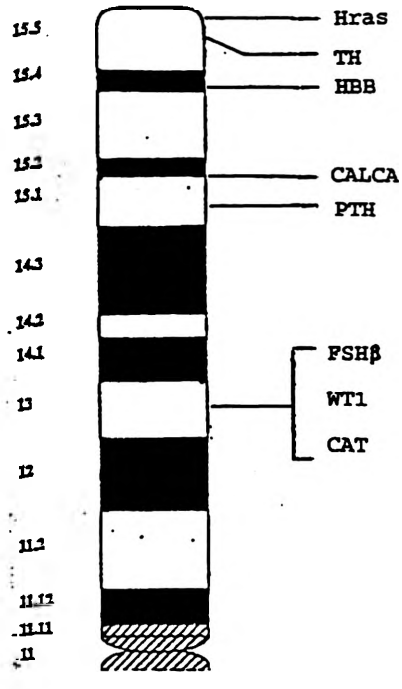


Figure 1: A map of the short arm of chromosome 11 (11p) indicating the location of candidate genes in the region 11p13 and 11p15

The region 11p13-p15 has also attracted great interest as it harbours two TSG loci implicated in the development of the embryonal rhabdomyosarcoma Wilms' tumour (WT) and Beckwith-Wiedemann syndrome (BWS). Wilms' tumour of the kidney or nephroblastoma, is a tumour of embryonic origin and is derived from metanephric blastermal tissues of the developing kidney that have failed to undergo the normal maturation process. The condition is associated with a high incidence of congenital abnormalities including Wilms' tumour development, genitourinary abnormalities and mental retardation (WAGR) syndrome (Breslow *et al.*1982).

Cytogenetic studies on Wilms' tumour initially revealed an 11p- in the region 11p13 (Kaneko *et al.*1981) and subsequently, variations in 11p have been reported for approximately 25% of all WTs studied. The breakpoints of these structural changes involved the regions 11p13 and 11p15 and thus it was speculated that two WT loci, one in each of these regions plays a role in this disorder. The WT TSG *WT1* has been mapped to the region 11p13 (Gessler *et al.*1990) and a second putative WT TSG has also been mapped to the region 11p15 and may be related to the childhood BWS (Koufos *et al.*1989).

Beckwith Wiedemann syndrome, an autosomal dominant condition, is characterized by numerous growth abnormalities including unilateral exomphalos, macroglossia and gigantism. These patients show a predisposition to development of several childhood tumours including WT, adrenocortical carcinoma and rhabdomyosarcoma. Genetic evidence shows that the region 11p15.5 distal to the *HBB* locus is associated with BWS (Henry *et al.*1993). This region also contains the three contiguous genes *IGF-II*, *H19* and *INS*. These genes have been shown to be imprinted, that is both alleles being derived exclusively either maternally or paternally (Ferguson-Smith *et al.*1993; Ohlsson *et al.*1993). Genomic imprinting has attracted great attention because of the connection between abnormalities in the imprinting process and tumourigenesis. Imprinting of the IGF-II gene was first described in mice. Here, partial trisomy 11p15 resulted from paternal isodisomy where the IGF-II gene in the mouse was paternally imprinted resulting in a growth deficiency. Those mice inheriting the maternal allele were normal (DeCharia *et al.* 1991). Several human malignancies reveal a duplicated set of either paternal or maternal chromosomes such as that found in dermoid tumours of the ovary and hydatidiform moles. Oncogenesis however may also occur when the normal imprinting process is disrupted and a normally silenced imprinted gene is expressed. In Wilms' tumour, the *IGF-II* gene is found to be overexpressed and these tumours have been shown to synthesise increased amounts of IGF-II. In 1993, Feinberg showed that the expected imprinting of *IGF-II* and *H19* in WT was disrupted indicating a "relaxation" of the imprinted allele. The expression of H19 which is believed to have TSG functions, has been shown to be linked to IGF-II expression where a cell expressing increased

amounts of IGF-II expresses decreased amounts of H19 tumour suppressor product leading to a possible scenario for tumourigenesis. Some BWS patients have also demonstrated abnormalities of IGF-II imprinting in a number of cells. Loss of IGF-II imprinting has not been restricted to WT and BWS. Vu *et al.*1995 demonstrated complete or partial relaxation of IGF-II imprinting in uterine leiomyosarcomas and Gicquel *et al.*1994 suggested a role for *IGF-II* and the region 11p15 in adrenocortical tumours.

The role of genomic imprinting in relation to ovarian carcinoma remains to be elucidated. Because mutations in the *WT1* gene are not a prerequisite for tumourigenicity in certain WT patients (Slater *et al.*1992) another region possibly on 16q13 may predispose some of these patients to malignancy (Newsham *et al.*1995). This leads one to speculate on the role of *WT1* and of the region 11p15 in WT as possibly playing a role in the progression of these tumours. This hypothesis would strengthen the link between the progression of ovarian tumours and Wilms' tumour.

1.2 Fluorescence in situ Hybridisation (FISH)

1.2.1 Background

In 1969, Gall *et al.* described a method for the visualisation of specific nucleic acid sequences in morphologically preserved chromosomes, cells and tissue sections, using isotopically labelled RNA probes. This procedure came to be known as *in situ* hybridisation. Such a technique was able to localise abundant sequences such as those of DNA in polytene chromosomes or highly repetitive sequences on metaphase chromosomes. Molecular cloning methods were unavailable and thus *in situ* hybridisation was restricted to those sequences that could be purified and isolated by conventional biochemical methods for example, mouse satellite DNA, viral DNA and ribosomal RNA. There were however several drawbacks with this method of *in situ* hybridisation the main ones being that the protocols were long and laborious and that abundant target sequences were detected with relatively low resolution.

In 1981, Harper *et al.* described a method whereby DNA sequences of only a few base pairs in length could be detected on metaphase chromosomes. Such a technique required fairly short autoradiography exposure times coupled with statistical analysis. The technique also allowed the mapping of genes relative to chromosomal bands by combining hybridisation/detection protocols with standard binding protocols and therefore, this system became an integral part of genome mapping protocols.

Although its progress has been substantial, isotopic *in situ* hybdridization is restrictive due to long autoradiographic development times, extensive and arduous statistical analysis, the emitted isotopic signal has to be captured with an emulsion overlay which limits mapping precision and the track of radioactive decay also limits spatial resolution. The cost of the reagents and methods for the disposal of such radioactive waste has also become a significant problem.

In order to overcome the limitations of isotopic or radioactive *in situ* hybridisation, methods for non-isotopic *in situ* hybridisation were devised. In 1977, the use of an antibody system for the detection of RNA-DNA hybrids was described, and Broker *et al.*1978 described a biotin-avidin system to detect *in situ* hybridisation by electron microsocopy.

From these pioneering studies, two methods of non-radioactive *in situ* hybridisation emerged. These methods involved the use of fluorochromes as part of the detection system. The direct method involved the direct labelling or introduction of fluorochromes to DNA probes.

The indirect method involved the labelling of DNA probes with a modified hapten such as biotinylated deoxyuridine triphosphate (dUTP). This biotinylated dUTP replaced thymidine during nick translation of a DNA probe and detection of the desired sequence could be achieved using antibodies against the desired hapten coupled to a fluorochrome (Langer *et al.*1981).

The reliability of both methods depended on the modified probes ability to withstand and not interfere with the hybridisation and post hybridisation procedures. Since then, several hapten-antibody systems for use in Fluorescence *in situ* hybridisation were produced including the digoxigenin system (Boehringer Mannheim, Germany). Other chemical modification schemes include mercuration, sulphonation and acetylaminofluorescence (Lichter *et al.*1991a). The two systems most widely used are the biotin and digoxigenin systems.

In situ hybridisation on cytological material is considerably more difficult than that performed on membrane filters and Lawrence *et al.*(1988) described three main criteria that should be met for maximum results:

1) the target sequence should remain preserved and accessible to the probe
2) hybridisation kinetics should be standardised so as to allow the probe to bind to specific target sequences without non-specific binding to biological material
3) probe detection should be such that a signal is evident with minimal background noise

Biotinylation of DNA during nick translation was first described by Langer *et al.*(1981). This was accomplished through the synthesis of the allylamine derivative of dUTP which could be biotinylated using the active N-hydroxysuccinimide ester of biotin. The ability of anti-hapten antibodies to detect the biotin moeity in DNA duplexes is dependent on the length of the spacer arm. Several other biotinylated precursors have since been developed and have proved to be more efficient.

The digoxigenin (DIG) labelling system was developed by Boehringer Mannheim (Germany) and is based on a steroid isolated from digitalis plants. Digoxigenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms.

The detection of *in situ* hybridisation sequences is mediated by avidin or anti-avidin antibodies. In fluorescence *in situ* hybridisation, the avidin or antibody may be coupled to a fluorochrome such as fluorescent isothyocynate (FITC) or AMCA. Enzymatic detection methods employing horseradish peroxidase and alkaline phosphatase have the added advantage that the reaction can be prolonged in order to amplify signals and the signals do not fade rapidly. Fluorescence detection provides the highest

resolution possible with light microscopy however the signal may fade rapidly and thus requires rapid recording and observation thus the detection of single copy gene probes <2 kb is restricted to autoradiographic techniques. In the digoxigenin system, specific anti-DIG antibodies have been developed which bind specifically to DIG and several amplification protocols are available.

1.2.2 Probes

A wide variety of probes are available for use in FISH and they may be categorised according to the complexity of their target sequences. Originally, recombinant DNA libraries enriched in sequences from single chromosome types have been used to facilitate the selection of probes for molecular genetic studies and have also proved reliable for use in FISH.

1.2.3 Whole Chromosome Probes

Whole chromosome or chromosome-specific composite probes allow a specific painting of chromosomes or chromosomal regions. A concise and useful set of whole chromosome probes was produced by Gray *et al.*(1991). Here, inserts from complete-digest Hind III chromosome-specific recombinant DNA Charon 21A phage libraries were subcloned into Bluescribe plasmids. The phage libraries were produced from chromosomes of a single type purified by fluorescence-activated cell sorting.

1.2.4 Repeat Sequence Probes

These are probes comprising chromosome specific repetitive DNAs which occur in clusters mainly in centromeric or other heterochromatic regions such as satellite and alphoid DNAs. They are generally small probes which may be used when the target sequences are present in high copy number. They encompass a range of probes which specifically recognise repeat sequences at the centromeric region of chromosomes and are useful in the identification of chromosomes or chromosome copy number in clinical material.

1.2.5 Gene-Specific Probes

The identification of loci involved in genetic diseases has allowed the development of a set of probes for FISH which specifically recognise these regions. Initially, studies with such probes allowed the detection of target sequences ranging from 15 to 500 kilobases (kb) (Tkachuk *et al.*1991).

Selection of the ideal probe to be used in FISH depends on the size of the targeted DNA sequence, as it correlates closely with hybridisation efficiency. Successful hybridisation means that the probe should recognise both chromatids of both chromosome

homologues on metaphase chromosomes. The efficiency by which probes recognise target sequences decreases with a decrease in probe complexity.

Cloning vectors used for the preparation of these probes include large insert phages, plasmids, cosmids and yeast artificial chromosomes (YACs). Phage vectors and plasmids contain inserts ranging from a few hundred base pairs up to 15 kb+ and may label from 50-75% of their target sites. Cosmid probes may contain inserts of 25-40kb and may label up to 90% of their target sites. They are thus important and informative, greatly reducing the required statistical analysis.

Most of the functional genetic units of higher organisms span large segments of DNA. Because of this, there arose a need to develop probes that could recognise regions of DNA covering many megabases and thus techniques were developed for isolating fragments of mammalian chromosomes as YACs. YACs were first used to isolate fragments of human DNA ranging from 100-1000 kb (Burke *et al.*1987). Techniques for the isolation and cloning of YACs have since been refined and have been successfully applied in FISH studies and may be potentially applied in long-range physical mapping of human chromosomes.

1.2.6 Chromosomal *in situ* Suppression Hybridisation

Most genomic DNA fragments of higher organisms contain ubiquitously occurring interspersed repetitive sequences (IRS) for example, Alu-elements and L-1 elements. These IRS's result in additional background signals throughout the genome of higher organisms and non-specific background noise during FISH reactions. Protocols have been designed to suppress the hybridisation signals from these repetitive sequence elements using competitor DNA (Lichter, *et al.* 1988). Human Cot 1 DNA is selectively enriched in highly repetitive DNA and is used as competitor DNA.

1.2.7 Applications of FISH

Classical cytogenetics employs chromosomal staining techniques in order to identify specific chromosomal abnormalities. These techniques, although providing information about the whole genome, provides little information about abnormalities in DNA regions of a few kilobase pairs or less. In addition, the chromosomal origins of marker chromosomes may be ambiguous and thus complex karyotypes may not be successfully analysed. FISH analysis may be applied to both metaphase and interphase analysis.

1.2.8 Metaphase Analysis

Conventional cytogenetic techniques require that cells generate well-spread and high quality metaphase chromosomes in order for a successful karyotypic analysis to be performed. The same applies to metaphase analysis using FISH where quality metaphase chromosomes are required to generate appropriate signals.

FISH analysis of metaphases has been extremely successful especially in the area of screening for numerical abnormalities. In individuals with Down's syndrome, a whole chromosome paint probe of chromosome 21 may successfully detect trisomy 21 in cells from a patient with the syndrome. This technique has been even more useful in the detection of structural abnormalities and specific translocation events. The t(9;22) translocation in chronic myeloid leukaemia (CML) may be detected using dual or FISH with chromosomes 9 and 22 or single colour FISH using either chromosome 9 or 22 in patients suspected of having CML.Structural abnormalities such as subtle deletions and inversions may be difficult to detect using this approach. More accurate detection of the t(9;22) may be further accomplished by using whole chromosomal library probes specific for the *BCR* gene and *ABL* gene loci. The two probes are labelled with different fluorochromes and the BCR-ABL fusion may be detected on the short Ph chromosome using a dual FISH detection system. The use of FISH to elucidate translocation events has been used extensively in the field of leukaemia (Becher *et al.*1991; Spelemann *et al.*1991) and amplification events in human tumours have also been analysed successfully (Avivi *et al.*1992).

Lichter *et al.*1990 states that the location of probes along chromosomes may be determined to within a few megabases. Using a specific probe, deletions of the region targeted by the probe can be detected due to loss of the hybridisation domain. Amplification events may be detected by the increase in intensity, area of hybridisation domain or by counting the number of resolvable hybridisation sites (Tkachuk *et al.*1991).

The introduction of a chromosomal stain to the FISH protocol allows the position of certain probes to be determined and subsequently mapped, relative to a certain chromosomal band. The chromosomal counterstain 4,6 Diamidino-2-phenylindole 2HCl (DAPI) is stable under light excitation over a long time and its emission spectrum doesn't overlap with those of other fluorochromes. It does not interfere with the hybridisation procedure as it is introduced post-hybridisation and its affinity for AT-rich DNA sequences confers to this dye the property to produce G-bands. The red counterstain propidium iodide alone does not produce banding but cells that have been exposed to bromodeoxyuridine (BRDU) may produce chromosomal bands on metaphase after staining with this dye.

The use of CISS hybridisation together with digital imaging microscopy has allowed mapping of segments with speed and precision (Lichter *et al.*1990). Probes may be ordered along a chromosome by using mapping co-ordinates defined by the fraction of the distance between the signal and a reference point (usually chosen to be the end of the short arm of the chromosome pter) to the total length of the chromosome, that is, FLpter (fraction length of chromosome with fraction length pter). The reliability of a FLpter map is dependent on the condensation state of the chromosome a greater resolution being obtained with elongated prometaphase chromosomes.

1.2.9 Interphase Analysis

One drawback with metaphase analysis, is the requirement for cells to be grown *in vitro*. This is time consuming, labour intensive and generally, growth conditions which apply to one specimen may have to be adjusted to suit another. With interphase analysis, only a few hundred representative cells are required for accurate results.

The concept of analysing chromosome aberrations in interphase nuclei started with the detection of numerical aberrations of the sex chromosomes by staining of the Barr and Y bodies. Since then, the technique has been successfully applied to the analysis of tumour specimens where a difficulty in producing metaphase chromosomes exists. Numerical abnormalities may be detected using chromosome-specific repeat sequence probes. These produce intense hybridisation signals which may easily be enumerated. They are especially successful in detecting aneuploidies of specific tumours such as gliomas, bladder, breast carcinomas and specific leukaemias.

In the area of bone marrow transplantation, repeat sequence probes for the Y chromosome have proved useful in assessing the success of bone marrow transplants. If a male recipient receives cells from a female donor, the frequency of residual male cells can be assessed quickly and accurately using this method. Using two-colour or dual FISH, interphase cells may be analysed for the detection of a successful transplant especially in female recipients of male donors using centromeric probes for X and Y chromosomes.

FISH with repeat sequences does not allow for the detection of structural abnormalities unless the target repeat sequence is involved. Whole chromosome probes are not as useful in interphase analysis as they are in metaphase analysis and therefore may not be applied in interphase identification of tumour cells where complex structural and numerical abnormalities are present as domain overlaps may be extreme (Tkachuk *et al.*1991).

AIMS OF THIS STUDY

The specific aim of this study is to unravel the significance of chromosome 11p involvement in ovarian carcinoma through analysis of two epithelial ovarian carcinoma cell lines, UWOV2 and UWOV2(Sf), which both harbour chromosome 11p abnormalities in the region 11p13-p15. Cell line UWOV2 was established in 1986 from ascitic fluid obtained from a 52 year-old female with ovarian cancer. Cell line UWOV2(Sf) was established from cell line UWOV2 after exposure of this cell line to serum-free conditions (Golombick *et al.* 1990).

Specific goals are to:

1. Collect and prepare DNA markers specific for the region 11p13-p15

2. Use these markers to map the breakpoint of the 11p abnormality using the technique of Fluorescence in situ Hybridisation.

3. Determine the involvement of this region in fresh tumour samples and compare these results to that of the cell lines.

SECTION 2.0

MATERIALS AND METHODS

2.1 CELL CULTURE AND CYTOGENETIC TECHNIQUES

2.1.1 Tissue Culture of Cell Lines

Two cell lines UWOV2 and UWOV2(Sf) were obtained from Terry Golombick, Department of Medicine, University of the Witwatersrand (Golombick *et al.* 1990). UWOV2 was propagated in RPMI 1640 medium (Highveld Biological, South Africa) with 2% Foetal Calf Serum (FCS) (Highveld Biological, South Africa) while the subline UWOV2(Sf) was maintained in RPMI 1640 medium without any exogenous protein or serum supplementation. Cultures were passaged at confluence and cultures were chosen at random and at no particular passage for the purposes of this study.

For the supported growth and proliferation of human cell lines in culture, it is necessary to supplement growth media with serum in order to supply yet undefined factors to satisfy yet undefined cellular functions. The effects of serum addition are numerous and under serum-supplemented culture conditions it is often difficult to distinguish biologically between the effects of non-specific 'nutritional' factors of serum and those of specific regulatory factors (Guilbert *et al.* 1976). In general, serum supplementation provides both attachment (Elsdale *et al.*1972) and growth factors (Antoniades *et al.*1982).

Tissue culture of human cell lines under serum-free conditions eliminates the non-specific effects of serum addition and allows investigation into the biological expression of cells especially in the field of growth factors. In the case of cell line UWOV2(Sf), valuable information has been obtained on the production of growth factors and other regulatory proteins and their involvement in the propagation of ovarian carcinoma (Golombick, Johannesburg, South Africa).

2.1.2 Tissue Culture of Fresh Tumour Material

Fresh tumour material was obtained from Dr. Franco Guidozzi, Department of Gynaecology and Obstetrics, Johannesburg General Hospital. Three specimens (T2, T4, and T5) were obtained and all tumours were epithelial in origin.

Using a scalpel, tumours were cut up into pieces of approximately $2mm^3$. Pieces were placed into $25cm^2$ flasks (Nunclon, Roskilde, Denmark) and then exposed to 0,3% Collagenase A (Boehringer Mannheim, Germany) in RPMI 1640 medium (GibcoBRL, U.S.A.) and incubated overnight at 37°C in 5% CO_2. The resulting cell suspension was gently shaken and then layered onto 100% Hypaque-Ficoll-1077 solution (Sigma Diagnostics, U.S.A.) and centrifuged at 1750rpm for 30 minutes. The upper phase was recovered and washed twice in Hank's Balanced Salts Solution (HBSS) (Highveld Biological, South Africa) at 1400rpm for 10 minutes each. Cells were enumerated using Trypan blue viability stain (Appendix A). Cells were resuspended in RPMI

1640 medium containing 15% Foetal Bovine Serum (FBS)(GibcoBRL, U.S.A.) and 5ml Penicillin G Sodium (10mg/ml)/Streptomycin Sulphate (10mg/ml)(Highveld Biological,South Africa) and then seeded into 25cm^2 flasks at a concentration of 2x10^6 cells/ml. Cultures were incubated at 37°C in 5% CO_2. All centrifugation steps were performed in a Beckman GS-6R benchtop centrifuge.

2.1.2.1 Collection and Transport of Specimens

Collection media for the transport of tumour specimens depends on the medium of choice. Several groups have used RPMI 1640, saline or Hanks Basic Salt Solution as the main transport medium while other groups report that dry collection and transportation is as effective, however, the time between receipt of and processing of the specimen has to be reduced significantly. Specimens received at room temperature or ambient temperature in collection medium appear to be the most common procedure (Lawce, 1994) while transport on ice (4°C) allows the specimen to remain viable for up to 24 hours.

The specimen is usually examined either by eye or by dissecting microscope depending on the size of the specimen. Areas of differing morphology are dissected out and cultured separately while tissue considered as necrotic or fat may be discarded. The tissue may then be rinsed in medium, saline or HBBS with or without antibiotics.

2.1.2.2 Cell Dissociation

The two principle methods used for the dissociation of ovarian tumours include the mechanical dissociation method and the enzymatic dissociation method. Generally, the more soft, friable tumours are mechanically disaggregated by pressing the cells through sieves of gradually reduced mesh, or by forcing the cells through a syringe or needle.

Kusyk *et al.* 1979, introduced a technique which employed the enzyme collagenase in the disaggregation of tumour material for cytogenetic studies. Collagenase in a non-cytotoxic hydrolytic enzyme which is isolated from *Clostridium hystolyticum* and operates optimally in isotonic medium of pH range 6.5 to 7.8. It acts by dissolving connective tissue without damaging cell surface structure. Sandberg *et al.* 1986, observed that tumours treated with 200U/ml of collagenase for 16-24 hours in culture medium yielded a larger number of metaphases with analysable chromosomes (in 80% of tumours tested) than tumours treated with higher concentrations of collagenase (1000U/ml) for a shorter period of time.

For more resilient tumours, treatment with warm trypsin (0.25%) for a short period of time helps in dissociating the tissue prior to collagenase treatment. This method however should not be employed with epithelial cells as it may be damaging to them,

while trypsin remains ineffective in dissociating more fibrous material and connective tissue.

The removal of contaminating red blood cells (RBCs) from dissociated cell cultures may be achieved by treatment with ammonium chloride (NH_4Cl_2) for 10 minutes at 4°C (Golombick *et al.* 1990). This procedure eliminates RBCs but no other contaminating stromal cells. It is particularly effective for ascitic fluid specimens. This method however usually requires the specimen to undergo several treatments and thus the risk of tumour cell loss is increased with each centrifugation step.

After enzymatic dissociation, cells are usually rinsed to decrease or remove enzymatic activity and then directly cultured (Lawce. 1994). In this present study, the method of Ficoll separation was an innovative idea the aim of which was to obtain as pure a cell sample as possible without the presence of contaminating 'normal' cells.

2.1.2.3 Culture Media

The most commonly used medium for the growth of ovarian tumour cells is RPMI 1640 medium. Other media include McCoy's 5A, MEM and Changs media. All growth media contain a mixture of vitamins, glucose, buffered salts and amino acids. Antibiotics and fungicides are added to prevent bacterial and fungal contamination. Foetal bovine serum (FBS) is often used in preference to FCS as it is much richer and conducive to growth conditions than FCS which may be toxic to cells under incorrect conditions (Stewart *et al.* 1986).

2.1.2.4 Incubation

Incubation temperature should be maintained between 36.8°C and 37.5°C. The gas mixture is not as important but 5% CO_2 in ambient air is common practice.

2.1.3 Cell Culture of Ascitic Fluid

Ascitic fluid from ovarian tumours was obtained from Dr. Franco Guidozzi, Department of Gynaecology and Obstetrics, Johannesburg General Hospital. Four ascitic fluid specimens were obtained (AF-1, AF-2, AF-3 and AF-5). Ascitic fluids AF-2 and AF-5 were aspirated from patients from which tumours T2 and T5 were obtained.

The fluid was centrifuged at 1400rpm for 10 minutes and the resulting pellet was resuspended in RPMI 1640 medium. The suspension was layered onto 100% Hypaque-Ficoll-1077 solution and centrifuged at 1750rpm for 30 minutes. The upper phase was recovered and washed twice in Hank's Balanced Salts Solution.

Cells were enumerated using Trypan blue viability stain (Appendix A). The pellet was resuspended in RPMI 1640 medium containing 15% FBS and 5ml Penicillin G Sodium (10mg/ml)/Streptomycin Sulphate (10mg/ml)(Highveld Biological,South Africa) and then seeded into 25cm² flasks at a concentration of $2x10^6$ cells/ml. Cultures were incubated at 37°C in 5% CO_2. All centrifugation steps were performed in a Beckman GS-6R benchtop centrifuge.

2.1.4 Metaphase Arrest

Flask cultures of UWOV2 and UWOV2(Sf) were grown until sub-confluent while fresh tumour and ascitic fluid cultures were grown for 16-24 hours and then exposed to arresting solution (6µl/ml) (TC Arresting Solution, Difco Laboratories, U.S.A.) for 2 hours at 37°C in 5%CO_2.

The main arresting agent of choice is Colcemid. Colcemid is a synthetic compound which prevents spindle formation arresting cell division at mitosis while promoting dispersion of the arrested metaphases. Its naturally occurring analogue colchicine acts in a similar way, however, prolonged exposure to this compound leads to marked chromosomal contraction and condensation resulting in poorer definition of the chromosomes.

The time of mitotic arrest in non-synchronised cultures depends on the tumour cell cycle doubling time as well as on the concentration of arresting solution used.

2.1.5 Cell Cycle Synchrony

In order to increase the yield of metaphases obtained from cell line UWOV2(Sf), an attempt was made to synchronise cultures using methotrexate (MXT). The method used assumes that the doubling time of UWOV2(Sf) cells follows that of normal human peripheral blood lymphocytes. Sub-confluent cultures were exposed to MXT (25µg/ml) (Lederle IV 50, S.A. Cyanamid (Pty) Ltd., South Africa) for 17 hours at 37°C. The MXT was removed and cells were washed briefly in Phosphate buffered saline (PBS) (Appendix A).

Two releasing agents were used to investigate their efficacy in influencing metaphase yield. The first releasing agent used was thymidine. Cultures were exposed to RPMI 1640 medium containing thymidine (0.0048µg/ml) (Fluka BioChemika, U.K.) and incubated for 5 hours at 37°C . Arresting solution was then added for 1 hour. The second releasing agent used was Bromodeoxyuridine (BRDU). Cultures were exposed to RPMI 1640 medium containing BRDU (10µl/ml)(5mg/ml stock)(Sigma Diagnostics, U.S.A.) and incubated for 6 hours at 37°C. Arresting solution was then added for 1 hour.

Methotrexate (MXT) is a folic acid antagonist. Folic acid in its active form is an enzyme which is important in several cellular metabolic reactions as well as in the synthesis of thymidine. Addition of MXT to cells slows down thymidine synthesis thus blocking DNA replication at the G_0 phase of the cell cycle. The subsequent addition of thymidine-rich culture medium to the cells allows them to complete replication and to proceed to mitosis in synchrony. This improves the mitotic index and the quality of direct harvests.

The use of BRDU as a releasing agent has several benefits. BRDU, a thymidine analogue, induces chromosomal elongation and improves definition of chromosomal bands (Yunis, 1976) binding preferentially to G-C-rich areas. However, cultures treated with BRDU may not be G-T-G banded as the heterochromatin structure is drastically altered and thus the banding method of choice would be the R-banding (reverse banding) technique. Release time for tumour cultures differ according to the type of tumour and its cell cycle.

2.1.6 Culture of Human Peripheral Blood Lymphocytes

Cultures of human peripheral blood lymphocytes were established as a source of control material for Fluorescence *in situ* Hybridisation (FISH) studies.

Fresh blood was collected from volunteer subjects. 0.5ml of blood was seeded into 8.5ml of RPMI 1640 medium supplemented with 15% FCS and antibiotics (as described in section 1.2). Cultures were incubated at 37°C in 5% CO_2 for 43 hours. MXT (25µg/ml) was added to the cultures and cells were incubated for 17 hours at 37°C in 5% CO_2. Cultures were centrifuged at 1400rpm for 10 minutes at room T° to remove the MXT. The pellet was resuspended in fully supplemented RPMI 1640 medium containing thymidine (0.0048µg/ml) and cultures were incubated for 5½ hours at 37°C in 5% CO_2. Arresting solution (6µl/ml) was added and the cultures were incubated for half an hour at 37°C in 5% CO_2.

All centrifugation steps took place in a Beckman GS-6R benchtop centrifuge.

2.1.7 Harvesting of Cell Cultures

After metaphase arrest, the supernatant was removed from flask cultures and cells were exposed to 0.25% trypsin in PBS (Difco Laboratories, U.S.A.) for 5-15 minutes or until cells lifted off the flask. More strongly adherent cells were mechanically dislodged by gently hitting the side of the flask. Cells were resuspended in Hank's Balanced Salts Solution and washed once by centrifugation at room T°.

Human peripheral blood lymphocyte cultures were removed from incubation and centrifuged at 1400rpm for 10 minutes.

The pellets from flask and blood lymphocyte cultures were resuspended in 10ml of 0.75M KCl and incubated for 20 minutes for cell lysis to occur. The KCl was removed by centrifugation at room T° and cells were fixed by the addition of 10ml of ice-cold methanol/acetic acid fixative (3:1) and washed by centrifugation at 4°C. The resulting pellet was resuspended in ice-cold fixative and incubated at -20°C in preparation for slidemaking. All centrifugation steps were at 1400rpm for 10 minutes in a Beckman GS-6R benchtop centrifuge.

In situ harvests are accomplished with hypotonic solutions of various concentrations of sodium citrate, KCl, NaCl or cancer hypotonic solution (CHS - a mixture of KCl, EGTA and Hepes buffer)(Lawce, 1994). The effect of the hypotonic solution acts synergistically with the colchicine in swelling the cells and dispersing the metaphase chromosomes.

Fixative is added to the hypotonic solution briefly to stop its action. The methanol:acetic acid fixative is made up fresh just before use and several successive fixative washes effectively removes any remaining debris. By washing several times, the adherence of cells to glass slides as well as their staining efficiency is greatly enhanced. Methanol, being highly volatile, aids in the flattening and dispersion of chromosomes while the chromosomal structure remains unaffected by the presence of acetic acid.

2.1.8 Slide making

Cells stored at -20°C were washed for a minimum of five times in ice-cold fixative by centrifugation at 1400rpm for 10 minutes at 4°C. The pellet was resuspended in a small volume of fixative and stored on ice. Microscope slides, cleaned overnight in 100% ethanol, were dried and stored in a dry dust-free place. Slides were flooded with ice-cold fixative and 15µl of cell suspension was dropped onto the slide from a height of ±20cm. The slide was steamed briefly (4-6 seconds) and then placed flat in a humid chamber (±45% humidity) to dry for 5 minutes followed by air drying for 5 minutes.

The slides were dehydrated in an ethanol series, 5 minutes each of 70%,90% and 100% ethanol. Slides for banding were stored at room T° for 7 days and slides to be used in Fluorescence *in situ* Hybridisation (FISH) were sealed in a slideholder containing silica gel and stored at -20°C until needed.

Methods of slidemaking vary between different tumour specimens. Flooding the slide with ice-cold fixative, dropping the specimen

from a height of approximately 20cm and steaming proved to be the best method for chromosome spreading in this study.

2.1.9 Giemsa Banding Technique using Trypsin (GTG Banding)

Slides were exposed to 0.005% trypsin (Difco Laboratories,U.S.A) in PBS. The duration of exposure to trypsin varied depending on slide age, slide condition, atmospheric conditions and enzyme activity and thus exposure time varied between banding sessions but was carefully standardised for each individual session. Generally, 7-day old slides were exposed to trypsin for ±20 seconds. The trypsin was inactivated in 8% FCS in PBS, washed briefly in PBS and stained with 6% giemsa (BDH Laboratory Supplies,U.K.) in 6.8 buffer (Appendix A) for 2½ minutes. Slides were rinsed in distilled water and air-dried.

Slides were viewed by brightfield microscopy using an Olympus BH2-RFCA microscope and cells were photographed using AGFA Agfapan APX 100 film.

Prior to banding, slides are enzymatically treated with trypsin, a proteolytic enzyme which enhances the basic chromosome structure of the metaphases. Giemsa stain, a methylene blue-eosin based stain, is a complex mixture of dyes containing the main ingredient thiazin. The mechanism of band formation is not fully understood, but it is thought that the thiazin molecules "stack" alongside the DNA only in AT-rich areas. The thiazin then binds to eosin forming a magenta complex. The resulting banding pattern on chromosomes is due to the chromatin in these bands interacting ionically with the thiazin dyes by means of hydrophobic bonding forces. This results in each chromosome having its own unique banding pattern. This is only a proposal for the action of Giemsa in chromosome staining and several other theories have been proposed (Comings, 1975; Sumner, 1980; van Duijn *et al.* 1985).

2.2 PLASMID PROBES

2.2.1 COLLECTION OF PLASMID PROBES

2.2.1.1 Chromosomally Enriched Plasmid Probes

Chromosomally enriched plasmid library probes, originally obtained by our laboratory, were received from Joe Gray, San Fransisco, U.S.A. in the form of MAX Efficiency DH5α transformed *E.coli* cells with ampicillin resistance (Gray *et al.*1991). Cells were stored in 60% glycerol at -70°C.

2.2.1.2 Centromeric Alphoid Repeat Probes

Centromeric alphoid repeat probes, originally obtained by our laboratory, were received from Frank Spelemann, Belgium, in the form of MAX Efficiency DH5α *E.coli* competent cells. Centromeric probe 3 was represented by an insert size of 2.1 kilobases (kb) in the vector pSP65. Centromeric probe 11 was represented by an insert size of 0.85 kb in the vector pSP64. Both probes were ampicillin resistant. Cells were stored in 60% glycerol at -70°C.

2.2.1.3 Single Copy Gene Probes

The human globin β gene probe, mapping to the region 11p15.5 on chromosome 11, was obtained from Corné Strydom, Department of Medical Biochemistry, Wits Medical School, in the form of transformed bacterial cells. This probe was represented by an insert size of 7.4 kb in the vector pSPβ and this probe was used in FISH studies.

The probe IGF-II representing the human insulin growth factor II gene mapping to the region 11p15.5, had been obtained from Melissa Little, Edinburgh, Scotland, (Schneid *et al.*1989). This probe was represented by an insert size of 660 base pairs (bp) in the vector pGEM4 and was used in slot blot assays for gene dosage studies.

2.2.2 PLASMID PROBE PREPARATION

2.2.2.1 Bacterial Transformation

MAX Efficiency and SE Efficiency DH5α E.coli cells were used in this procedure. The DH5α cells, stored at -70°C, were thawed on ice and dispensed into 50 µl aliquots. Aliquots not used were freeze-dried using 100% ethanol and dry ice and stored at -70°C. 60ng of plasmid DNA from the probe IGF-II was added to a 50µl cell suspension and left on ice for 30 minutes. Cells were heat shocked for 20 seconds at 37°C and then left on ice for 2 minutes. The cells were then inoculated into 500µl of Luria-Bertani (LB) broth (Appendix B) without antibiotics and shaken for 60 minutes at 37°C. Agar plates (Appendix B) containing 50µg/ml ampicillin (100mg/ml stock solution) were inoculated with 50µl and 450µl of broth respectively and incubated overnight at 37°C. These plates were subsequently used as a source of inoculation for liquid cultures.

The ability to transfer plasmids into bacterial cells has become an integral part of molecular biology. The protocol described above is based on the observation that *E. coli* cells and DNA productively interact in an environment of divalent cations at low temperatures. The divalent cations referred to is a solution

of $CaCl_2$ which effectively renders the bacterial cells competent, that is, enables them to take up high molecular weight double-stranded intact DNA. Competence varies with the physiological state of the cells and is highest at the middle phase of exponential growth, rapidly declining to a minimum later on. The way in which $CaCl_2$ influences bacterial transformation is not fully understood. Two hypotheses are that:

(a) $CaCl_2$ causes DNA to precipitate onto the outside of bacterial cells
(b) the cationic solution induces some kind of change in cell wall properties that improves DNA binding

Actual uptake of DNA into competent cells is achieved by briefly raising the temperature in a process known as 'heat shock'. The optimal times of heat shock are partly a function of the surface to volume ratio of the cell suspension and the thermal conductivity of the tubes used.

The above protocol is simple but relatively inefficient where transformation efficiency is concerned. In order to achieve maximum transformation efficiency, several additional steps may be employed including (i) the addition of monovalent cations to the transformation buffer (ii) treatment of cells with solvents and sulphydryl reagents and (iii) growth of cells in medium containing elevated levels of magnesium (10-20mM).

2.2.2.2 Plate Inoculation and Plasmid DNA Amplification

The chromosomally enriched plasmid probes specifically chosen for the purpose of elucidating the chromosomal origin of the chromosome 11p+ abnormality were those which represented chromosomes commonly involved in abnormalities found in ovarian carcinomas. These corresponded to human chromosomes 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 21, 22, X.

The centromeric alphoid repeat probes chosen for the purpose of this study were specific for human chromosomes 3, 8 and 11.

Bacterial cells were streaked onto agar plates containing ampicillin 50µg/ml (stock: 100mg/ml) and grown overnight at 37°C. Colonies were inoculated into liquid culture consisting of 250ml LB with ampicillin (50µl/100ml) (stock:100mg/ml) and grown overnight at 37°C on an orbital shaker until bacterial growth had reached log phase. For chromosomally enriched plasmid probes and centromeric probes, a minimum of six streak plates were used to inoculate liquid cultures while for single copy gene probes, a single colony was chosen for inoculation.

For the preparation of chromosomally enriched plasmid libraries, colonies on plates were pooled so as to give a better representation of fragments from the chromosome under study. In

the case of single copy gene probes, a single colony was chosen so as to avoid selection for ampicillin-resistant mutations.

2.2.2.3 Plasmid Extraction

Bacterial cells were pelleted at 5000rpm for 15 minutes at 4°C. Cells were resuspended in lysosyme solution containing 2mg/ml lysosyme (Boehringer Mannheim, Germany) in Lysosyme Buffer (25mM Tris-Hcl pH 7.5, 10mM EDTA, 15% sucrose) and placed on ice for 20 minutes. To this, 12ml of 0.2M NaOH, 1% SDS was added. Cells were mixed by inversion and placed on ice for 10 minutes. 7.5 ml of sodium acetate pH 4.6 was added. Cells were mixed by inversion and placed on ice for 10 minutes. Cells were centrifuged at 15000rpm for 15 minutes at 4°C. The supernatant was filtered through a nylon filter and transferred to a clean tube. An equal volume of ice-cold isopropanol was added and the mixture was centrifuged immediately at 12000rpm for 15 minutes. The pellet was lyophylised and resuspended in 2ml of TE (Tris-HCl,EDTA pH 7.6). All centrifugation steps took place in a Beckman Model J2-21 centrifuge. Each sample was made up to 4ml by the addition of 2ml of TE pH 7.6. To each sample, 4g of Cesium Chloride (CsCl) and 320 µl of ethidium bromide (stock:10mg/ml in H_2O) was added. Samples were mixed, placed into ultracentrifuge tubes and centrifuged in a Beckman Ultracentrifuge at 45000rpm for 18-24 hours at 4°C. The plasmid DNA band was collected and washed 4 times with an equal volume of isoamylalcohol to remove the ethidium bromide. The sample was then dyalised in TE pH 7.6 for half an hour and stored at -20°C.

The procedure described above is based on the alkaline lysis procedure of Maniatis *et al.* 1989

Lysosyme is an acid muramidase enzyme which attacks glycosidic bonds in the murein structure of bacterial cell walls. Its activity is enhanced and modulated by the lysosyme buffer which provides a hypotonic environment for the cells. The chelating agent EDTA enhances lysosyme activity while the sucrose prevents cells from lysing immediately and allows the production of osmotically fragile protoplasts.

Addition of the ionic detergent SDS results in cell lysis while NaOH raises the pH to 12.0-12.5 effectively denaturing non-supercoiled bacterial DNA while supercoiled plasmid DNA remains intact. The addition of sodium acetate allows denatured strands to reaggregate into a tangled mass which is insoluble and precipitates out during centrifugation. This yields a clear lysate consisting almost entirely of plasmid DNA. During this step, the action of SDS and sodium acetate renders proteins and RNA insoluble facilitating their removal by centrifugation.

Cesium chloride gradient centrifugation is based on the observation that cesium and chloride ions are forced towards the bottom of a tube on centrifugation of such solutions at high speeds. The downward ionic migration is counterbalanced by diffusion creating a concentration gradient. DNA centrifuged in such a solution will form bands at distinct points in the gradient depending on its buoyant density. DNA will migrate to a point equal to a buoyant density of $1.7g/cm^3$ while RNA having a higher buoyant density will sediment at the bottom of the tube.

The incorporation of ethidium bromide allows differentiation of supercoiled DNA from linear DNA. Ethidium bromide binds to DNA molecules of linear DNA intercalating between base pairs causing partial unwinding of the double helix. This leads to a decreased buoyant density. Bacterial chromosomal DNA, being supercoiled does not unwind as easily and therefore the ethidium bromide cannot bind as effectively resulting in an even decreased buoyant density compared to that of plasmid or linear DNA. The result is that the two types of DNA will sediment at different rates. Using ultraviolet light, the relative positions of the bands can be seen and a pure plasmid DNA preparation can be extracted.

2.3 YEAST ARTIFICIAL CHROMOSOME (YAC) PROBES

2.3.1 COLLECTION OF YAC PROBES

Twelve YAC probes mapping to the region 11p14.3-15.5 were obtained from Denis LePaslier (CEPH - Centre d'Etude du Polymorphisme Humain,France) libraries. The gene assignments of the YACs can be seen in table I

YAC	Human Genome Mapping Locus Assignment*
892g9	D11S922
785e5	D11S1318, D11S1323, D11S1338
847a12	D11S1323, D11S1338
954f4	D11S909, D11S932
933b8	D11S1329, D11S1346, D11S2069
928d1	D11S1315, D11S1346, D11S2069
922e1	D11S1315, D11S926, D11S1334, D11S1348, D11S1349
879b10	D11S1315, D11S1334
770c6	D11S861, D11S1307, D11S926
966e8	D11S902, D11S921
845a3	D11S899, D11S1308
855b1	D11S1308

Table I: The Human Genome Mapping regional assignments of CEPH YAC probes to the chromosomal region 11p15.5-14.3

*** van Heyningen *et al.*** 1995

2.3.1.1 YAC Amplification and DNA Extraction

YAC cultures were inoculated into 3ml of AHC medium (Appendix B) and grown overnight at 30°C on an orbital shaker (±150rpm). Glycerol stocks were made, by addition to 2ml of culture and of 20% glycerol. Cell stocks were stored at -70°C.

The remaining 1ml of culture was inoculated into 250ml of AHC medium and grown for 36 hours at 30°C (150rpm) or until the medium turned pink. This indicates that the yeast cells have reached log phase of growth. Cultures were then centrifuged and the resulting cell pellet was weighed to ensure that

approximately 1g dry weight of yeast cells was obtained per sample.

Cells were harvested by washing twice with 10ml of 40mM EDTA/90mM β-mercaptoethanol by centrifugation at 3000rpm for 10 minutes. The cells were resuspended in 2ml of SCE (1M sorbitol, 0.06M EDTA, 0.1M sodium citrate pH 7.0) containing 1:125 dilution of β-mercaptoethanol and 5µl of lyticase (Boehringer Mannheim,Germany). After incubation at 37°C for 2 hours, the spheroplasts were centrifuged at 3000rpm for 10 minutes and resuspended in 0.7ml of 50mM Tris(pH 7.4), 25mM EDTA, 500mM NaCl, 3mM 2-mercaptoethanol, 1% SDS. Cells were incubated at 65°C for 15 minutes, vortexing intermittently.

A phenol/chloroform extraction was performed on each sample by the addition of ½volume of phenol and ½volume of chloroform (chloroform:isoamylalcohol 24:1). Samples were centrifuged at 3000rpm for 15 minutes between each extraction. The final wash consisted of an equal volume of chloroform.

An equal volume of isopropanol was added to the aqueous phase and incubated at room T° for 15 minutes. The sample was microfuged for 15 minutes at room T° and the pellet rinsed in 200µl of cold 70% ethanol and air dried.

The pellet was resuspended in TE (pH 7.6) and 2 units of DNAse-free-RNAse (Boehringer Mannheim, Germany) was added. After a 2 hour incubation at 37°C with intermittent agitation, the DNA was precipitated with isopropanol as described above.

The pellet was resuspended in 0.3ml TE (pH 7.6) and the DNA was precipitated by the addition of 2½ volumes of ice-cold 100% ethanol. Samples were placed at -70°C for 15 minutes and then microfuged at room T° for 15 minutes. The pellet was rinsed with cold 70% ethanol and air dried. The pellet was resuspended in TE pH 7.6 and stored at 4°C. All centrifugation steps took place in a Beckman GS-6R benchtop centrifuge and a Heraeus Biofuge 15 microfuge.

The procedure described above is based on the method from Cold Spring Harbour (Dr.Turc Carel, Nice, France)

For the preparation of Yeast Artificial Chromosomes for use in *in situ* hybridisation, several protocols are available. The standard technique for YAC DNA isolation involves the agarose plug method in which yeast cells are embedded in agarose in order to maintain the integrity of the yeast chromosomes. The spheroplasting and lysing steps are performed within these agarose plugs and the desired YAC clones are analysed and isolated by Pulsed-Field Gel Electrophoresis. Using this method, DNA may also be labelled within the agarose plug and the agarose

is subsequently removed during the DNA purification stage by elution through Sephadex G50 spun columns. This method is labour intensive and time consuming and the DNA yield is relatively low usually providing enough DNA for only three labelling reactions (Selleri *et al.*1991).

Yeasts exhibit a relatively high rate of mitotic recombination and because of this, artefactual clones arise due to intrafragment rearrangement or interfragment chimaera formation (Oliver *et al.*1994) which may also be influenced by long incubation and preparation protocols and by exposure to toxic chemicals employed during the transformation procedures.

The method described in this present study is rapid, providing high quality total genomic yeast liquid DNA and therefore, the probability of mitotic recombination events is greatly reduced.

The most commonly used media for the growth of YACs are YEP-D, synthetic complete medium (such as AHC) and the dropout mix (which is enriched with amino acids and is used mainly for growth of the auxotrophic strains). The yeast strain of interest is grown in the desired medium to the desired density. The density should exceed 1g dry weight of yeast cells as cell loss is encountered during the numerous preparation procedures. Inoculation of cultures should be carried out under sterile conditions as no antibiotics nor fungicides may be added to the culture medium.

In order to obtain a pure yeast chromosomal DNA preparation, the spheroplasting step has to be carefully defined for each individual strain. As a pre-treatment to spheroplasting, slowly growing log phase yeast strains are exposed to EDTA and β-mercaptoethanol which conditions the cells for spheroplasting and assures reproducibly rapid spheroplast formation. This is important as direct exposure of cells to the SCE solution may cause the cells to lyse too quickly. The SCE medium provides an osmotically stable environment for the spheroplasts preventing cell lysis. It is important that incubation during the spheroplasting stage is carried out without agitation.

Several enzymes may be used for spheroplasting. These include Glusulase, Oxalyticase, Zymolyase and Lyticase. Lyticase is secreted by *Arthrobacter luteus* and may yield a clearer lysate than other enzymes (Scott *et al.*1980). The rate of cell lysis is dependent on several factors the most important ones being the strain of yeast and the concentration of lytic enzyme. Generally, the slower growing yeast strains require more enzyme and longer incubation periods compared to log phase or rapidly growing strains. Cell lysis is accomplished by incubation in low salt solutions and low concentrations of SDS.

2.4 ESTIMATION OF DNA YIELDS

In order to quantitate the yield of DNA from both plasmid and YAC preparations, two methods were employed to ensure that an accurate estimation was obtained. This was done to ensure that the correct amount of DNA was employed which would influence further steps leading to the use of this DNA in Fluorescence *in situ* Hybridisation.

2.4.1 Agarose Gel Electrophoresis

A 1% agarose gel was prepared in 1xTAE Buffer (Appendix A) containing 3µl/100ml ethidium bromide (stock:10mg/ml in H_2O). After 5µl of each sample was mixed with 1µl of loading dye, they were loaded into wells and electrophoresed for 1 hour at 80V (±120mA). Lambda DNA (250ng/µl)(Boehringer Mannheim, Germany), at various known concentrations was electrophoresed alongside the samples. After electrophoresis, the gel was visualised under UV transillumination and photographed.

2.4.2 Spectrophotometry

DNA yield was also determined spectrophotometrically using a Beckman Module-65 Spectrophotometer. Readings were taken at 260nm and 280nm respectively and the concentration of plasmid DNA was determined by using the formula:

$$\text{DNA yield } (\mu g/\mu l) = \frac{\text{spec. reading x O.D. x dilution factor}}{1000}$$

O.D. = Optical Density where 1 O.D.= 50 for DNA at 260nm

For the most accurate results, the two methods of DNA quantification described above should both be employed. Using spectrophotometry, readings should be taken at 260nm and 280nm in the ultraviolet range of the spectrum. At 260nm, the amount of double-stranded DNA may be determined where an O.D. of 1 is equal to approximately 50µg/ml. The purity of a DNA sample may be determined by calculating the ratio between readings at 260nm and 280nm, that is $O.D._{260}/O.D._{280}$ (this reading should be in the range of 1.8-2.0). The drawback with this method of quantification is that concentrations of DNA lower than 250ng/ml cannot be detected accurately (Maniatis *et al.*1989)

Agarose gel electrophoresis incorporating ethidium bromide is a more sensitive quantitative method. The strength of the fluorescence of ethidium bromide under ultraviolet light allows DNA samples to be compared with that of a series of standards. As little as 1-5ng of DNA may be detected using this method.

2.5 PROBE LABELLING

Before labelling, plasmid DNA was treated with DNAse-free-RNAse (Boehringer Mannheim,Germany) (10µl/100µl) for 1 hour at 37°C. The DNA was then precipitated by the addition of $1/_{20}$vol of sodium acetate pH4.6 and 2½vol ice-cold ethanol. The sample was then microfuged for 15 minutes at 4°C and the pellet rinsed with cold 70% ethanol and air-dried. The pellet was resuspended in 100µl of TE (pH7.6).

Plasmid and YAC DNA was labelled with either biotin-16-dUTP or digoxigenin-11-ddUTP (Boehringer Mannheim,Germany) by nick translation. For each reaction, 2µg of template DNA per 100µl of reaction mixture was used and the labelling reaction was carried out as follows:

Reaction Mixture:

2µg	template DNA
10µl	10x nick translation buffer (0.5M Tris-HCl pH8.0, 50mM $MgCl_2$, 0.5 mg/ml bovine serum albumin)
10µl	0.1M β-mercaptoethanol
10µl	10x nucleotide stock*
0,5µl	DNAse I (1:1000 in distilled water) (stock: 3mg/ml in 0.5ml 0.3M NaCl+ 0.5ml glycerol)
1µl	DNA polymerase

The reaction mix was made up to 100µl with ddH_2O

* Biotin/nucleotide stock		* Digoxigenin/nucleotide stock	
dCTP	0.5mM	dCTP	0.5mM
dGTP	0.5mM	dGTP	0.5mM
dATP	0.5mM	dATP	0.5mM
dTTP	0.1mM	dTTP	0.375mM
biotin-16-dUTP	0.4mM	digoxigenin-11-ddUTP	0.125mM

The reaction was incubated in a water bath at 15°C for 2 hours. A 7µl aliquot was then denatured by boiling for 3 minutes, cooled on ice for 3 minutes and then loaded into wells of a 2% agarose gel prepared in 1xTAE (Appendix A) with 3µl/100ml ethidium bromide (stock: 10mg/ml in H_2O). DNA molecular weight marker VI (Boehringer Mannheim,Germany) was electrophoresed alongside the samples. The samples were electrophoresed at 100V (±140mA) for half an hour. The gel was visualised under UV transillumination and photographed.

Fragment sizes of between 200 and 500 nucleotides were considered suitable for use in Fluorescence *in situ* Hybridisation.

The labelling reaction was stopped by the addition of $1/_{10}$ volume of stop mix (0.1M EDTA pH8.0, 1% SDS) and heated at 68°C for 15 minutes on a heating block and then stored at 4°C.

The procedure described above is a modification of the method described by Langer *et al.* 1981.

Nick translation of DNA is based on the action of *Eschericia coli* DNA polymerase I. This enzyme adds nucleotide residues to the 3' hydroxyl terminus on a strand of DNA 'nicked' by the enzyme DNAse I. It also possesses 5'→3' exonucleolytic activity and thus simultaneously removes nucleotides from the 5' end and adds nucleotides to the 3' end. This facilitates movement of the 'nick' along the DNA (Kelly *et al.* 1970).

The rate at which nucleotides are incorporated depends on the activity of DNAse I and the type of DNA template used (Maniatis *et al.* 1989). By carrying out the labelling reaction at lower temperatures (±15°C), the activity of DNAse is reduced which prevents the degradation of DNA when extended labelling periods are required. This allows more efficient incorporation of the labelled nucleotides (Koch *et al.*1986). In order to optimise the concentrations of DNAse for labelling, varying concentrations of DNAse may be added to DNA samples keeping all other parameters constant.

Nick translation buffer is important for maintaining an optimal physiological environment in which the enzyme can function. Alterations in the pH of the buffer or ionic strength (provided by $MgCl_2$) could decrease enzyme activity.

Termination of the labelling reaction is achieved by the addition of EDTA. EDTA inactivates enzymes indirectly by binding to Mg^{2+} ions. Short incubation at 65°C is sufficient to heat inactivate the enzyme.

2.5.1 Purification of Labelled DNA

Labelled DNA was purified by centrifugation through Sephadex G-50 spin columns in order to remove the unincorporated nucleotides.

The stamp was removed from 1ml syringes and a plug of silanized glass wool was packed at the bottom of the syringe to a height of 2-3mm. The column was packed with buffered Sephadex G-50 resin (Sephadex G-50 dispersed in column buffer: 10mM Tris-HCl

pH8.0, 1mM EDTA, 0,1% SDS and autoclaved) to the 1ml mark. The column was centrifuged at 3000rpm for 5 minutes until the resin packed to the 1ml mark. The column was washed three times with 100µl of column buffer by centrifugation at 3000rpm for 5 minutes.

An eppendorf tube was then placed under the syringe and the labelled DNA was loaded onto the column and spun at 3000rpm for 5 minutes. The eluted DNA sample was removed and stored at -20°C. All centrifugation steps took place in a Beckman GS-6R benchtop centrifuge.

The procedure described above is based on the method of Lichter,P; Cremer, T. 1991)

This method of gel filtration chromotography is used to separate high molecular weight DNA from smaller molecules as well as separating unincorporated labelled dNTP's from DNA labelled by nick translation. Sephadex G50 is a commonly used gel matrix, the porosity of which is suitable for purifying DNA larger than 80 nucleotides in length. Smaller fragments of DNA and unincorporated nucleotides are retained in the pores of the gel (Maniatis *et al.*1989). The incorporation of SDS into the column buffer is necessary because the biotinylated probe might stick in the column due to hydrophobic biotin groups (Lichter, P.; Cremer, T. 1991).

2.6 FLUORESCENCE 'IN SITU' HYBRIDISATION (FISH)

All labelled plasmid and YAC DNA probes, were tested with FISH. Before labelled probes were hybridized onto test slides, they were hybridized onto control slides of human peripheral blood lymphocyte metaphase cells in order to test for non-specific hybridisation and also probe preparation and labelling quality.

Test slides used for the purposes of this study included cultures from cell lines UWOV2, UWOV2(Sf), fresh tumour sample T5 and ascitic fluid culture AF-5.

The method described below is a modification of the method of Lichter, Heidelberg, Germany.

2.6.1 Slide Preparation

Slides stored at -20°C were thawed for 30 minutes at 4°C followed by 30 minutes at room T° before being used for FISH.

The slides were treated with 100µl of DNAse-free-RNAse (Boehringer Mannheim,Germany)(20µl/80µl 2xSSC) per slide, for 30 minutes at 37°C, followed by three washes of 5 minutes each in

2xSSC (Appendix A). Slides were dehydrated in an ethanol series, 5 minutes each of 70%, 90% and 100% ethanol.

Slides were then exposed to pepsin (Boehringer Mannheim,Germany)(50µl/100ml in 0.01N HCl)(pepsin stock: 10%) for between 10 and 15 minutes at 37°C depending on the age of the slide. Slides were rinsed briefly in PBS and then washed for 5 minutes with 1xPBS, 50mM $MgCl_2$ in distilled water followed by a 5 minute wash with 1xPBS, 50mM $MgCl_2$, 4% paraformaldehyde (Appendix A) in distilled water.

Slides were washed for 5 minutes in 1xPBS and then dehydrated in an ethanol series 5 minutes each of 70%, 90% and 100% ethanol. Slides were stored at 60°C in preparation for slide denaturation.

Slides were denatured in denaturation solution:

5ml 20xSSC
5ml 0.5M phosphate buffer (Appendix A)
5ml distilled water
35ml deionized formamide

Denaturation of the slides was at 70°C for exactly 2 minutes, the timing of which was critical. This was followed by immediate dehydration in an ice-cold ethanol series 5 minutes each of 70%, 90% and 100% ethanol.

The pre-treatment of metaphase slides with RNAse serves to remove any endogenous RNA that may be present in the preparation. This step however is not critical and might even negatively influence the quality of results obtained (Lawrence *et al.*1988). The precise action of HCl on chromosomal structure is not known but it may aid in the extraction of proteins and in the hydrolysis of target sequences which effectively increases the signal to noise ratio. For the effective penetration of probe molecules, cells are mildly digested with pepsin. The concentrations of pepsin are adjusted according to the source of the material, age of slide and purity of the metaphase cells (Lichter, Heidelberg, Germany). Proteinase K may be used in place of pepsin. Pinkel *et al.* 1986, stated that for optimal digestion, the concentration of proteinase K is adjusted so that almost no phase contrast microscopic image remains on a dry slide.

During the paraformaldehyde washing step, cells are fixed *in situ* which maintains the morphology of the target sequences. This step is important especially if several pre-treatment steps are employed which risks altering chromosome morphology.

For the successful slide denaturation, it is important that all parameters are carefully defined. DNA denatures in 0.1-0.2M Na^+ at 90°C-100°C. The addition of an organic solvent such as

formamide, reduces the thermal stability of double-stranded DNA allowing separation of the double helix at lower temperatures. This reduction in the melting temperature of DNA occurs in a linear fashion with a 0.72°C reduction for every % of formamide. Such a procedure for denaturation requires a shorter time which has a less adverse effect on chromosome morphology which may be altered at higher temperatures.

2.6.2 Probe Preparation

For each FISH reaction, the amount of labelled probe used, varied with the kind of probe. In a 12µl volume of final hybridisation mixture, 2-20ng of centromeric probe DNA or 80-120ng of cDNA (with insert size >2kb) was used. For chromosomally enriched plasmid probes, 150-500ng of labelled DNA was used while for YAC probes, 500ng-1µg of labelled DNA was used for each FISH reaction.

The method of chromosomal in situ suppression (CISS) hybridisation was used for chromosomally enriched plasmid probes, total genomic single copy gene probes and YAC probes. This method involves the addition of competitor DNA to the probe during the probe preparation step, the function of which is to suppress the hybridisation signal from ubiquitous repeated sequences such as Alu and Kpn elements (Lichter *et al.* 1990).

Probes were precipitated using the following mix:

competitor DNA *
1µl salmon testes DNA (Sigma Diagnostics, U.S.A.)
1/20vol sodium acetate pH4.6
2½vol ice-cold ethanol
* Human cot 1 DNA (GibcoBRL,U.S.A.)
For YAC probes, 3ug/50ng of labelled probe was used
For single copy probes, 60ng/50ng of labelled probe was used

The mixtures were incubated at -70°C for 15 minutes and then microfuged for 15 minutes at 4°C. The pellet was washed with cold 70% ethanol by microfuging for 5 minutes at 4°C and then air-dried. The pellet was resuspended in 6µl of deionized formamide and shaken on a vortex for ±30 minutes. To this, 6µl of dextran sulphate (20% in 4xSSC) was added and the probe was dissolved at 37°C for 30 minutes.

The probe DNA were denatured on a hotplate at 76°C for 5 minutes and then pre-annealed for 15 minutes at 37°C and then immediately placed on ice.

During probe preparation, lyophilised probes are initially dissolved in deionized formamide because DNA that has been

modified with either biotin or digoxigenin, introduces hydrophobic residues to the DNA which dissolve more easily in formamide than in aqueous solutions (Lichter, P.; Cremer, T. 1991b).

2.6.3 Hybridisation

The denatured probe mixture was added to the denatured slides. (Often, especially in the case of dual or two-colour FISH, several probes were combined together at this stage and hybridized onto a single slide. In such cases, however, the volumes of the hybridization mix for each individual probe was adjusted so that the final volume of the combined hybridisation mixture did not exceed 16µl).

A coverslip was placed over the hybridisation area and the edges of the coverslip were sealed with rubber cement and allowed to dry on a hotplate at 37°C for a few minutes. Slides were then placed in a moist chamber overnight at 37°C. (In some cases, the hybridisation was left for up to 48 hours which improved the binding efficiency).

After hybridisation, coverslips were removed and the slides were washed in 50% formamide/2xSSC for 10 minutes at 42°C followed by three washes each of 5 minutes in 50% formamide/2xSSC. Slides were then washed three times each of 5 minutes in 1xSSC (kept at 60°C) at 42°C.

Several parameters affect the hybridisation kinetics of *in situ* hybridisation. Generally, DNA renatures at about Tm -25°C and is fairly independent of pH in the range pH5-9. Phosphate buffers of pH6.5-7.5 are thus frequently used in hybridisation mixtures. The rate of renaturation also depends on the concentration of Na^+ ions in the hybridisation buffer. These ions interact electrostatically with phosphate groups of DNA nucleotides and decrease the electrostatic repulsion between the two strands of DNA, stabilizing it. For this reason, lower Na^+ ion concentrations are preferred. The presence of citrate in the hybridisation buffer prevents the action of free divalent cations which may strongly stabilise the DNA double helix.

The action of deionized formamide has been previously described (section 7.1). Its presence decreases the rate of hybridisation and lowers the melting temperature of DNA so that hybridisation may take place at lower temperatures over a longer period of time. By varying the formamide concentration during the hybridisation step, the specificity of centromeric alphoid repeat probes for their target sequences may be increased. This results in a lowering of background signal noise.

Dextran sulphate in aqueous solution is strongly hydrated. Such hydration prevents probe DNA from accessing water which has the

effect of concentrating the probes and increasing their reannealing rates.

The quality of the target preparation is also an important factor in determining hybridisation efficiency. Hybridisation intensity has been shown to decrease with increased storage of the slides in air as the chromosomes remain compact (Pinkel *et al.*1986) while any residual cytoplasm around metaphase cells will limit probe penetration.

2.6.4 Immunodetection

After hybridisation, the duplexes formed will be a result of perfectly matched sequences and also result from sequences that are less homologous. Before immunodetection can be carried out, post-hybridisation washes are performed on the slides. These washes serve to remove any probe that has hybridized to the less homologous sequences so as to reduce background noise. This may be done by varying the stringency of the washes, through manipulation of the salt concentration, formamide concentration and temperature.

During this step, the specificity of centromeric alphoid repeat probes for their target sequences may be increased by increasing the stringency of the washes (Lichter*et al.* 1991a).

2.6.4.1 Immunodetection of Biotin-labelled Probes

For the detection of biotinylated probes, fluorescein isothiocynanate (FITC) coupled to avidin was used. In order to enhance the signal, an amplification step employing a biotinylated anti-avidin antibody was used followed by the addition once again of avidin-FITC.

Slides were blocked by the addition of 100µl of blocking solution [4xSSC; 3% Bovine Serum Albumin (BSA); 0.1% Tween 20] to the area of hybridisation. A coverslip was applied and the slides were incubated at 37°C in a moist chamber for 30 minutes.

The coverslip was removed and 100µl of avidin-FITC (Vector Laboratories Inc., U.S.A.) [diluted 1:1000 in detection buffer (4xSSC; 1% BSA; 0.1% Tween 20)] (stock concentration of avidin-FITC: 200µg/ml)was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C for 30 minutes in the dark.

Coverslips were removed and slides were washes three times of 5 minutes each in 4xSSC,0.1% Tween 20 (freshly prepared) at 42°C in the dark.

After washing, 100µl of biotinylated anti-avidin antibody (Vector Laboratories Inc.,U.S.A.)(diluted 1:100 in detection buffer) (stock concentration: 200µl/ml) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C for 30 minutes in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in 4xSSC,0.1% Tween 20 at 42°C in the dark.

After washing, 100µl of avidin-FITC, diluted as described above, was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C for 30 minutes in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in 4xSSC,0.1% Tween 20 at 42°C in the dark.

Slides were counterstained with 4,6 Diamidino-2-phenylindole 2HCl (DAPI)(200ng/ml in 2xSSC) for 15 minutes on an orbital shaker in the dark at room T°.

Slides were washed for 2 minutes in 2xSSC,0.05% Tween 20 in the dark at room T° and then mounted in 30µl of Citifluor mounting medium (1% glycerol in PBS).

The signal provided by the avidin-FITC conjugate may be enhanced by repeating the above avidin-FITC/anti-avidin protocol until the desired signal is achieved. However, this procedure increases the background noise level as each amplification step provides approximately a six-fold increase in intensity, each avidin molecule having about six fluorescein molecules attached to it (Pinkel *et al.*1986).

2.6.4.2 Immunodetection of Digoxigenin-labelled Probes

In the detection of digoxigenin-labelled probes, two different protocols were investigated employing two different fluorochromes both emitting a red signal. Protocol 1 involved the use of the fluorochrome Rhodamine, while protocol 2 involved the use of the fluorochrome TRITC. The experimental procedure for the two protocols is the same.

Slides were blocked by the addition of 100µl of blocking solution TNB [0.1M Tris-HCl pH7.2; 0.15M NaCl; 0.5% blocking reagent (Boehringer Mannheim,Germany)] to the area of hybridisation. A coverslip was applied and the slides were incubated at 37°C in a moist chamber for 30 minutes.

The coverslip was removed and 100µl of mouse monoclonal anti-Dig

antibody (Boehringer Mannheim,Germany)(diluted 1:200 in TNB)(stock concentration: 200µg/ml) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C for 30 minutes. This step was the same for both protocols 1 and 2.

Coverslips were removed and slides were washed three times of 5 minutes each in TNT (0.1M Tris-HCl pH7.2; 0.15M NaCl; 0.05% Tween 20- freshly prepared).

Protocol 1: After washing, 100µl of sheep anti-mouse Ig-DIG (Boehringer Mannheim,Germany)(diluted 2:100 in TNB)(stock concentration: 200µg/ml) was added to the slides.

Protocol 2: After washing, 100 µl of rabbit anti-mouse TRITC antibody (Sigma Diagnostics, U.S.A.)(diluted 2:100 in TNB)(stock concentration: 200µg/ml) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in TNT at 42°C in the dark.

Protocol 1: After washing, 100µl of anti-dig-RHODAMINE antibody (Boehringer Mannheim,Germany)(diluted 2:100 in TNB)(stock concentration: 200µg/ml) was added to the slides.

Protocol 2: After washing, 100µl of goat anti-rabbit TRITC antibody (Sigma Diagnostics, U.S.A.)(diluted 4:100 in TNB)(stock concentration 200µg/ml was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated in a moist chamber at 37°C in the dark.

Coverslips were removed and slides were washed three times 5 minutes each in TNT at 42°C in the dark.

Slides were counterstained with 4,6 Diamidino-2-phenylindole 2HCl (DAPI)(200ng/ml in 2xSSC) for 15 minutes on an orbital shaker in the dark at room T°.

Slides were washed for 2 minutes in 2xSSC;0.05% Tween 20 in the dark at room T° and then mounted in 30µl of Citifluor mounting medium (1% glycerol in PBS).

2.6.4.3 Immunodetection For Dual FISH

The technique of dual FISH or two-colour FISH refers to the simultaneous hybridisation of both biotin-labelled probes and digoxigenin-labelled probes to the same slide. Immunodetection of these labels proceeded as described in section 6.4.2. but

antibodies specific for the two labels were prepared together in the same detection buffer and concentrations were adjusted according to the number of slides. The biotin immunodetection was applied with digoxigenin immunodetection protocols 1 and 2.

Slides were blocked by the addition of 100µl of blocking solution TNB to the area of hybridisation. A coverslip was applied and the slides were incubated at 37°C in a moist chamber for 30 minutes.

After washing, 100µl of mouse monoclonal anti-Dig antibody + avidin-FITC (diluted 1:200 and 1:1000 respectively in TNB) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated at 37°C in a moist chamber for 30 minutes in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in TNT at 42°C in the dark.

After washing, 100µl of sheep anti-mouse Ig-DIG + biotinylated anti-avidin antibody (diluted 2:100 and 1:100 in TNB respectively)
or 100µl of rabbit anti-mouse TRITC + biotinylated anti-avidin antibody (diluted 2:100 and 1:100 in TNB respectively) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated at 37°C in a moist chamber in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in TNT at 42°C in the dark.

After washing, 100µl of anti-dig-RHODAMINE antibody + avidin-FITC (diluted 2:100 and 1:1000 respectively in TNB) or 100µl of goat anti-rabbit TRITC + avidin-FITC (diluted 4:100 and 1:1000 respectively in TNB) was added to the slides. A coverslip was applied to the hybridisation area and slides were incubated at 37°C in a moist chamber in the dark.

Coverslips were removed and slides were washed three times of 5 minutes each in TNT at 42°C in the dark.

Slides were counterstained with 4,6 Diamidino-2-phenylindole 2HCl (DAPI)(200ng/ml in 2xSSC) for 15 minutes on an orbital shaker in the dark at room T°.

Slides were washed for 2 minutes in 2xSSC;0.05% Tween 20 in the dark at room T° and then mounted in 30µl of Citifluor mounting medium (1% glycerol in PBS).

2.7 FLUORESCENCE MICROSCOPY

Slides were viewed by fluorescence microscopy using an Olympus BH2-RFCA microscope. FITC signals were detected using a FITC filter. Rhodamine and TRITC signal were detected using a TRITC filter and for dual FISH a triple filter was used. Cells were photographed using Scotch CHROME 640-T Colour Slide film.

2.8 GENE DOSAGE STUDIES

This study was undertaken to determine whether or not candidate genes involved in the translocation on chromosome 11 were either deleted, present in normal dosage or amplified. For the purposes of this study, DNA extraction and Slot Blot Assay techniques were employed.

Cell lines UWOV2 and UWOV2(Sf) were tested in this study. Human peripheral blood lymphocyte DNA was used as a control.

The probe used in this study was the human IGF-II probe mapping to chromosome 11p15.5. As a control, the β-actin gene probe pHFβA-1 was used. This cDNA probe represents a 1.9-2.1 kb insert of the human fibroblast cytoplasmic β-actin gene mapping to chromosome 7 (Ponte, H.*et al.*1984).

2.8.1 DNA Extraction

Cell cultures of UWOV2 and UWOV2(Sf) were grown until confluent. The supernatant was removed and cells were exposed to 0.25% trypsin in PBS at 37°C for 5-15 minutes until the cells lifted off the flask. Cells were washed in Hank's Balanced Salts Solution by centrifugation at 1400rpm for 10 minutes at room T°.

Human peripheral blood was collected and layered onto a 100% Hypaque-Ficoll-1077 solution (Sigma Diagnostics ,U.S.A.) and centrifuged at 1750rpm for 30 minutes in a Beckman GS-6R benchtop centrifuge. The A band was collected and washed twice in Hanks Balanced Salt Solution.

The pellets were resuspended in Amniocyte Buffer (50mM Tris-HCl pH7.6, 100mM NaCl, 1mM EDTA pH8.0, 0.5% SDS). To this, 0.05mg of proteinase K (Boehringer Mannheim,Germany) was added and cells were incubated at 56°C for 4 hours.

2.8.1.1 Phenol/Chloroform Extraction

After incubation, 0.5 vol of phenol (Appendix A) and 0.5 vol of chloroform (chloroform:isoamylalcohol 24:1) was added. Tubes were shaken and centrifuged at 3000rpm for 15 minutes at room T°. The aqueous phase was retained and the extraction procedure was repeated for a minimum of three washes until the debris at the

interphase had been removed. An equal volume of chloroform was added to the aqueous phase. The tubes were shaken and centrifuged at 3000rpm for 5 minutes at room T°. The aqueous phase was removed and the DNA was precipitated by the addition of $1/_{20}$vol of sodium acetate pH4.6 and 2½vol ice-cold ethanol. The mixture was incubated at -70°C for 15 minutes and centrifuged at 5000rpm for 15 minutes. The pellet was rinsed in 70% cold ethanol and the pellet was air-dried.

The pellet was resuspended in TE pH7.6 and stored at 4°C.

2.8.2 SLOT BLOT ASSAY

2.8.2.1 DNA Preparation

DNA samples of UWOV2, UWOV2(Sf) and peripheral blood lymphocytes were adjusted so that the final concentration for each sample was the same. Aliquots of 75ng, 150ng, 300ng and 600ng of DNA were prepared and made up to a final volume of 200µl in 10N NaOH + 0.5M EDTA.

2.8.2.2 Blot Preparation

Hybond-N paper (Amersham, U.K.) was cut to the exact dimensions of the blotting apparatus. For each probe that was to be tested, a single membrane was prepared. In addition to the membrane, two pieces of Whatmann filter paper were prepared having the same dimensions as the membrane. The filter paper and membrane were dipped into 20xSSC and then placed into the blotting apparatus. A vacuum was passed through the membrane until all excess liquid had been removed.

2.8.2.3 Blotting Procedure

The 200µl DNA samples were denatured by heating on a hotplate at 95°C for 5 minutes and then loaded into wells of the blotting apparatus. A vacuum was passed through the membrane and then the wells were washed three times by the addition of 400µl of 20xSSC and between each wash, a vacuum was passed through the membrane. After this, the blots were removed, air-dried and then baked at 80°C for 2 hours.

2.8.2.4 Probe Labelling

Probes IGF-II and β-actin were prepared by placing 50ng of probe DNA into a 45µl volume of TE pH 7.6.

Labelling of probe DNA with P^{32} was carried out using the Rediprime random primer labelling kit (Amersham, U.K.). Briefly, 45µl of probe DNA was added to 5µl of [32-P] dCTP and mixed by

gently pipetting. The tubes were incubated for 10 minutes at 37°C in order for the label to be incorporated. The labelled probes were purified by elution through Sephadex G-50 columns as described in section 2.5.1.

The samples were then tested for radioactivity using a Geiger counter.

2.8.2.5 Prehybridisation of the Blots

Blots were prehybridised in prehybridisation solution [5xSSPE (Appendix A), 5x Denhardt's solution, 0.5% (w/v) SDS, pre-heated to 65°C]. To this, 0.5ml of a 1mg/ml solution of denatured salmon testes DNA (Sigma Diagnostics, U.S.A.) was added. Denaturation of the salmon testes DNA was by boiling for 5 minutes and then chilling on ice. Blots were prehybridized for 1 hour at 65°C.

2.8.2.6 Hybridisation

Blots were removed from the prehybridization solution and placed into hybridisation bags (GibcoBRL, U.S.A.) and hybridisation solution [5xSSPE, 5x Denhardt's solution, 0.5% (w/v) SDS, 0.5ml denatured salmon testes DNA (1mg/ml) was added.

Labelled probes were denatured by boiling for 5 minutes and were then added to the hybridisation mixture. The bags were sealed and incubated overnight at 65°C.

2.8.2.7 Post-hybridisation Washes

After hybridisation, blots were removed and washed twice in 2xSSPE, 0.1% (w/v) SDS at room T° for 10 minutes each followed by one wash in 1xSSPE, 0.1% (w/v) SDS at 65°C for 15 minutes.

2.8.2.8 Autoradiography

After washing, blots were sealed in plastic and autoradiography was carried out by exposure to X-ray film at -70°C for one week.

SECTION 3.0

RESULTS

3.1 Cytogenetic Analysis of Cell Line UWOV2(Sf)

Previously, cytogenetic analysis of cell line UWOV2(Sf) revealed the presence of two marker chromosomes harbouring p arm abnormalities being on chromosomes 1 and 11 (Golombick *et al.*1990). The chromosomal origin of this additional material was not fully elucidated. This present study confirms these previous reports but the chromosome 1 abnormality appeared to involve the q arm.

In this study, cytogenetic analysis of G-T-G banded slides revealed several other structural and numerical clonal abnormalities which were present in every cell analysed (Figure 2). The structural abnormalities included a 1q+, 7q-, 8q+, 11p+, 14q+ and 16q-. The numerical abnormalities observed included trisomy 5, trisomy 8, trisomy 12, trisomy 14, partial trisomy 7, monosomy 15, monosomy 18, and monosomy X. Several marker chromosomes of unknown origin were also observed. Cytogenetically, the chromosomal origins of the 1q+ and 8q+ abnormalities were unclear while the 14q+ appeared to be composed of chromosome 14 material.

The chromosomal origin of the 11p+ abnormality could not be determined cytogenetically, however, the breakpoint of the abnormality appeared to involve the region 11p15. The representative karyotype for this cell line as determined by this study is as follows:

```
50-56,X, -X, add(1)(q42), t(3;10)(p12-14;q11), +5,
         del(7)(q21q36), +8, add(8)(q23), add(8)(q23),
         der(11)t(11;?)(p15;?), +12, add(14)(q32), -15,
        del(16)(q24), -18, mar
```

3.2 Cytogenetic Analysis of Parent Cell Line UWOV2

Previously, cytogenetic studies on the parent cell line UWOV2 revealed the presence of an 11p+ abnormality considerably larger than that found in the subline UWOV2(Sf). The chromosomal origin of this marker was unknown (Golombick *et al.*1990). Cytogenetic analysis of G-T-G banded slides in this present study revealed the additional material to be composed entirely of chromosome 3 (Figure 3) thus implying a t(3;11) translocation. Cytogenetically, the breakpoints of this translocation appeared to involve the regions 11p15 and 3p12-14 respectively. It was not possible however, to determine whether or not the chromosome 11 abnormalities in the two cell lines arose independently of one another.

Full karyotypic data on this cell line was difficult to obtain as cells were often polyploid in number and clumped making an accurate analysis difficult and thus a representative karyotype was not obtained.

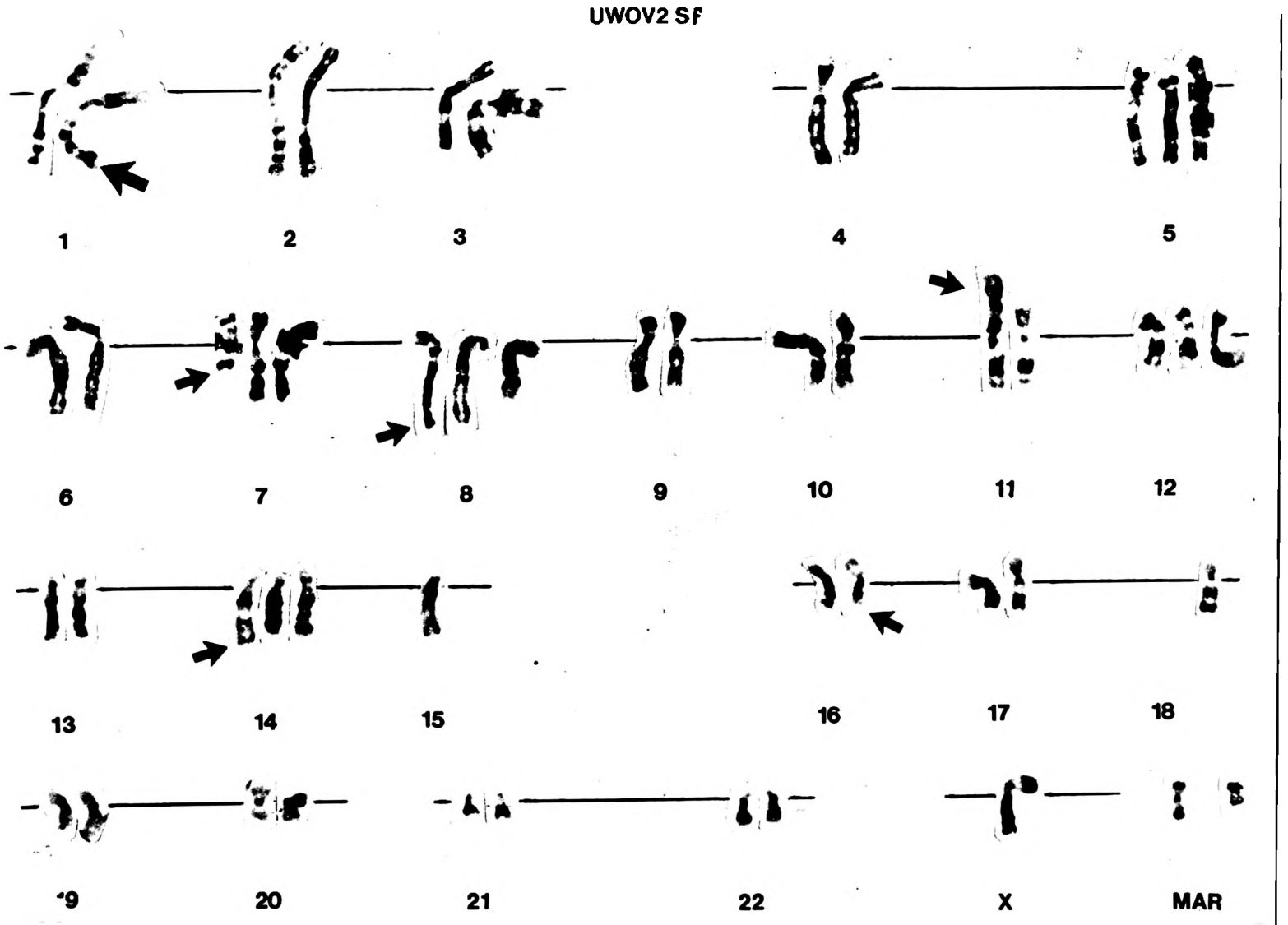


Figure 2: G-T-G-banded karyotype of a metaphase cell from cell line UWOV2(Sf) showing the clonal structural abnormalities (arrows).

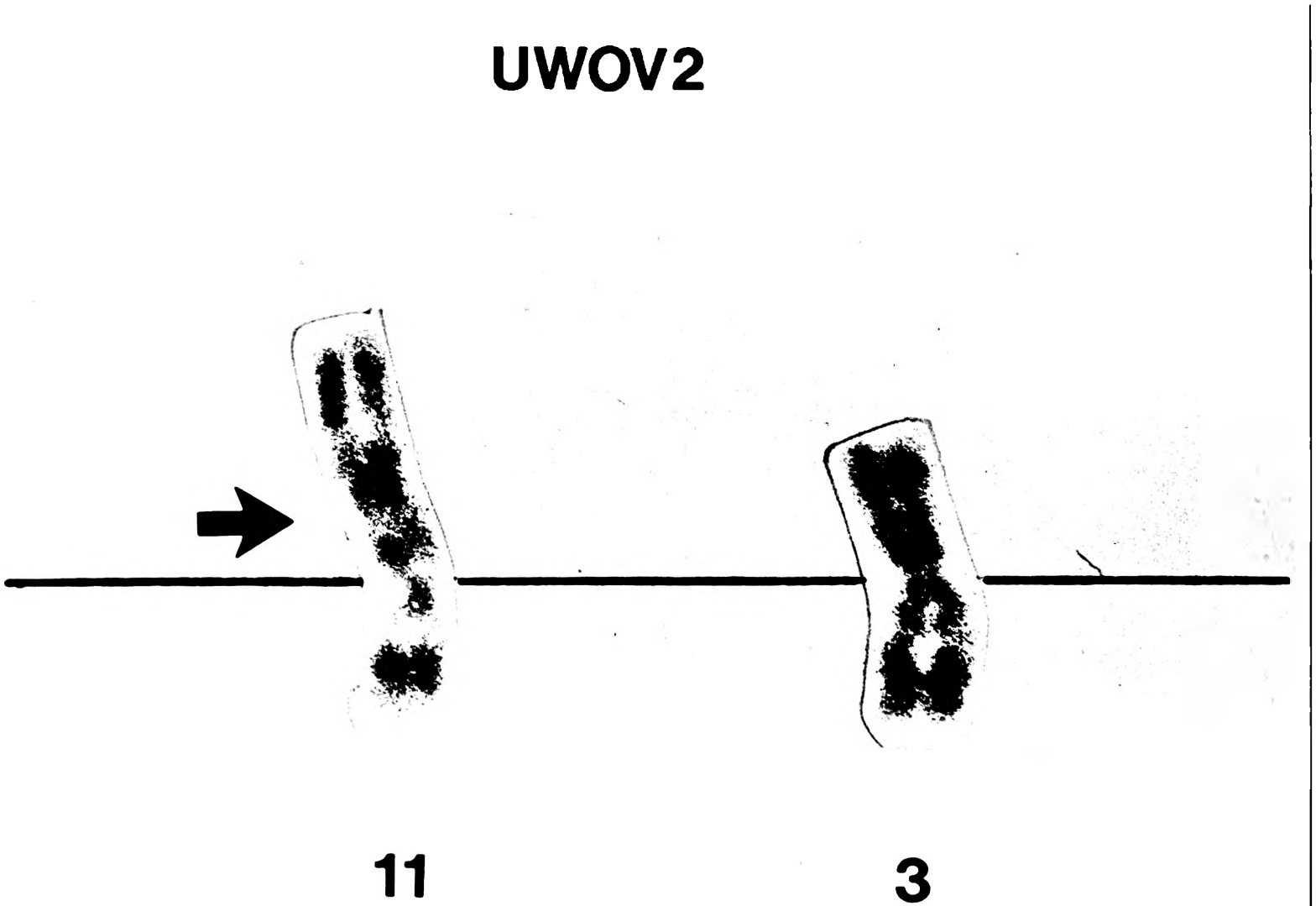


Figure 3: Partial G-T-G-banded karyotype from cell line UWOV2 showing the chromosome 11 abnormality der(11)t(3;11) and a cytogenetically normal chromosome 3.

3.3 Cytogenetic Analysis of Fresh Tumour Material

Karyotypic information on fresh tumour material used in this study was difficult to obtain. In the cases of tumours T2 and T4, no information was obtained due to difficulties in isolating a pure cell culture. Firstly, after 16-24 hours in culture there appeared to be an overgrowth of fibroblast cells. Such contamination by fibroblast cells can usually be removed by differential trypsinization but such a procedure requires long-term culture conditions which could have introduced further genetic variation into the cells. Secondly, the seeding density of tumour cells is important in selecting for a pure cell population. In the cases of tumours T2 and T4, the seeding densities were low due to an insufficient amount of specimen material. Thus all metaphase cells analysed from these tumours appeared to be cytogenetically normal and of fibroblastoid origin.

Cytogenetic analysis of tumour T5 revealed a clonal abnormality involving a deletion on the long arm of chromosome 6. The abnormality appeared to involve the region 6q21. A single giant marker chromosome of unknown origin was also present in several but not all cells (Figure 4a) while additional markers were observed in other cells (Figure 4b). Cytogenetically, chromosome 11 appeared normal and was not involved in any structural or numerical abnormalities. The representative karyotype for tumour T5 is as follows:

44,XX, del(6)(q21q27) 2-3 mar

3.4 Cytogenetic Analysis of Ascitic Fluid Cultures

Ascitic fluid cultures AF-1, AF-2, AF-3 and AF-5 showed an abundance of fibroblastoid-like cells after 24 hours in culture (Figures 5a-d). Harvests of AF-1, AF-2 and AF-3 did not yield any cytogenetically analysable metaphases.

Ascitic fluid culture AF-5 was the only cytogenetically analysable culture yielding a perfect karyotype of 46 XX in all cells analysed (results not shown). This could be representative of a normal fibroblast cell.

3.5 FISH Studies on Cell Line UWOV2(Sf)

Results of FISH studies using chromosome libraries to identify the chromosomal origin of the 11p+ abnormality are summarised in Table II. In each case, a minimum of twenty cells were analysed for consistency. The result of FISH studies using the chromosome 11 library probe may be seen in Figure 6.

The FISH results showed that the marker chromosome 1q+ abnormality was derived from chromosome 1 material (results not shown). The trisomy 5 and trisomy 12 observed cytogenetically were confirmed in these FISH studies. FISH results using the chromosome 7 library revealed that the chromosome 7q deletion did not appear to be as a result of a translocation event, as the deleted portion appeared to be lost from the cell line (results not shown).

CHROMOSOME LIBRARY	HYBRIDISATION SIGNAL WITH MARKER CHROMOSOME 11	OTHER CHROMOSOMAL ABNORMALITIES
chromosome 1	-	1q+
chromosome 3	+	der(10)t(3;10) der(8)t(3;8) der(11)t(3;8;11)
chromosome 5	-	+5
chromosome 6	-	
chromosome 7	-	partial trisomy 7; 7q-
chromosome 8	+	+ 8; der(8)t(3;8) der(11)t(3;8;11)
chromosome 9	-	
chromosome 10	-	der(10)t(3;10)
chromosome 11	+	der(11)t(3;8;11)
chromosome 12	-	+12
chromosome 13	-	
chromosome 14	-	+14; 14q+
chromosome 15	-	-15
chromosome 16	-	16q-
chromosome 17	-	
chromosome 19	-	
chromosome 21	-	
chromosome 22	-	
chromosome X	-	-X

Table II: Results showing the hybridisation signal between the chromosomal libraries and the marker chromosome 11 as determined by Fluorescence *in situ* hybridisation studies with cell line UWOV2(Sf).

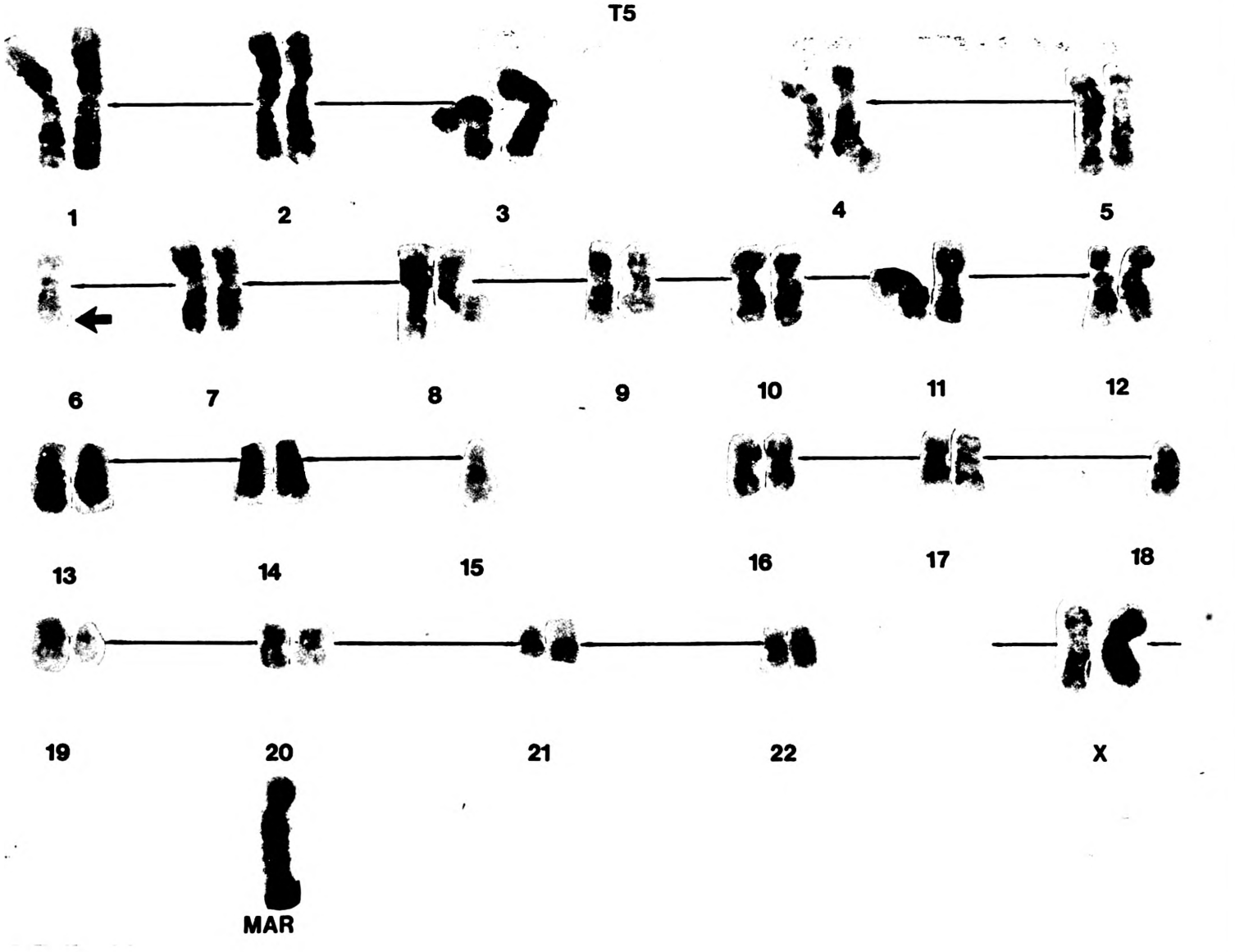


Figure 4a: G-T-G-banded karyotype of a metaphase cell from specimen T5 showing the abnormality del(6)(q21q27) (arrow) and a single giant marker chromosome (MAR).

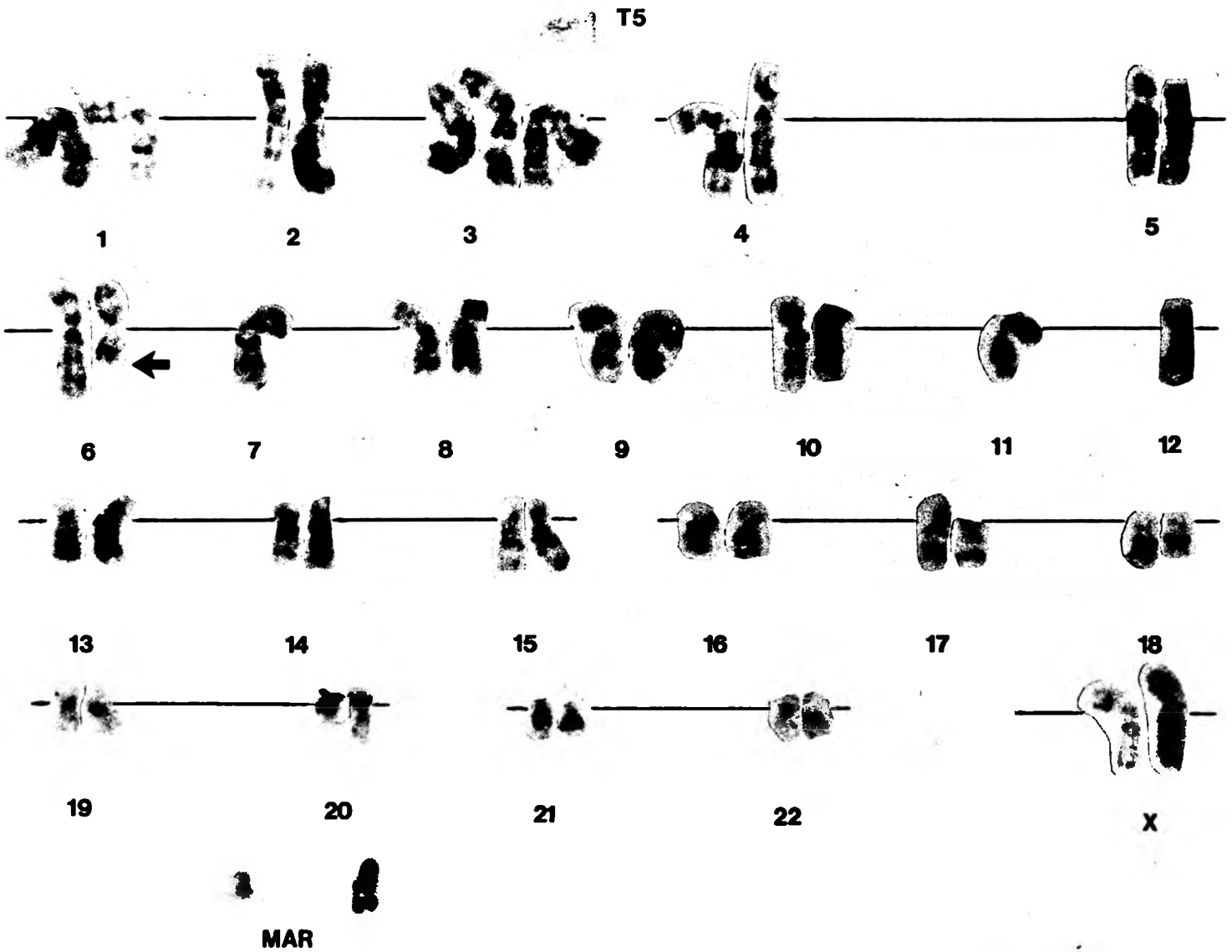


Figure 4b: G-T-G-banded karyotype of a metaphase cell from specimen T5 showing the abnormality del(6)(q21q27) (arrow). Two marker chromosomes are present.



Figure 5a: Cells from ascitic fluid culture AF-1 showing a fibroblastoid-like morphology (Giemsa stain x400).



Figure 5b: Cells from ascitic fluid culture AF-2 showing a fibroblastoid-like morphology (Giemsa stain x400).

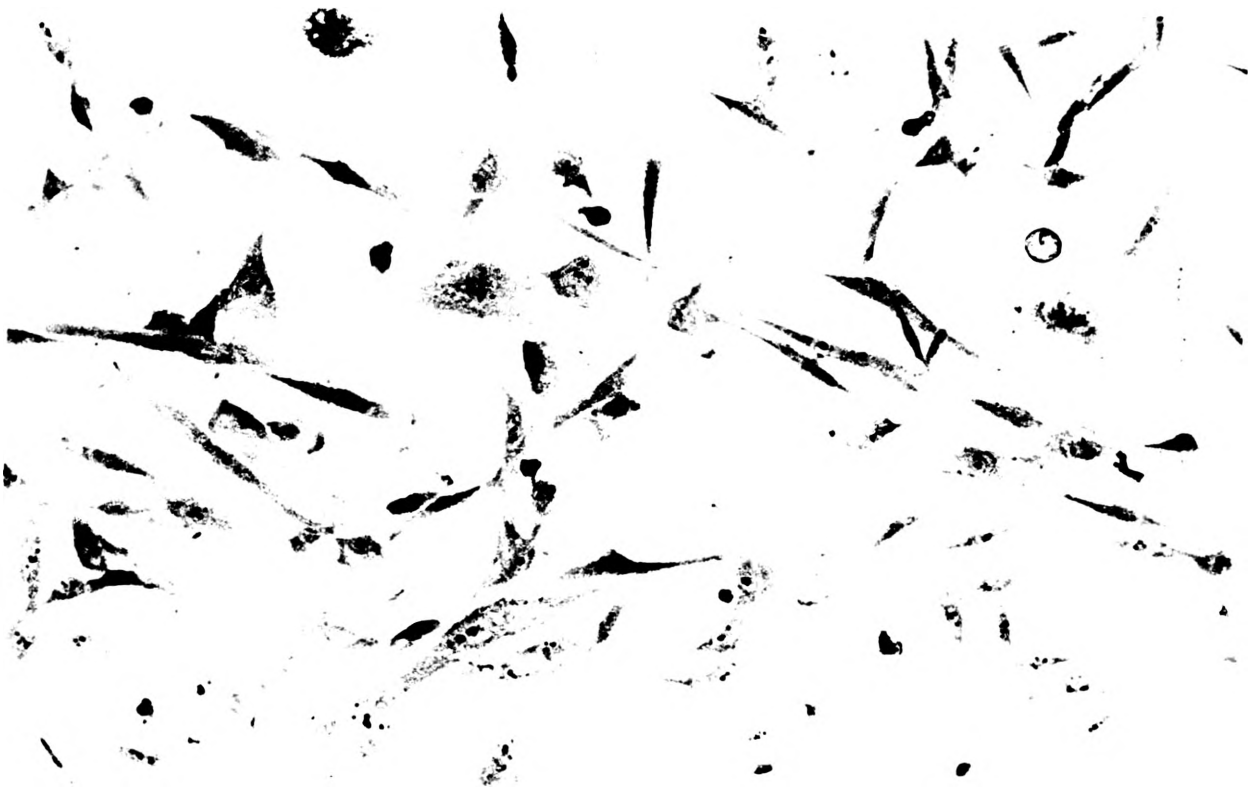


Figure 5c: Cells from ascitic fluid culture AF-3 showing a fibroblastiod-like morphology (Giemsa stain x400).



Figure 5d: Cells from ascitic fluid culture AF-5 showing a fibroblastoid-like morphology. Multinucleated cells are present (arrow) (Geimsa stain x400).

Studies using the chromosome 3 library probe showed that chromosome 3 was involved in several structural abnormalities including positive hybridisation to the proximal portion of the chromosome 11p+ abnormality. The additional material on chromosome 8 (as detected cytogenetically) was found to be of chromosome 3 origin, and the t(3;10) observed cytogenetically was also confirmed using FISH (Figure 7).

Analysis of chromosome 8 revealed that it was involved in a translocation event with chromosome 3 and hence the 8q+ detected cytogenetically. This t(3;8) was present in two of the three copies of chromosome 8 present in each cell. After screening using single colour FISH, dual FISH was used to confirm the t(3;8) event. It also showed positive hybridisation to the distal portion of the chromosome 11p abnormality (Figure 8).

The 11p+ abnormality was thus found to consist of derivatives from chromosomes 3 and 8 indicating a complex translocation t(3;8;11) the distal portion of the marker being of chromosome 8 origin and the proximal portion being of chromosome 3 origin (figure 9). The breakpoint of the t(3;11) could not be accurately determined from this screening study while the t(3;8) appeared to involve the regions 8q22-24 on chromosome 8 and 3q12-21 on chromosome 3.

The chromosome 14q+ abnormality showed positive hybridisation with the chromosome 14 library probe (figure 10) and appeared to involve the region 14q32. Chromosome 16 was consistently deleted in the region 16q24 in all cells analysed (results not shown).

Based on the FISH results obtained in this study, a revised karyotype of cell line UWOV2(Sf) may be described as follows:

50-56,X, -X, add(1)(q42), der(10)t(3;10), +5, del(7)(q21q36), +8, der(8)t(3;8)(q12-21,q22-24), der(8)t(3;8)(q12-21;q22-24), der(11)t(3;8;11)(p12-14;q22-24;p15), +12, add(14)(q32), -15, del(16)(q24), -18, mar

3.6 FISH Studies on Cell Line UWOV2

After the chromosomal origin of the 11p+ abnormality in cell line UWOV2(Sf) was elucidated, the abnormality in the parent line UWOV2 was screened using chromosome 3 and chromosome 8 library probes. This abnormality was found to be composed entirely of chromosome 3 material and was negative for chromosome 8 material, thus confirming the cytogenetic findings. Chromosome 3 did not appear to be involved in any other abnormalities and two normal copies of chromosome 3 were present in each cell in addition to the t(3;11).

A chromosome 8α centromeric probe was used to determine the copy number of chromosome 8 in this cell line. FISH studies revealed

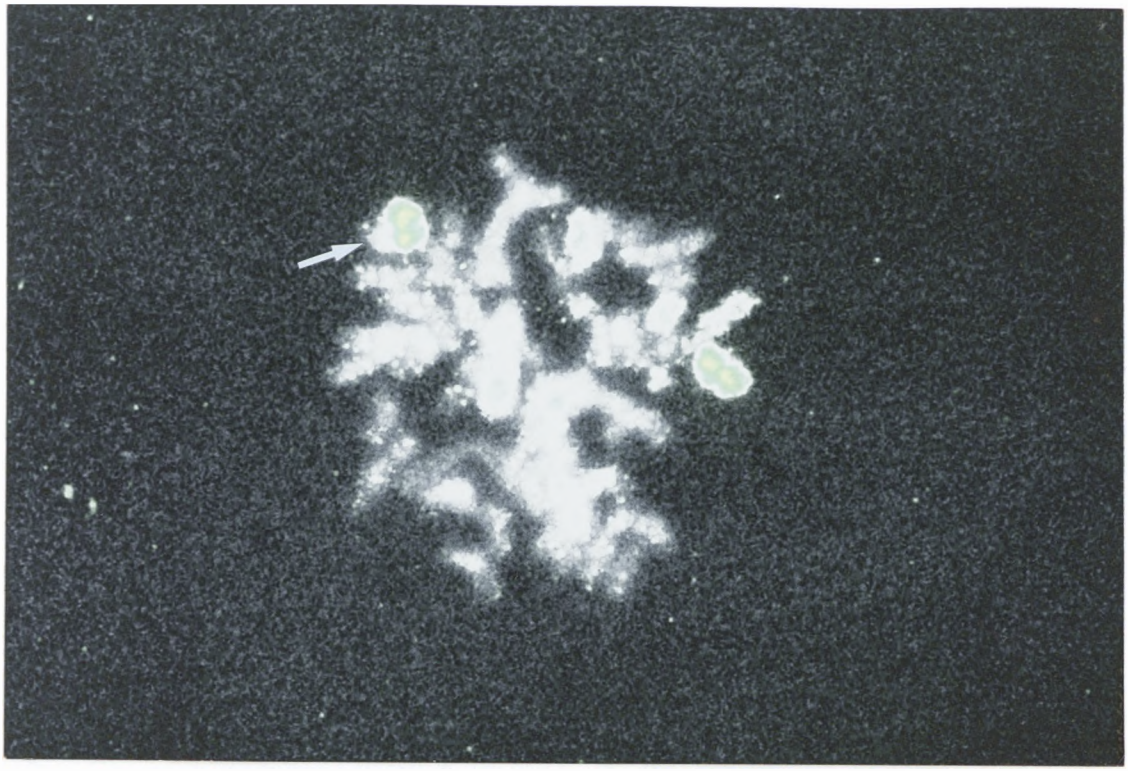


Figure 6: Biotinylated whole chromosome 11 library probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing the 11p+ abnormality (arrow) and a normal chromosome 11 (DAPI counterstain x1000).

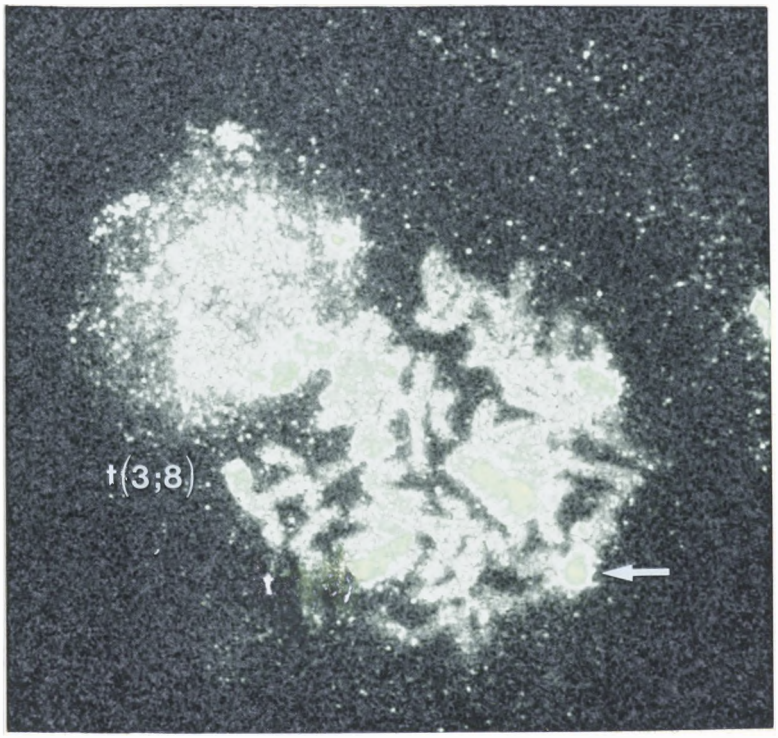


Figure 7: Biotinylated chromosome 3 library probe and biotinylated chromosome 11 centromeric probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing positive hybridization of chromosome 3 material to the distal portion of the 11p+ abnormality (arrow). Several other structural abnormalities involving chromosome 3 are present (DAPI counterstain, FITC filter x1000).

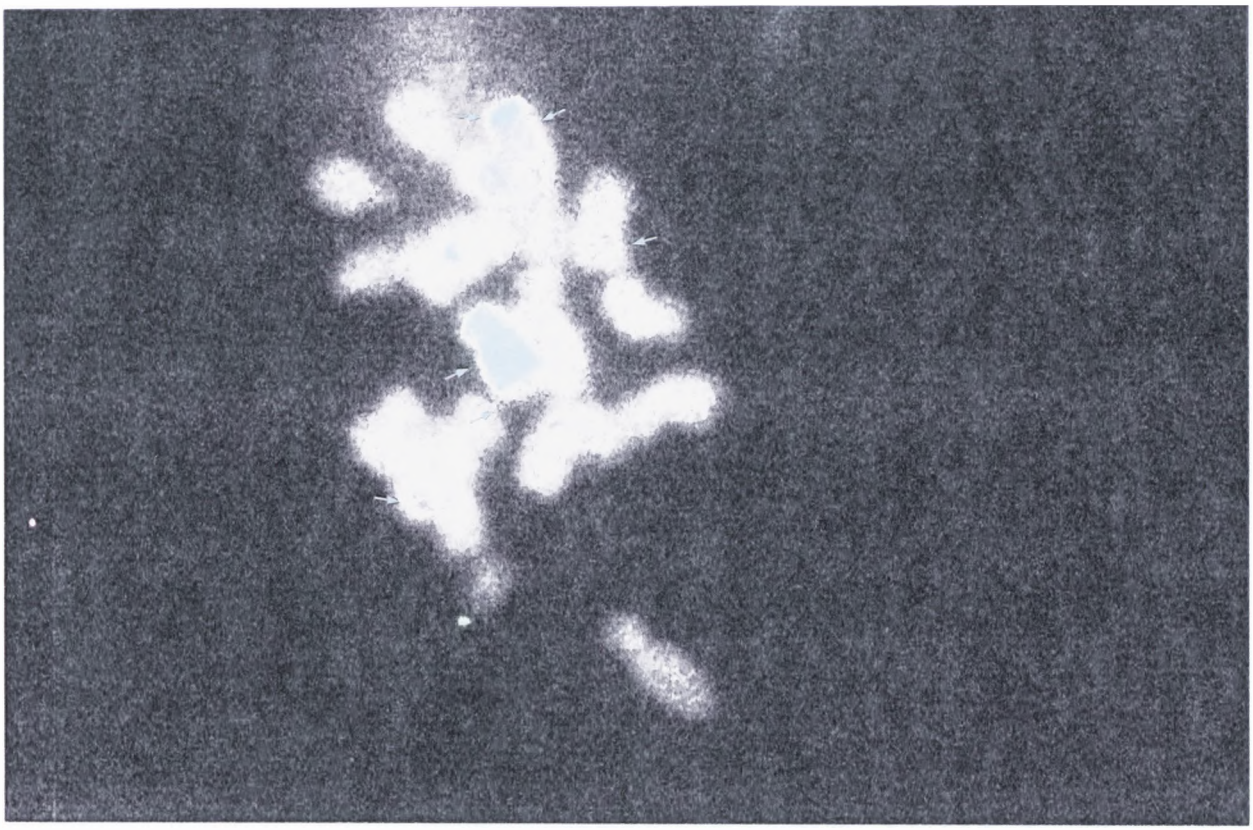


Figure 8: Dual FISH with biotinylated chromosome 8 library probe and DIg-labelled chromosome 3 library probe on a metaphase cell from cell line UWOV2(Sf) showing the t(3;8) and positive hybridization of the chromosome 8 probe and feint hybridization of the chromosome 3 probe to the distal portion of the 11p+ abnormality (arrows) (DAPI counterstain, triple filter x1000).

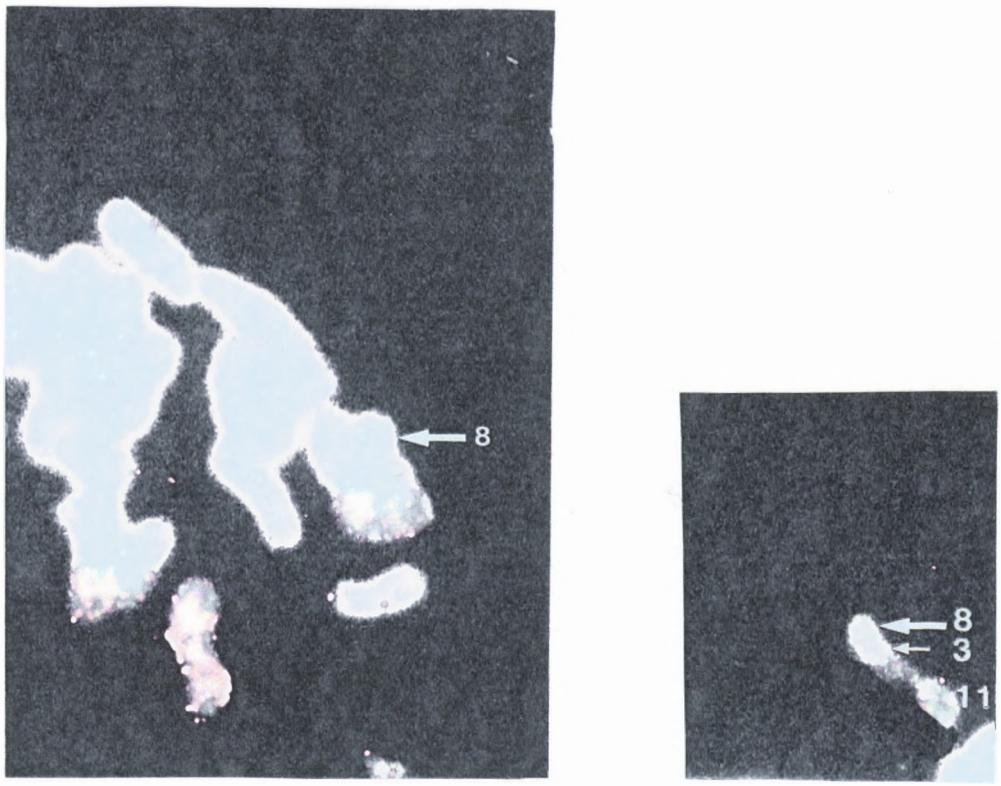


Figure 9: Dual FISH with a chromosome 8 library probe (FITC signal) and a chromosome 11 library probe (Rhodamine signal) to a metaphase cell of cell line UWOV2(Sf) showing chromosome 8 material on the distal portion of the 11p+ abnormality (DAPI counterstain, triple filter x1000).

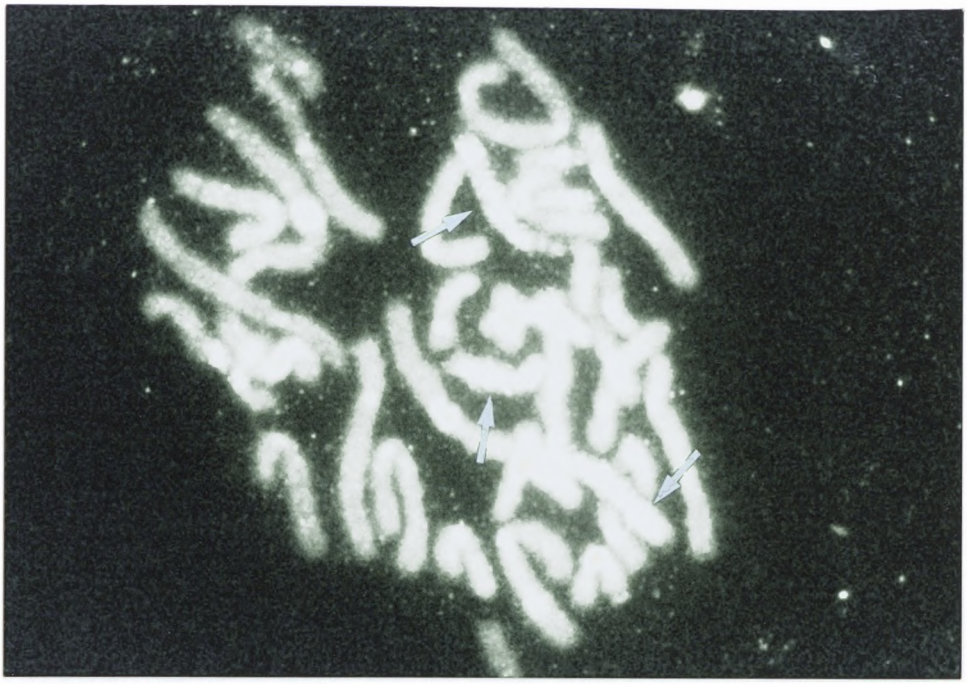


Figure 10: Biotin-labelled chromosome 14 library probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing trisomy 14 (arrows) and 14q+ (DAPI counterstain, FITC filter x1000).

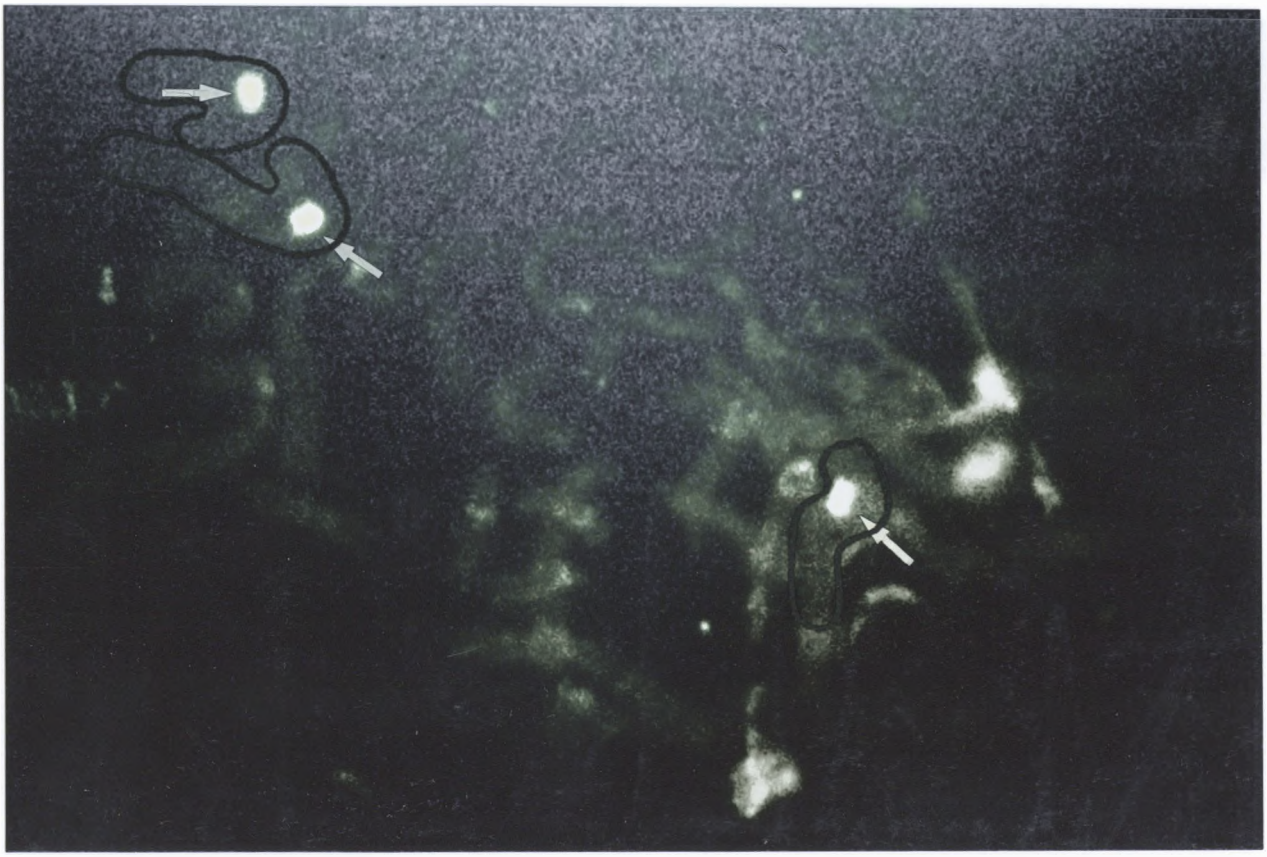


Figure 11: Biotin-labelled chromosome 8 centromeric probe hybridized to a metaphase cell of cell line UWOV2 showing three cytogenetically normal copies of chromosome 8 (arrows) (DAPI counterstain, FITC filter x1000).

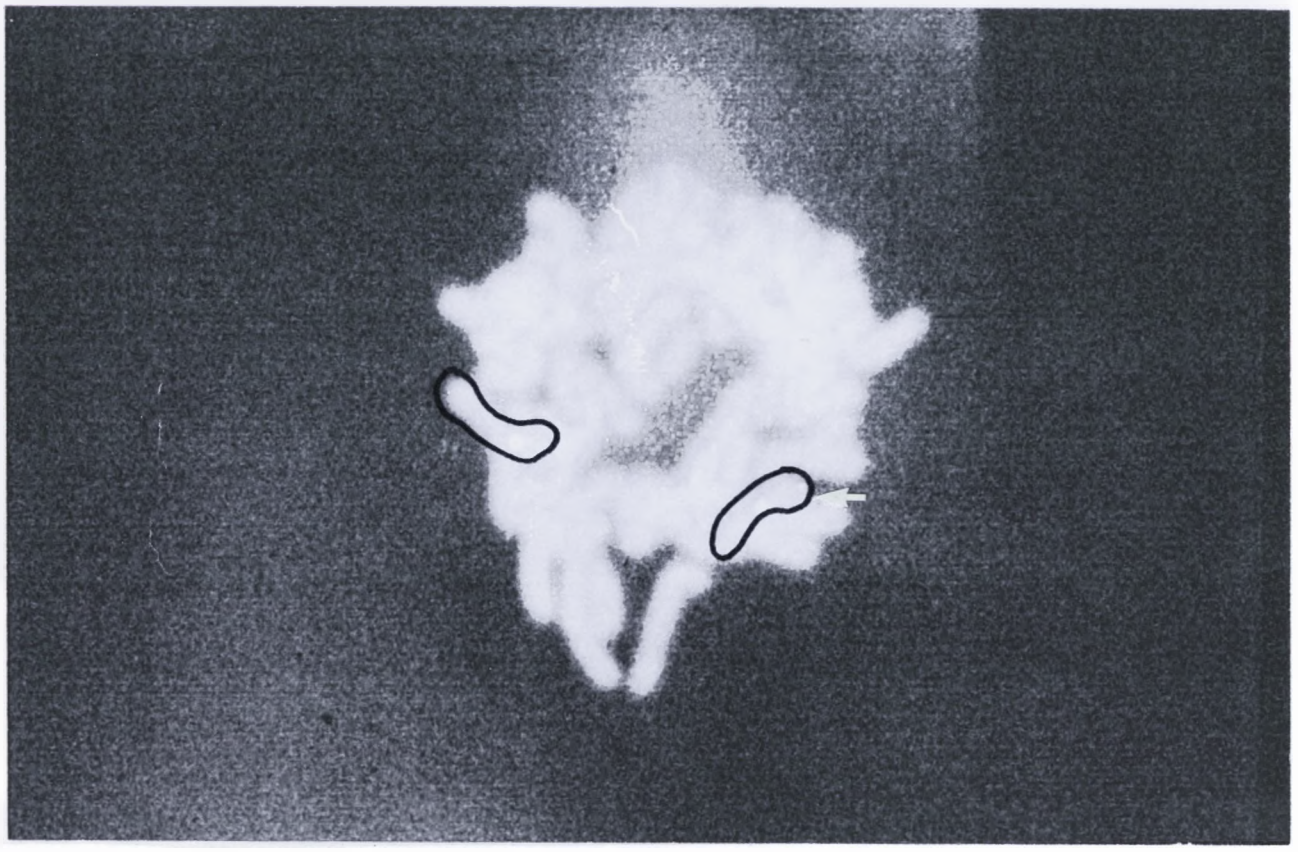


Figure 12: Biotin-labelled chromosome 11 centromeric probe and pSBβ gene probe hybridized to a metaphase cell of cell line UWOV2(Sf) showing positive hybridization of pSBβ to the normal chromosome 11 (arrow) (DAPI counterstain, triple filter x1000).

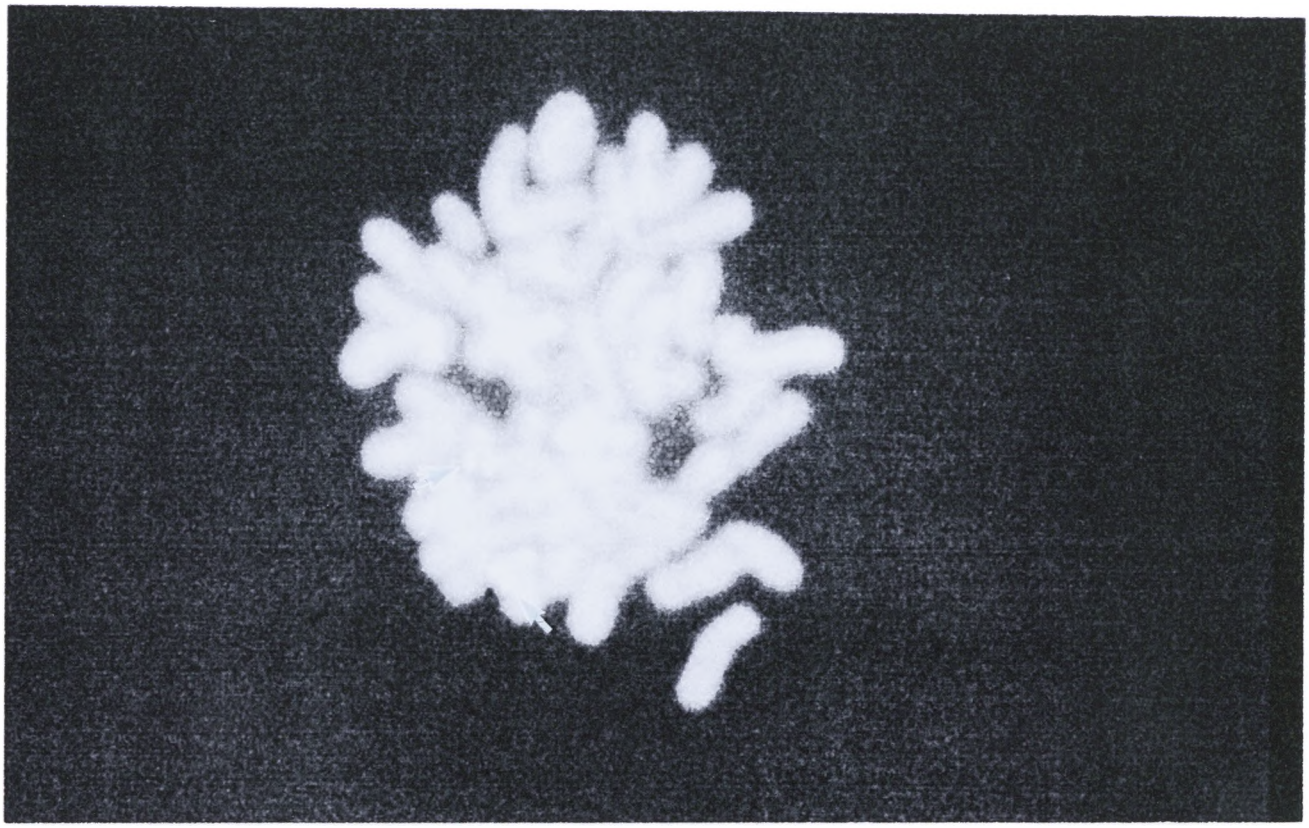


Figure 13: Biotin-labelled YAC probe 847a12 hybridized to a normal metaphase cell showing FITC signals at the distal portion of chromosome 11p15 (arrows) (DAPI counterstain, triple filter x1000).

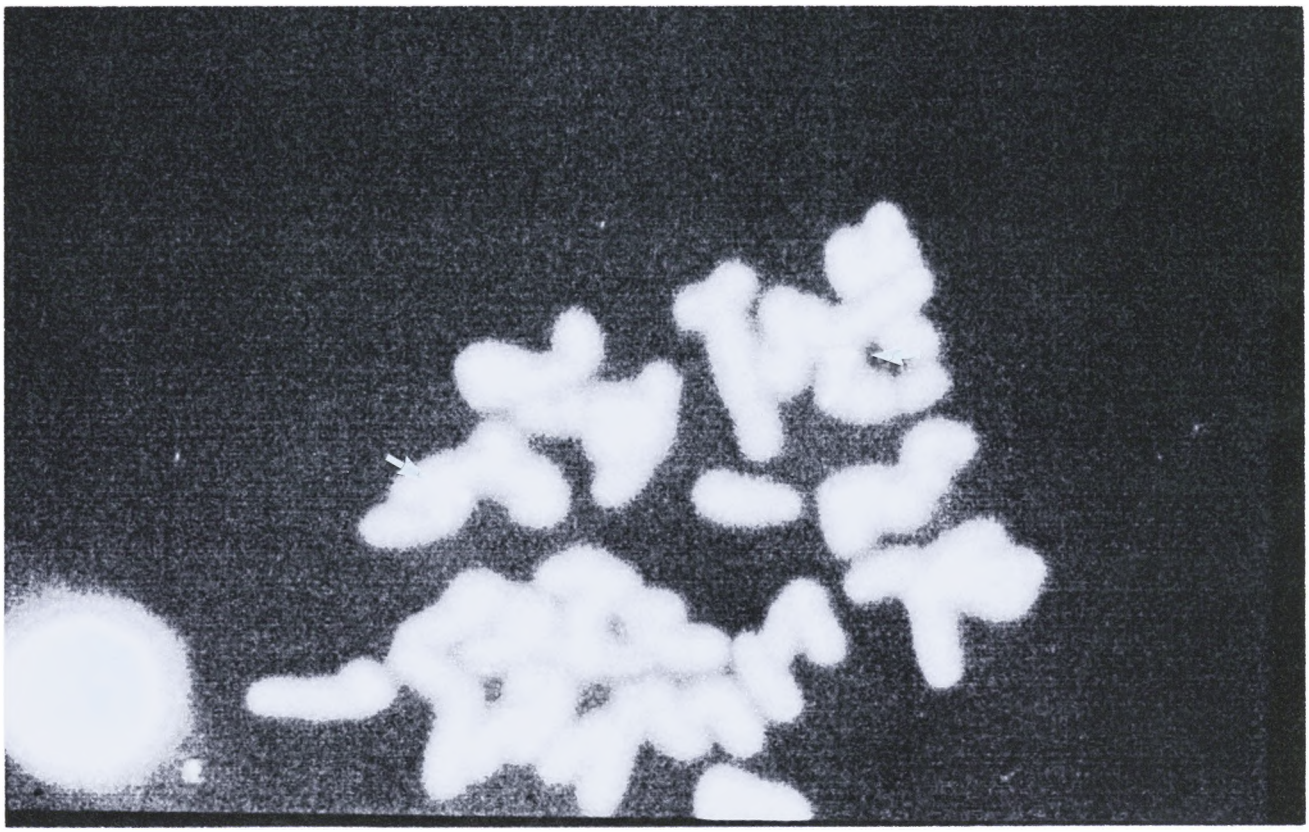


Figure 14: Biotin-labelled YAC probe 954f4 hybridized to a normal metaphase cell showing FITC signals at the distal portion of chromosome 11p15 (arrows) (DAPI counterstain, triple filter x1000).

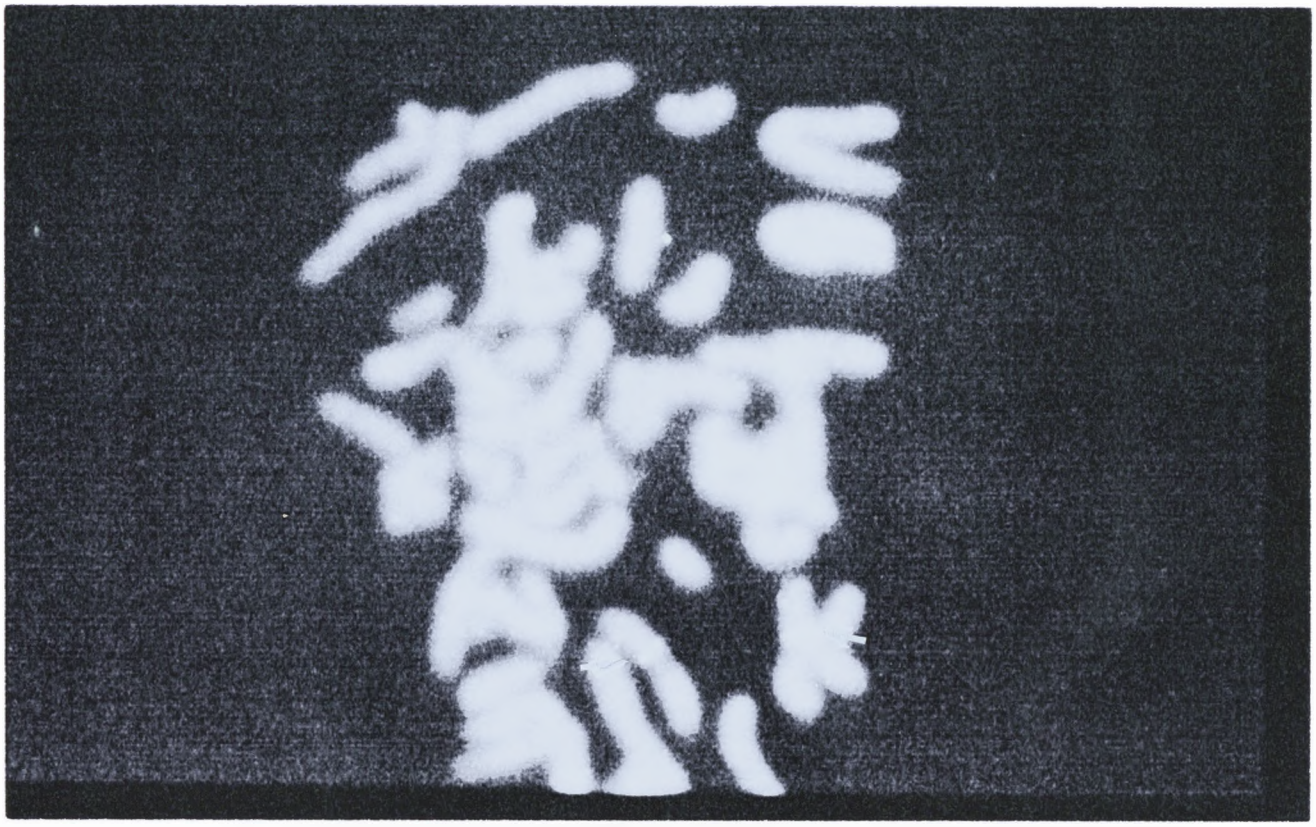
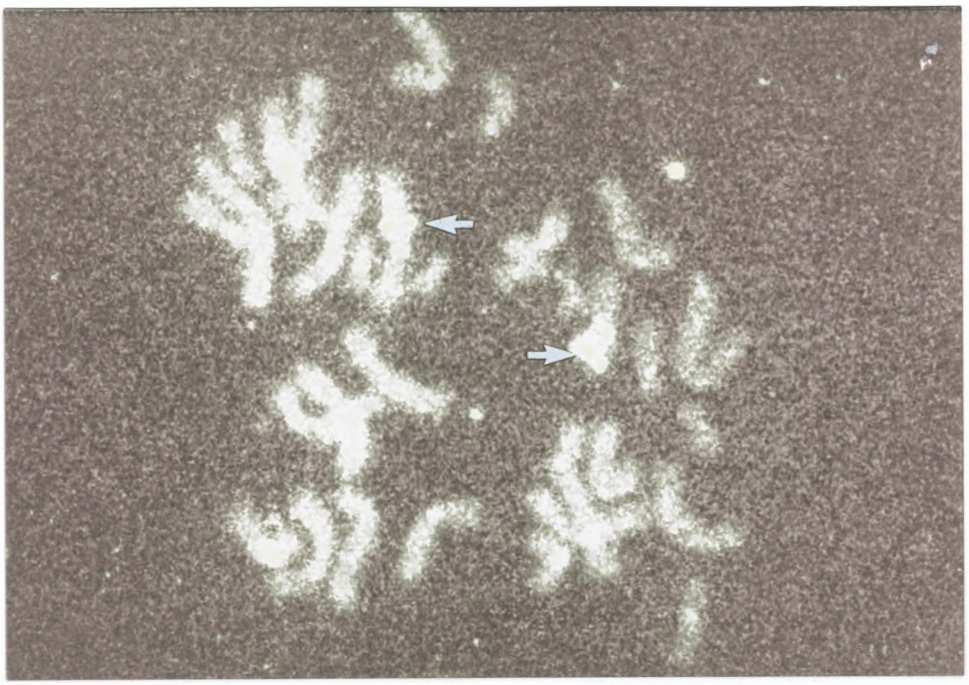
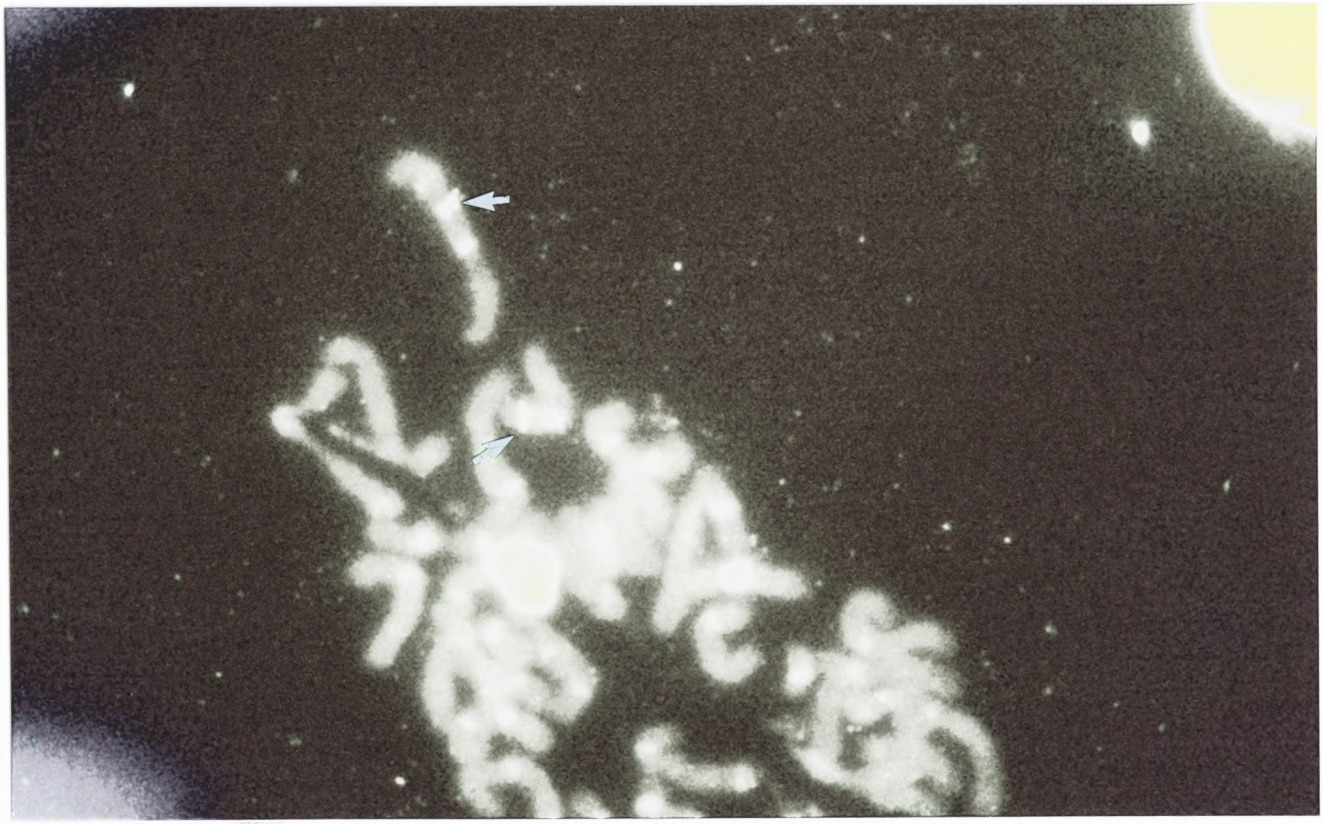


Figure 15: Biotin-labelled YAC probe 845a3 hybridized to a normal metaphase cell showing FITC signals at the region 11p15.1 (arrows) (DAPI counterstain, triple filter x1000).





Figures 16a & b: Metaphase cells of cell line UWOV2(Sf) showing hybridization of biotin-labelled YAC probe 845a3 to the chromosome 11p+ abnormality (arrows) and to the normal chromosome 11 (DAPI counterstain, FITC filter x1000).

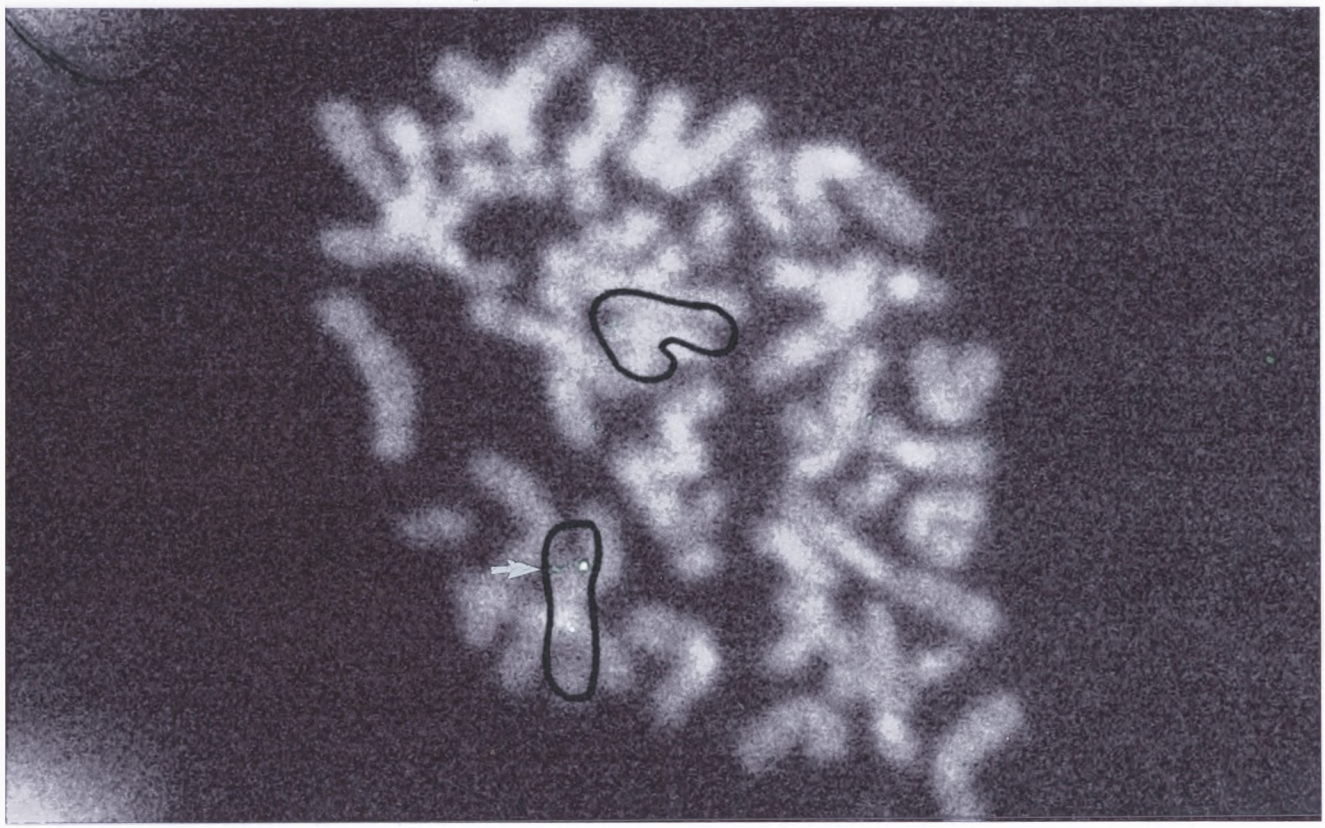


Figure 17: Metaphase cell of cell line UWOV2(Sf) showing hybridization of the biotin-labelled YAC probe 966e8 to the normal chromosome 11 but not to the chromosome 11p+ abnormality (arrows) (DAPI counterstain, triple filter x1000).

there to be three copies of chromosome 8 which appeared cytogenetically normal as revealed by DAPI counterstaining (Figure 11).

3.7 Mapping of the Breakpoint in Cell Line UWOV2(Sf) Using FISH Studies

Initially, mapping of the t(3;11) on chromosome 11, proceeded in a pter→pcen direction. Using FISH, the single copy gene probe pSBβ mapping to the region 11p15.5 showed feint positive hybridisation to the normal chromosome 11 homologue, but no signal was detected on the marker chromosome 11 suggesting that the breakpoint of the t(3;11) lay proximal to this region (Figure 12).

CEPH YAC probes 892g9, 785e5, 847a12 and 954f4 were used for mapping. Results on control slides using YACs 847a12 and 954f4 revealed strong signals in the region 11p15 with no non-specific hybridisation (Figures 13 and 14). FISH studies on cell line UWOV2(Sf) showed no signal on the marker chromosome 11 (results not shown) indicating that the breakpoint lay proximal to the region 11p15.4. The signal intensity of the different YAC probes differed considerable due to the quality of the probes and the quality of the target cells.

Using YAC probe 845a3 and the more distal YAC probe 966e8, FISH results on control slides using these probes revealed signals in the region 11p14.3-15.1 (figure 15) with no non-specific hybridisation. Results with probe 845a3 are not shown.

FISH studies on cell line UWOV2(Sf) with YAC 845a3 showed hybridisation to both the marker chromosome 11 and its normal homologue (Figures 16a & b). FISH studies with the more distal YAC 966e8 showed no hybridisation to marker chromosome 11 but a signal was detected on the normal homologue (Figure 17). From these results it was deduced that the breakpoint of the t(3;11) lay in the region between and/or incorporating the regions spanned by YACs 845a3 and 966e8. This corresponds to the region 11p14.3-15.1 according to the physical integration map of Bickmore *et al.*1995 (figure 22). A map of chromosome 11p showing the regional allocations of the various probes used in this study can be seen in figure 23.

Dual FISH using the two probes 845a3 and 966e8 on interphase cells of UWOV2(Sf) confirmed the above results (Figures 18a & b).

3.8 Mapping of the Breakpoint Region in Cell Line UWOV2 Using FISH Studies

Based on the mapping results obtained with cell line UWOV2(Sf), YACs 845a3 and 966e8 were also hybridized to cell line UWOV2 to determine whether or not the breakpoints were the same. Results

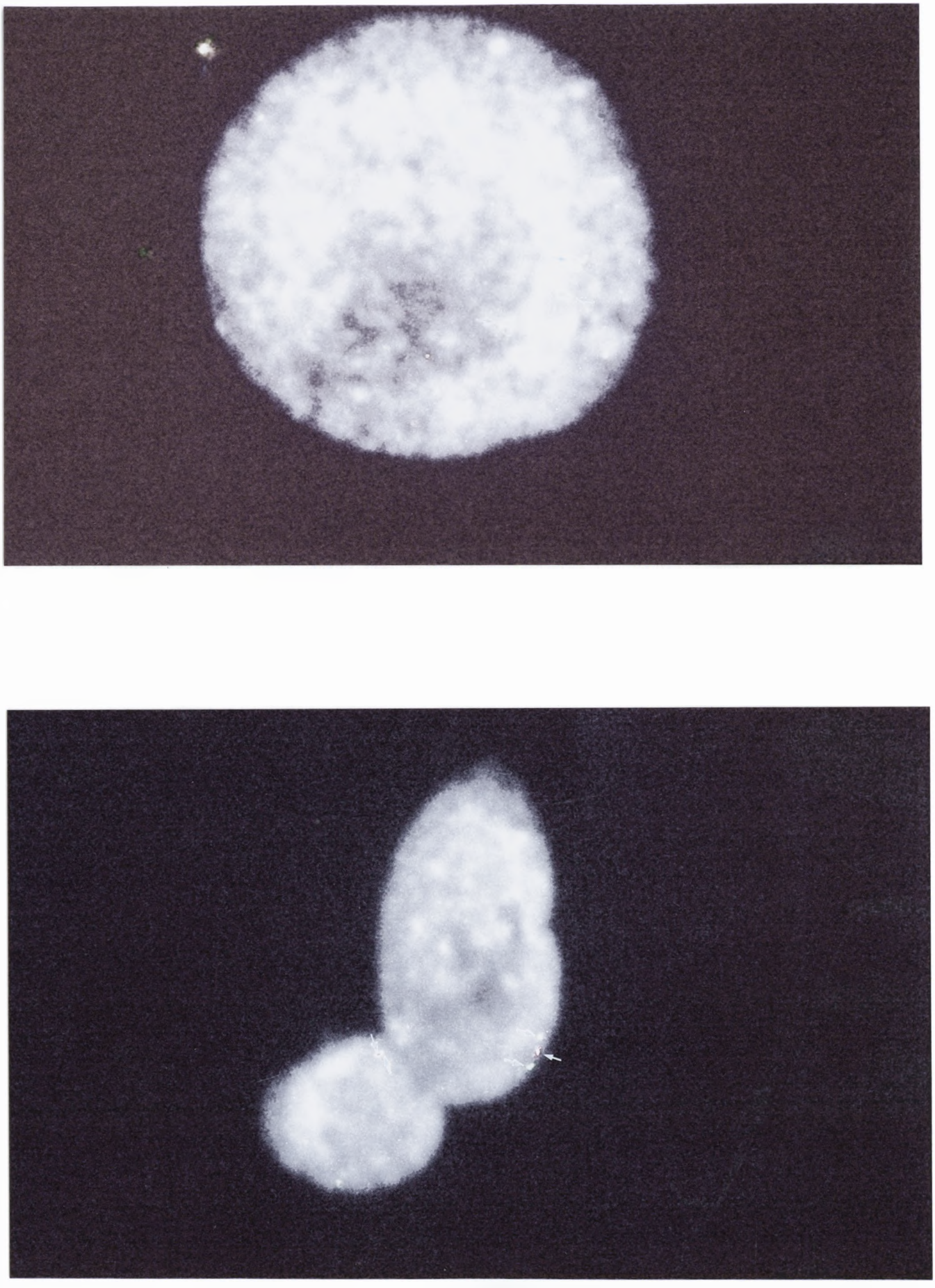


Figure 18(a & b): Dual FISH on interphase cells of cell line UWOV2(Sf) with YAC probe 845a3 (FITC signal) and 966e8 (Rhodamine signal) (arrows) (DAPI counterstain, triple filter x1000).

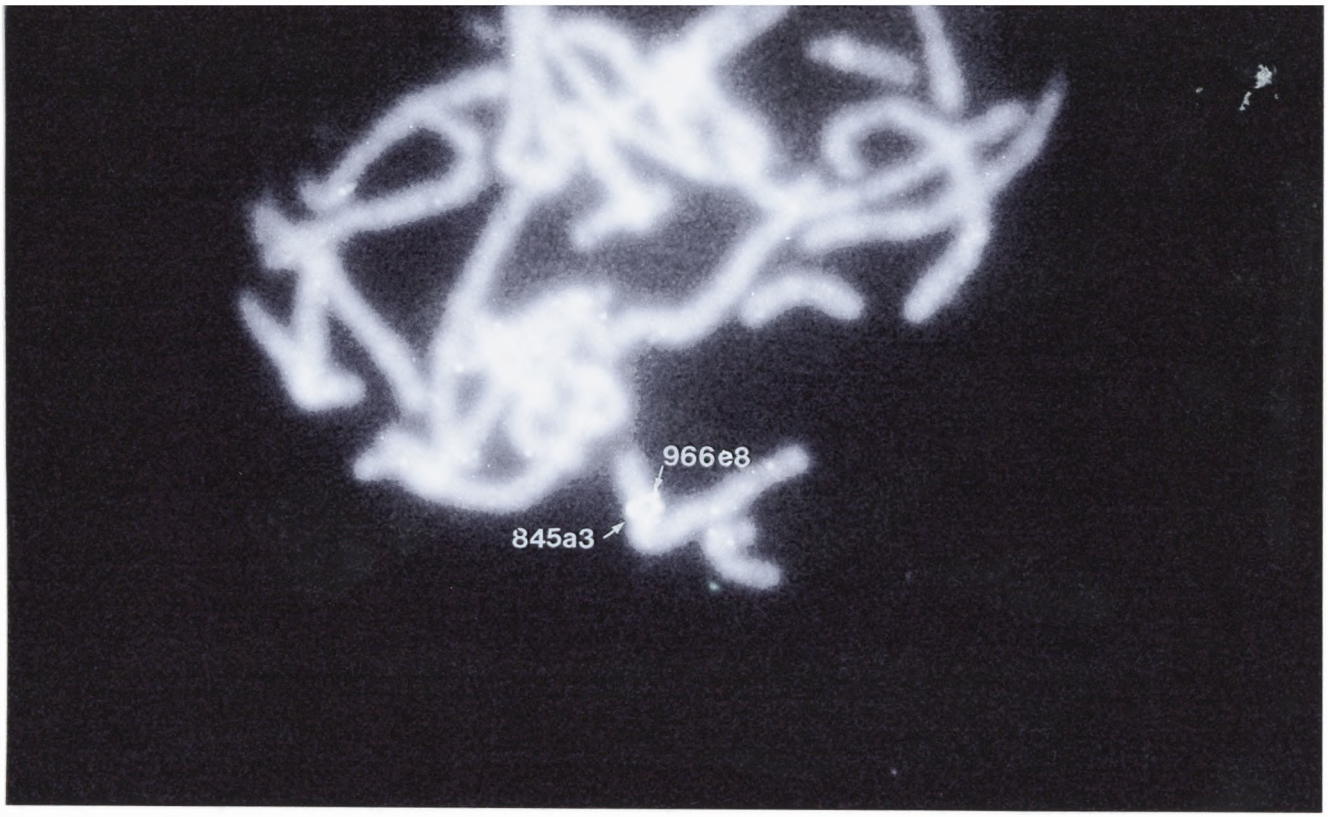


Figure 19: A hypodiploid metaphase cell of specimen T5 showing dual FISH with YAC probes 845a3 (FITC signal) and 966e8 (Rhodamine signal) (DAPI counterstain, triple filter x1000).



Figure 20: A hypodiploid metaphase cell of specimen AF-5 showing dual FISH with YAC probes 845a3 (FITC signal) and 966e8 (Rhodamine signal) (DAPI counterstain, triple filter x1000).

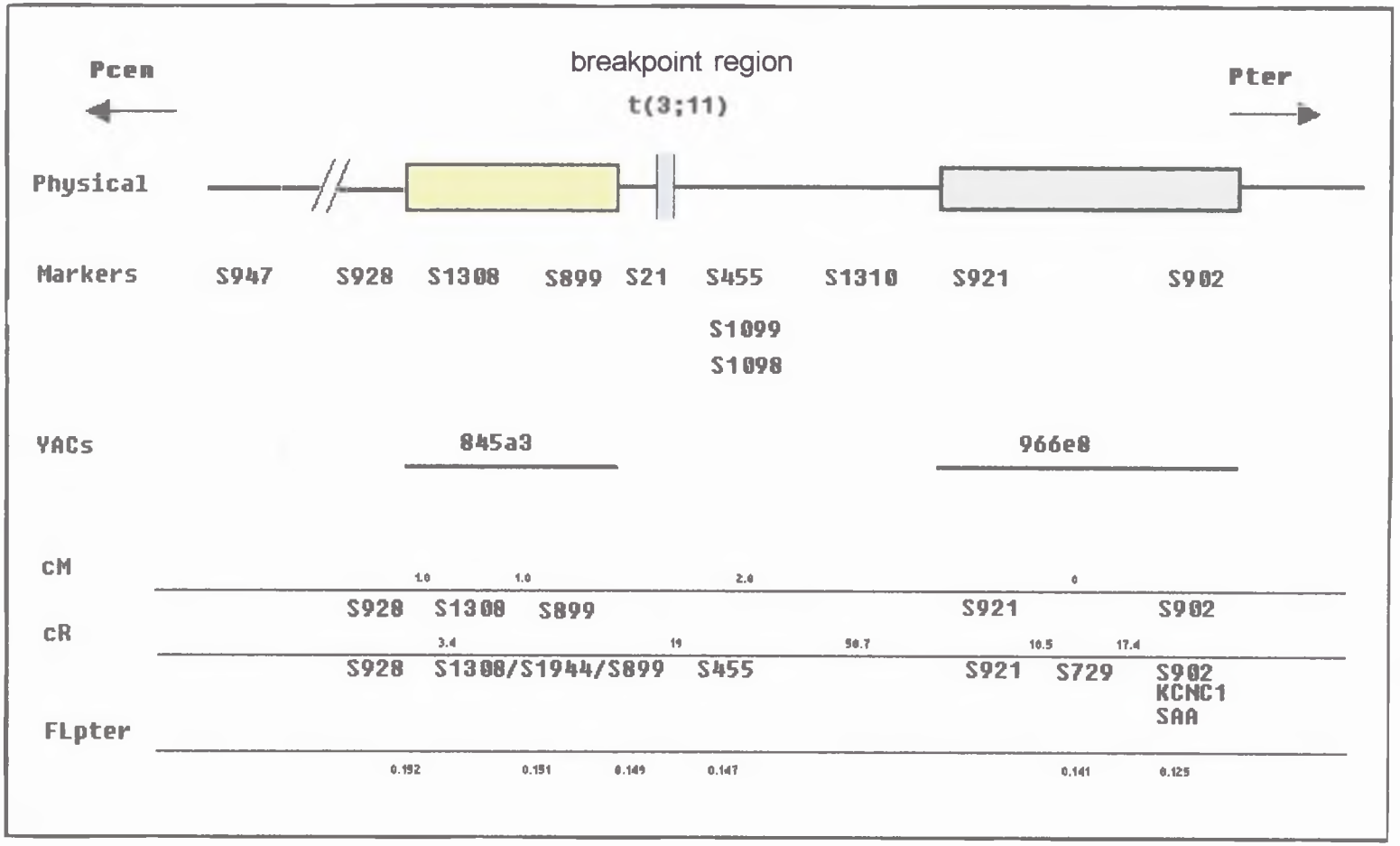


Figure 22: A physical integration map of a portion of the region 11p14.3-p15.1 indicating the possible position of the t(3;11) breakpoint region on chromosome 11 in cell lines UWOV2 and UWOV2(Sf) relative to the two YAC probes 845a3 and 966e8 (Reproduced from Bickmore et al. 1995).

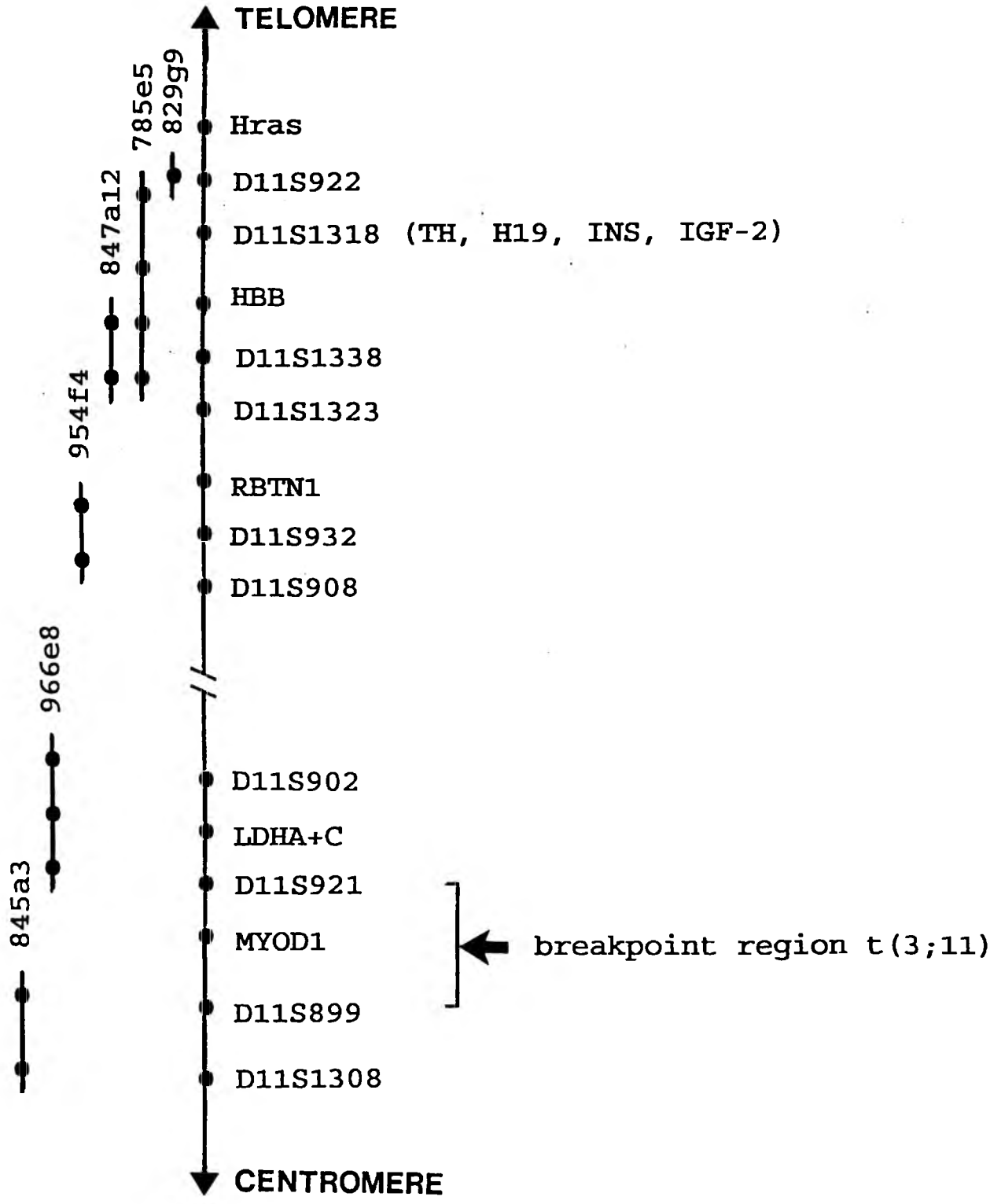


Figure 23: A map of the distal portion of chromosome 11p15 showing the position of the YAC probes used in this study relative to various DNA markers. The region of the t(3;11) breakpoint is indicated (Reproduced from James et al. 1994).

showed that probe 845a3 hybridized to both the marker chromosome 11 and its normal homologue while hybridisation of probe 966e8 yielded a signal from the normal chromosome 11 only (results not recorded due to rapid fading of the signal). From this it can be assumed that the t(3;11) breakpoints in the parent cell line and its subline lie in the same region 11p14.3-15.1.

3.9 FISH Analysis of Fresh Tumour Material

Cytogenetic analysis of tumour T5 revealed two cytogenetically normal copies of chromosome 11. Results of dual FISH using YAC probes 845a3 and 966e8 showed no detectable abnormalities involving the region 11p14.3-15.1 (Figure 19). Results of dual FISH using YAC probes 845a3 and 966e8 on ascitic fluid culture AF-5 showed no abnormalities in this region (Figure 20). In both cases, cells were hypodiploid and in all cells analysed, only one homologue of chromosome 11 was present. All of these homologues however yielded a normal result.

3.10 Gene Dosage Studies

Results of gene dosage studies can be seen in Figure 21. In figure 21a, hybridisation of probe IGF-II to cell line UWOV2(Sf) (lane 1) and UWOV2 (lane 3) yielded weaker signals compared to the control DNA (lane 2). Although differences in signal intensity between control and cell line DNA could be discerned, this was not evidence enough to support the possibility that the region incorporating the IGF-II gene locus on 11p15.5 was lost from cell lines UWOV2 and UWOV2(Sf) on marker chromosome 11.

Figure 21b shows results with the β-actin gene probe. A significant difference in signal intensity could not be accurately discerned.

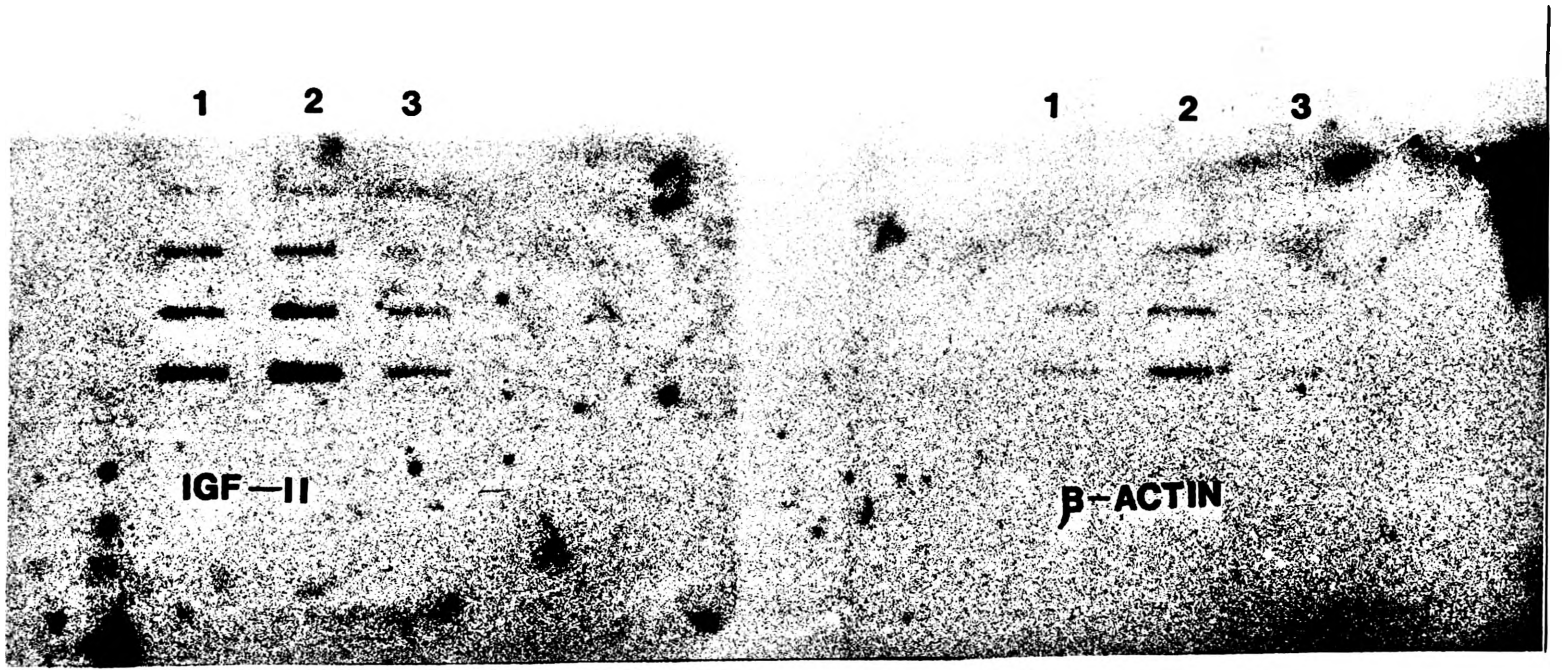


Figure 21: Autoradiograph of slot blot assays with probes IGF-II (a) and β-actin (b). Lane 1 indicates cell line UWOV2(Sf) DNA, Lane 2 indicates control DNA and Lane 3 indicates cell line UWOV2 DNA.

SECTION 4.0

DISCUSSION

The genetic changes occurring during epithelial ovarian cancer are not well understood although the role of many known cellular oncogenes and tumour suppressor genes have been investigated. This study served to define more accurately the role that chromosome 11 abnormalities play in the pathogenesis of epithelial ovarian carcinoma. Using FISH techniques, the breakpoint of a complex translocation in two ovarian cancer cell lines was mapped successfully in a process that proved to be both rapid and reliable.

4.1 Cytogenetic Analysis of the 11p Abnormality in Cell Lines UWOV2 and UWOV2(Sf)

The nature of the chromosome 11p abnormality in cell line UWOV2 could not be clearly determined cytogenetically. The difficulty in identifying the abnormality in this study and in that of Golombick *et al.*1990, may have been in part due to the inability to detect the chromosome 3 centromere. Metacentric chromosome centromeres may become unstable and lose their functionality during translocation events. The dicentric nature of the abnormality could thus not be seen.

4.2 FISH Analysis of Cell Lines UWOV2 and UWOV2(Sf)

Using the dual FISH detection system and DAPI banding in conjunction with YAC probes, the t(3;11) breakpoint region in cell line UWOV2(Sf) could be assessed quite rapidly. Through conventional cytogenetic methods, the chromosomal origin of this complex translocation could not be determined. The technique of screening with chromosomal libraries may be applied to all types of human tumour cells exhibiting complex karyotypes. Using FISH, we were also able to trace the clonal evolution of the 11p+ marker chromosome from the parent line to the subline.

In this study, several of the results could not be graphically depicted. This may have primarily been due to a) rapid fading of the fluorescent signal b) technical problems encountered during photographing the results and c) inconsistencies in the hybridisation efficiency of the various whole chromosome library probes - this phenomenon was clearly outlined by Gray *et al.*1991. These problems were encountered especially during the dual FISH hybridisation experiments where the result could not be amplified after signal loss. Such a situation thus required the specific experiment to be repeated.

Problems were encountered with the batch of Rhodamine obtained from the manufacturer who agreed that problems of rapid signal fading had been encountered by themselves and other observers.

The two dual FISH protocols employing either Rhodamine or TRITC together with FITC appeared to be equally successful however, the Rhodamine signals tended to fade much more rapidly, for

reasons described above, while the TRITC which provided more unwanted background staining, tended to be more stable under epifluorescent microscopy.

The presence of the marker 11 chromosome harbouring the t(3;11) abnormality in the parent cell line UWOV2 and the t(3;8;11) abnormality in the subline UWOV2(Sf) leads us to speculate that the t(3;8) in the subline was an acquired abnormality as a result of *in vitro* cell culture under serum free conditions. This hypothesis may be supported by the observation that UWOV2 revealed three copies of chromosome 8 which appeared cytogenetically normal (Golombick *et al.*1990). The growth advantage conferred by the t(3;8) on UWOV2(Sf) remains to be elucidated. This region however is of interest as it harbours the *c-myc* proto-oncogene on chromosome 8q whose fusion with a yet undefined gene on chromosome 3q may directly or indirectly lead to the production of a known growth factor or novel growth factor which may allow propagation of this cell line under serum free conditions.

From the FISH results, it may be assumed that loss of chromosome 11 material distal to 11p15.1 occurred on marker chromosome 11 in both cell lines UWOV2 and UWOV2(Sf). This assumption however could not be confirmed using slot blot assays which yielded an ambiguous result.

The involvement of chromosome 3 in this translocation has numerous implications regarding observations made in genitourinary cancers and the breakpoint region involved here has been implicated in several other human cancers. Besides being implicated in contributing to the malignant phenotype in ovarian carcinomas, cytogenetic abnormalities and molecular genetic changes involving this chromosome have been implicated in the development of lung (Brauch *et al.*1990), and renal cell carcinomas (Latif *et al.*1993). Several reports have detailed these chromosomal changes in ovarian carcinoma (Teyssier *et al.*1987; Tanaka *et al.*1989) and LOH studies have revealed common regions of deletion on 3p (Ehlen, T; Dubeau, L.1990). These reports have mainly detailed loss of heterozygosity studies, unbalanced translocations and numerical abnormalities involving chromosome 3 and the regions on chromosome 3 which are implicated in the translocation events observed in the cell lines in this study.

Deletions and unbalanced translocations involving the regions 3p13-21 (Pejovic *et al.*1992), 3p10-23 (Thompson *et al.*1994) and 3p21-26 (Jenkins *et al.*1993) were described (figure 24). Thompson *et al.*1994, found that a + del(3p) was the only abnormality present in a patient with stage I ovarian cancer suggesting a role for this chromosome in early tumour progression in some patients. Bello *et al.*1990 describes a case in which the sole karyotypic abnormality in an ovarian cancer patient was trisomy 3. These findings may be extrapolated to

those of our study where trisomy 3 may have been present *in vivo* before UWOV2 and subsequently the subline UWOV2(Sf) may have acquired the t(3;11) event.

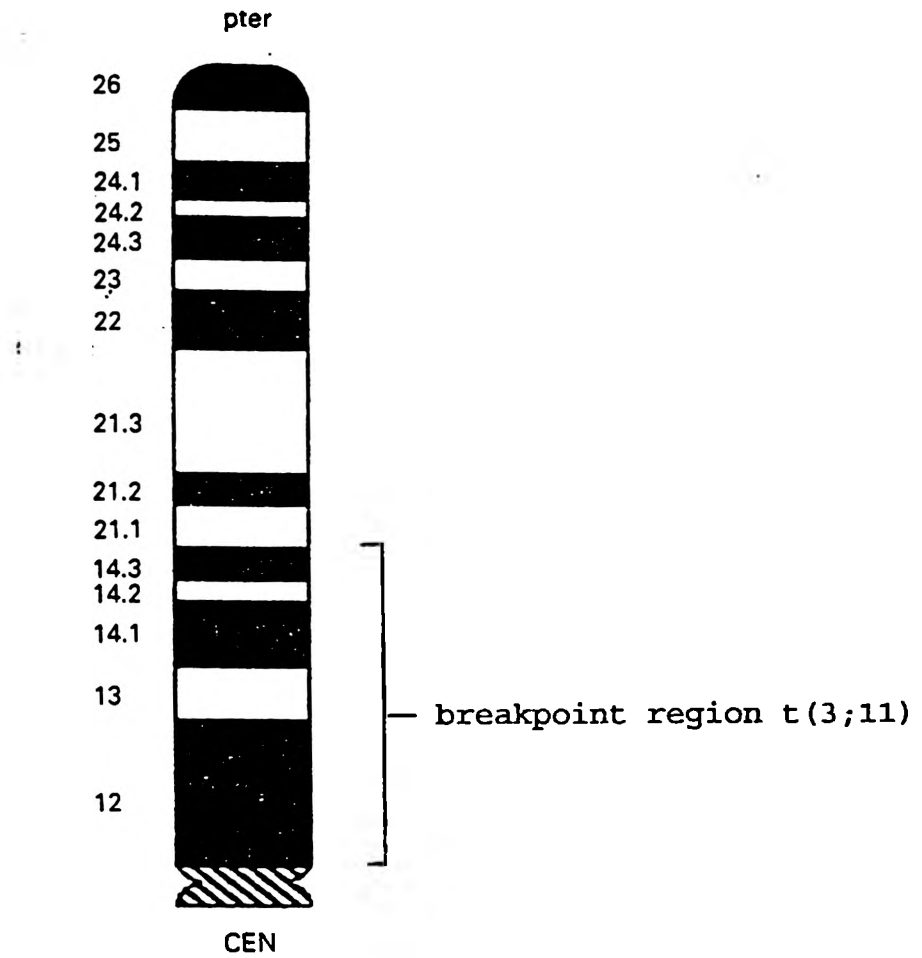


Figure 24: A map of the short arm of chromosome 3 indicating the various regions implicated in ovarian carcinoma. The position of the t(3;11) breakpoint is indicated.

The loss of chromosome 3p sequences has been shown to be the most characteristic and possibly the first genetic event in the development of non-papillary renal cell carcinoma (RCC). Initially it was though that the gene responsible for RCC had been found (Latif *et al.*1993) and the putative TSG, the *VHL* gene was cloned and assigned to the region 3p25-26. Alterations in this gene are responsible for von Hippel-Lindau disease, a dominantly inherited familial cancer syndrome and the most common form of RCC (Maher *et al.*1991). Other studies however point to the regions 3p12-14 and 3p21 as harbouring a second or other putative TSGs (Sanchez *et al.*1994) implying that several genes may be involved in this condition. The region 3p14.2 involves the t(3;8) translocation breakpoint implicated in both

familial and sporadic forms of RCC and this translocation event has been hypothesised to affect the expression of an RCC TSG or oncogene (Boldog *et al.*1993). Huebner *et al.*1995 implicated other yet undefined loci in the region 3p12-14 responsible for RCC. Kovacs *et al.*1995 state that these studies suggest that the mechanism of tumourigenesis may be due to several events:

- a cluster of TSGs are present in the region 3p12-14
- a translocation event has a positional effect on a TSG in the region of the breakpoint
- the breakpoint directly affects a TSG in the region

Allele-specific chromosome 3 deletions have also been shown to occur at an early stage in the pathogenesis of lung carcinoma. The most common changes in small cell and non-small cell lung carcinomas involve loss of genetic material at three distinct regions located at 3p25, 3p21.3 and 3p14→cen (Brauch *et al.*1990; Thiberville *et al.*1995). The *VHL* gene shows few mutations in lung carcinoma and therefore there may be another recessive oncogene at the 3p25 region. Hung *et al.*1995 showed that 3p allelic loss occurs at the very earliest preneoplastic stage in lung cancer.

The connection between the breakpoint on 3p12-14 in cell lines UWOV2 and UWOV2(Sf) and those described above are purely speculative. Although the exact breakpoint region of the t(3;11) in this study, remains to be mapped one cannot rule out a common TSG or cluster of TSGs in this region which may be necessary for the establishment of the malignant phenotype and may even be one of the first events in a series of genetic events leading to tumourigenesis. The frequent involvement of the region 3p12-p14 in studies described above and the involvement of this region in the breakpoints described in this study leads one to speculate that there may be a region of involvement common to the pathophysiology of these cancers.

Trisomy 12 has also been described in ovarian carcinoma often as being the sole abnormality in a variety of these tumours both epithelial and non-epithelial, benign and malignant (Pejovic *et al.*1992). This includes ovarian fibromas and serous cystadenomas. Jenkins *et al.*1993 states that the frequent occurrence of trisomy 12 in non-epithelial ovarian tumours and its absence from more differentiated epithelial tumours is evidence for it not being an early obligatory event unique to epithelial tumours and that clones harbouring trisomy 12 appear to have a selective advantage in culture. The *K-ras* proto-oncogene locus on 12p is frequently activated in ovarian carcinomas but appears to be unrelated to the presence of trisomy 12 (Yang-Feng *et al.*1991). Trisomy 12 has also been reported as being the sole abnormality in a case of Wilms' tumour and is quite prevalent in this condition (Wang Wuu *et al.*1990).

Structural and numerical abnormalities involving chromosome 14 are not commonly reported on in ovarian tumours. Besides the observations of Wake *et al.*1980, chromosome 14 has been reported to be involved in deletions and unbalanced translocations in several other reports on ovarian cancer (Jenkins *et al.*1993; Thompson *et al.*1994; Pejovic *et al.*1992). In our study, the additional material on 14q observed cytogenetically resulted from an amplification event (HSR) involving the region 14q31-32. This was confirmed by FISH studies. Its influence on cell line UWOV2(Sf) remains to be determined.

Cytogenetic analysis of the X chromosome in ovarian tumour specimens frequently identifies the loss of an X chromosome often at quite high levels. Thompson *et al.*1994 suggests that chromosome X loss may be a primary or early event in tumourigenesis. However, loss of an X chromosome may not be a prerequisite for tumourigenesis as seen in our study where tumour specimen T5 exhibited two cytogenetically normal copies of this chromosome. A different hypothesis is that selective inactivation of X chromosome genes by hypermethylation may contribute to the inactivation of a tumours suppressor gene/s (Laird *et al.*1994). The significance of the X chromosome in ovarian tumours remains to be determined.

4.3 Mapping Studies on Chromosome 11p

Chromosome 11p has been well studied and mapped especially in the region 11p13-p15.5 due to its involvement in Wilms' tumour, aniridia, genitourinary, mental retardation (WAGR) syndrome and the Beckwith-Wiedemann syndrome (BWS). Several physical maps of the region 11p13-15 have been published (Redeker *et al.*1994) and a radiation hybrid map is also available (James *et al.*1994). The problem in comparing information from these maps has been that they use different sets of DNA markers or landmarks in their construction. The mapping data are expressed using differing distance units based on the methods used for example, kilobases, centiMorgan (cM) or centiRays (cR). It is therefore difficult to relate information from one source with that from another.

In our study, the regional assignments of CEPH YACs 845a3 and 966e8 are in agreement with the physical integration map of Bickmore *et al.*1995 (figure 22) and the radiation hybrid map of James *et al.*1994 (figure 23). This study thus confirmed that the two markers D11S1308 and D11S899 spanned by YAC 845a3 lie proximal to the two markers D11S902 and D11S921 which are spanned by the YAC 966e8. The physical distance, with reference to the physical map of Bickmore *et al.*1995, between the most distal marker D11S899 (contained by YAC 845a3) and the most proximal marker of YAC 966e8, that is D11S921 corresponds to approximately 1 megabase. From the FISH results, it is apparent that the breakpoint of the t(3;11) lies within this region just distal to YAC 845a3 but not including it. This is an assumption

based on the observation that the signal intensity provided by this YAC on the marker chromosome 11 was as strong and the area covered appeared to be equal to that observed on the normal chromosome 11 homologue. One cannot however exclude the possible involvement of a smaller region contained within this YAC in this translocation.

Through Fluorescence in situ hybridisation analysis, YAC probes 845a3 and 966e8 appeared to be in close proximity to each other. Although the observed distance between the two YACs may appear small on metaphase chromosomes, the actual distance is much larger due to the conformational state of the DNA double helix. Lichter *et al.*1991a ordered probes on chromosome 11 in the 1 Mb range and states that this range may represent the border of resolution for metaphase mapping. Resolution may not be influenced only by chromosome length but also by variations of chromatin conformation at different chromosomal sites. Because of this, metaphase mapping may provide an ambiguous result in as far as measurements of distance between ordered probes is concerned. For the purposes of metaphase mapping, elongated prometaphase chromosomes are the ideal targets as the signals may then be better interpreted. The incorporation of a banding technique such as DAPI staining has allowed probes to be mapped with reference to the various chromosomal bands. Probes lying in the same latitude that is, those lying on the width of a chromosome on chromatids, may be better resolved on more condensed metaphase chromosomes. Mapping resolution may be further improved by analysis of chromosomal DNA in more decondensed states such as that found in interphase nuclei.

Dual FISH with YAC probes 845a3 and 966e8 on interphase cells revealed the two probes to be separated by a considerable distance while similar studies on elongated metaphase chromosomes showed the two signals to virtually coalesce into one. Interphase mapping may therefore prove to be a more reliable determinant of distance between ordered probes however, a large number of nuclei should be analysed as the decondensation states of the chromatin vary between nuclei. Besides this, only probes in close proximity to each other may be analysed with any amount of accuracy as the specific domains that each chromosome occupies within an interphase cell still remains to be determined.

The region on 11p15.1 in which the t(3;11) breakpoint lies has not been well studied and characterized. Although YAC contigs and cosmid probes mapping to this region are available (van Heyningen *et al.*1995) few known genes or disease loci have been assigned to this region. The region as a whole is of particular interest in terms of the genetics of urinogenital cancers especially the childhood rhabdomyosarcomas, Wilms' tumour and Beckwith-Wiedemann syndrome (Slater *et al.*1992). Although the *WT1* TSG at 11p13 was not shown to be altered in ovarian carcinomas (Viel *et al.*1994) the region 11p15 has been

implicated in the harbouring of a second putative Wilms' tumour TSG and its association with ovarian cancer is yet to be determined. Recently, three putative TSGs relative to Beckwith-Wiedemann syndrome associated chromosomal breakpoints have been mapped to 11p15.3 using FISH studies (Redeker *et al.*1995) and other studies have disclosed two clusters of breakpoints in the 11p15.4-11pter region (Henry *et al.*1989). From our results we speculate that a cluster of TSGs in the region 11p15 play an important role in the progression of these cancers and epithelial ovarian carcinoma.

4.4 Analysis of Fresh Tumour Material

The procedures employed in the cell culture of fresh tumour material and ascitic fluid proved to be suitable for most of the specimens and although only a few specimens yielded cytogenetically analysable metaphases, the ones that did, gave very informative results. The establishment of epithelial ovarian cancer cultures is usually problematic and sometimes the correct parameters have to be defined for each specimen. A drawback in this study was that the histopathology data for each specimen used was not obtained and therefore their classification according to the FIGO classification system was not established.

The cellular appearance of ascitic fluid cultures AF-1, AF-2, AF-3 and AF-5 were fibroblastoid in morphology and they were thus assumed to be normal fibroblast cells which had contaminated the preparation. Fresh tumour samples T2 and T4 assumed a fibroblastoid-like morphology while T5 appeared to form a compact cobblestone monolayer characteristic of epithelial cells and epithelial ovarian tumour cells in culture. Although it may be assumed that the presence of fibroblasts in culture may have been due to contamination, studies have shown that epithelial phenotypes which the ovarian epithelial tumour cells exhibit *in vivo* may modulate to an atypical fibroblast-like form *in vitro*, gradually under standard culture conditions (Auersperg *et al.*1984) or rapidly in response to epidermal growth factor (Siemens *et al.*1988). The appearance of epithelial cells in culture has also been shown to be influenced by the medium on which they are grown. The ability of these cells to modulate into a fibroblastoid-like appearance shows that these cells are far more complex and versatile than would be expected. Their mesodermal origin and their close relationship to stromal cells appears to confer on these cells the capacity to express a certain amount of plasticity by modulating between epithelial and fibroblastoid phenotypes as they are able to undergo epithelio-mesenchymal conversion upon interaction with specific substrates. The exact identity of these cells may have been determined by testing for ovarian cancer-specific antigens.

Tumour T5 was the most informative of all the cultures. The only clonal abnormality - clonality being defined as two or more cells harbouring a specific defect - appeared to be a del(6q) involving the region 6q21. Abnormalities involving the long arm of chromosome 6 have been extensively described in ovarian carcinomas (Pejovic *et al.*1992; Thompson *et al.*1994). Wake *et al.* 1980 showed that in short term culture of ovarian seropapillary cystadenocarcinomas from nine patients, five had the reciprocal translocation t(6;14)(q21;q24) and four had a 6q- marker chromosome. The results were of considerable interest as they represented the first clear indication of a structural chromosome specificity in any carcinoma. This 6q- was not limited to serous tumours and had previously been detected in a range of histologically different subtypes (Van der Riet-Fox *et al.*1979). Woods *et al.*1979, detected a similar 6q- marker in three out of four cell lines isolated from ovarian seropapillary adenocarcinomas, however previous studies on cell lines from similar carcinomas did not reveal the presence of this marker (Freedman *et al.*1978) and this was confirmed in a later study (Hill *et al.*1984). Therefore the role of the 6q- in tumour pathogenesis could not be fully established.

Loss of heterozygosity studies reported subsequently on chromosome 6q has suggested that this region harbours an ovarian cancer associated TSG(s) and three regions have been implicated: 6q24-27, 6q21-23 and 6q12-16 (Lee *et al.*1990, Mok *et al.*1995). The involvement of the region 6q21-23 in our study is significant considering the findings of Wake *et al.*1980 however, this region also harbours the proto-oncogene *c-myb*. Mok *et al.*1995 showed that this site may not be involved in the pathogenesis of borderline seropapillary carcinomas and Boyle *et al.*1995 implicated this region to be involved in benign tumours and endometroid tumours suggesting a possible involvement of this region early in the progression of these tumours. This finding is supported by the observation that the involvement of this region in later stage seropapillary carcinomas is significantly reduced.

The region 6q24-27 is the most frequent region of LOH in invasive tumours and is of particular interest as it harbours the oestrogen receptor gene site. Studies have demonstrated that this region displays LOH of up to 75% (Lee *et al.*1990; Saito *et al.*1992; Dodson *et al.*1993; Foulkes *et al.*1993). Mok *et al.*1995 demonstrated LOH at this region in only 29% of invasive tumours and found that this figure was significantly reduced in borderline tumours. This suggests the presence of a TSG which may be found in the vicinity of the oestrogen receptor gene but may not necessarily involve it directly as this locus did not appear to be deleted.

In tumour T5, the region 6q24-27 was lost in one homologue while deletions at this region on the normal homologue could not be detected cytogenetically. More information on the exact location

of the breakpoint region on 6q may be obtained by mapping studies using YAC probes in combination with FISH.

The del(6q) has also been reported in other malignancies. Menasce *et al.*1994 showed in patients with acute lymphoblastic leukaemia (ALL) and non-Hodgkins lymphoma a deletion in the region 6q16.3. Whether the same gene is involved or not in these two conditions is unknown.

The above studies thus point to the involvement of at least two regions on chromosome 6q that may be involved in the initiation and progression of different histological types of ovarian tumours.

The results with YAC probes 845a3 and 966e8 on T5 and AF-5 confirmed the cytogenetically normal appearance of chromosome 11 in these specimens, however, these results do not rule out the possibility of there being a subtle structural abnormality involving a gene in the region between the two probes that may contribute to the initiation or progression of the malignant phenotype. This may be assessed by further mapping and cloning of the breakpoint on chromosome 11p15.

SECTION 5.0

CONCLUSION

In this study, FISH proved to be a valuable technique in elucidating the nature of the 11p+ abnormality in both cell lines, as being the complex translocation t(3;8;11)(p12-14;q22-24;p15.1) in UWOV2(Sf) and the translocation t(3;11)(p12-14;p15.1) in UWOV2.

The success of Fluorescence *in situ* Hybridisation potentiates its application in the field of solid tumour work. The technique proved to be rapid and successful and thus solid tumours yielding complex karyotypes may be screened and the clonal development of structural and numerical abnormalities may be traced through the evolution of these tumours. An important area of application may be in the comparison between primary tumours and their metastases.

The results obtained in this study on the cell lines, highlight two chromosomal regions on two chromosomes critical to the pathophysiology of ovarian carcinomas and other human malignancies. The region on chromosome 11p15.1 remains to be mapped further using cosmid probes or other YAC probes spanning the region between D11S899 and D11S921. This would allow the critical region to be narrowed down even more thus providing a handle to clone the breakpoint region using FISH techniques.

In a similar way, the breakpoint on chromosome 3p12-p14 may be mapped using YAC probes specific for this region. From this study, it is evident that chromosome 3 abnormalities are critical to the development of ovarian carcinomas and thus the breakpoint in the region 3p12-p14 also remains to be mapped which might permit the elucidation of a gene(s) involved in the initiation of these tumours and other genitourinary tumours.

The 6q- abnormality found in the fresh tumour specimen is equally as important in light of the frequent occurrence of this anomaly in ovarian tumours often as being the sole abnormality in several cases reported. In this study, the nature of the 6q- may also be elucidated using FISH techniques. The exact role that chromosome 6q abnormalities play in the initiation these cancers is unknown, however, chromosome 3 and chromosome 6 events appear to be critical in the establishment of the malignant phenotype while chromosome 11 events appear to play a later role.

SECTION 6.0

APPENDIX

APPENDIX A

BUFFERS

$MgCl_2$ (1M)	Dissolve 203.3g of $MgCl_2.6H_2O$ in 800ml of H_2O. Adjust the volume to 1l with H_2O. Sterilize by autoclaving
Phosphate-buffered saline (PBS)	Dissolve 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 800ml H_2O. Adjust the pH to 7.4 with HCl. Adjust the volume to 1l with H_2O. Sterilize by autoclaving
20x SSPE buffer	Dissolve 175.3g NaCl, 27.6g $NaH_2PO_4.H_2O$ and 7.4g EDTA in 800ml H_2O. Adjust the pH to 7.4 with NaOH and adjust the volume to 1l. Sterilize by autoclaving
20x SSC buffer	Dissolve 175.3g NaCl and 88,2g sodium citrate in 800ml H_2O. Adjust the ph to 7.0 with NaOH. Adjust the volume to 1l with H_2O. Sterilize by autoclaving
1M Tris buffer	Dissolve 121.1g Tris base in 800ml H_2O. Adjust the pH to the desired reading by adding concentrated HCl. Adjust the volume to 1l with H_2O. Sterilize by autoclaving
50x TAE buffer	Dissolve 242g Tris base, 57.1ml glacial acetic acid and 100ml EDTA (pH 8.0) make up the volume to 1l with H_2O. Sterilize by autoclaving
Phosphate buffer pH 6.8	Dissolve 6g KH_2PO_4 and 5.1g of $NaH_2PO_4.12H_2O$ in 1l H_2O.Adjust the pH to 6.8. Sterilize by autoclaving.
0.5M Phosphate buffer for FISH	Prepare 0.5M $Na_2HPO_4.2H_2O$ and 0.5M $NaH_2PO_4.2H_2O$. Mix the two by using the latter buffer to pH the former buffer to pH 7.0. Sterilize by autoclaving

STAINS AND REAGENTS

Phenol preparation

Melt the phenol at 68°C. Add hydroxyquinolone to a final concentration of 0.1%.

To the melted phenol add an equal volume of buffer (0.5M Tris.HCl pH 8.0) at room T°. Stir the mixture on a stirrer for 15 minutes. Allow to settle into two phases. Aspirate the upper/aqueous phase using a glass pipette and discard.

Add an equal volume of 0.1M Tris.HCl pH 8.0 and repeat the above steps.

Repeat the above step until the pH of the phenolic phase is >7.8 (as measured with pH paper).

Add 0.1 vol of 0.1M Tris.HCl pH 8.0 containing 0.2% ß-mercaptoethanol and store in this form.

Paraformaldehyde 8%

Dissolve 40g of paraformaldehyde in 500ml of H_2O by gently heating on a stirrer. Add approximately 5 drops of 10N NaOH to aid the dissolution. After the paraformaldehyde is dissolved, adjust the pH to 7.0 by the addition of HCl. Filter through Wattman filter paper and store at 4°C.

Trypan Blue Stain (0.2%)

Trypan blue	0.2g
PBS pH 7.4	100ml

Filtered through Wattman filter paper

APPENDIX B

GROWTH MEDIA

Luria-Bertani broth (LB broth)

Bacto-tryptone	15g
NaCl	10g
Yeast Extract	15g

Dissolve in 1l H_2O. Sterilize by autoclaving.
For the preparation of LB agar plates add 12g/l commercial agar before autoclaving.

AHC Medium for YAC Preparation

Yeast nitrogen base without amino acids	0.67%
Acid-hydrolysed casein	1%
Adenine hemisulphate	20mg/l

Adjust the pH to 5.8 with about 70µl of 12N HCl for 1 litre. Sterilize by autoclaving.

To this add 50ml of 40% (w/v) D-glucose for 1 litre.

SECTION 7.0

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