Analysis of non-Hodgkin's lymphoma by conventional cytogenetics and fluorescence in-situ hybridisation

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Thesis submitted for the degree of Doctor of Philosophy

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For Joel, Zoë, Martin, Tom, Robin and Callum.
DECLARATION

I hereby declare, that no part of this thesis has previously been submitted in support of any other degree or qualification at this or any other university or institute of learning.
SUMMARY

Analysis of non-Hodgkin's lymphomas by conventional cytogenetics and fluorescence in-situ hybridisation

David William Hammond

Cytogenetic analysis was performed on 40 non-Hodgkin's lymphoma (NHL) node biopsies. Chromosomes X, 3 and 12 were the most frequently gained; of the much rarer monosomies, loss of chromosome 13 was most common. Structural abnormalities primarily involved chromosomes 14, 1, 18, 6 and 17. A markedly greater number of chromosome gains were associated with low-grade disease when compared to high-grade.

In order to obtain further information from the cytogenetic analysis of the NHL karyotypes, the fluorescence in-situ hybridisation (FISH) technique was applied to the series.

The activation state of additional X-chromosomes was examined and evidence that more than one X-chromosome was present in the active state in 4/9 cases was obtained. Further, in an apparent case of monosomy X, a marker was identified as an abnormal X-chromosome by chromosome painting.

Interphase FISH was applied to NHL cells and numerical chromosome changes were identified; this approach was also attempted on aged bone marrow smears from acute lymphocytic leukaemia patients, in order to test the utility of the technique on archival material.

Dual chromosome painting was used to elucidate the origins of add(14) chromosomes in 8 of the cases. In the control and two other cases the translocated material was demonstrated to be from chromosome 18, in two cases it was from chromosome 3 and in one case there was an insertion of chromosome 11 material. It was not possible to identify the origins of the translocated material in one NHL
and in the final case the apparent add(14) was demonstrated not to contain chromosome 14 material.

Structural abnormalities of chromosome 6 were investigated both by chromosome painting and by hybridisation of the MYB gene. The latter, which was initially mapped to 6q23 before hybridisation to NHL cells revealed previously unsuspected rearrangements.

One case contained extrachromosomal chromatin bodies that appeared to be double minute chromosomes (dmin), which FISH analysis demonstrated to be derived from the X-chromosome and contain centromere-associated DNA.

The significance of these results is discussed with reference to previously published series of NHL karyotypes.
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<td>aminomethylcoumarin acetic acid</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
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<tr>
<td>APAP</td>
<td>alkaline phosphatase anti alkaline phosphatase</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphate</td>
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<tr>
<td>BrdU</td>
<td>5-bromodeoxyuridine</td>
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<tr>
<td>CCD</td>
<td>charge coupled device</td>
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<td>CGH</td>
<td>comparative genomic hybridisation</td>
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<td>CISSH</td>
<td>chromosome in-situ suppression hybridisation</td>
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<tr>
<td>CLL</td>
<td>chronic lymphocytic leukaemia</td>
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<td>DAPI</td>
<td>diaminoPhenylindole</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DOP</td>
<td>degenerate oligonucleotide primed</td>
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<td>EDTA</td>
<td>disodium ethylenediaminetetraacetate</td>
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<td>FISH</td>
<td>fluorescence in-situ hybridisation</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IRS</td>
<td>interspersed repetitive sequences</td>
</tr>
<tr>
<td>ISCN</td>
<td>International System for Human Cytogenetic Nomenclature</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<tr>
<td>NHL</td>
<td>non-Hodgkin's lymphoma</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>RHH</td>
<td>Royal Hallamshire Hospital</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>Abbr.</td>
<td>Term</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TR</td>
<td>texas red</td>
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<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
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<tr>
<td>TSG</td>
<td>tumour suppressor gene</td>
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<tr>
<td>VNTR</td>
<td>variable number tandem repeat</td>
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<tr>
<td>WPH</td>
<td>Weston Park Hospital</td>
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<td>YAC</td>
<td>yeast artificial chromosome</td>
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GLOSSARY OF CYTOGENETIC TERMS

add Additional material of unknown origin
brackets, square [ ] Used to indicate clone size and number of cells
comma (,) Separates chromosome numbers, sex chromosomes and chromosome abnormalities
cp Composite karyotype
del Deletion
der Derivative chromosome
dmin Double minute chromosome
dup Duplication
i Isochromosome
idem Used to denote the stemline karyotype in subclones
ins Insertion
inv Inversion
mar Marker chromosome
p Short arm of the chromosome
p10 Short arm part of the centromere
parenthesis ( ) Surrounds structurally altered chromosome(s) and breakpoints
plus (+) Gain
q Long arm of chromosome
q10 Long arm part of the centromere
question mark (?) Questionable identification of a chromosome, chromosome arm or chromosome breakpoint
semicolon (;) Separates chromosomes and bands in structural rearrangements involving more than one chromosome
t Translocation
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CHAPTER ONE

INTRODUCTION
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1.1.1) Historical background

It is now over 100 years since the observations first linking chromosome abnormalities and cancer cells were recorded by von Hansemann, (reviewed in Chaganti, 1983). Theodor Boveri, writing in the early years of this century, proposed the earliest hypothesis that related the development of tumours to cellular genetics (reviewed in Hsu, 1987). He suggested that malignant cells were derived from normal cells and that the malignant phenotype was caused by factors within the cell rather than the environment. Furthermore, he proposed that each tumour originated from a single cell and that the tumour cell had an abnormal chromatin content. However, it was not possible to test these prophetic ideas for some decades. It was not until techniques using colcemid, a spindle poison, to collect mitotic cells and hypotonic treatment to swell those cells and therefore allow chromosome spreading to be devised (Hsu, 1952), that it became possible to analyse chromosomes in mammalian cells accurately.

Following the establishment of the normal diploid human chromosome number (Tjio and Levan, 1956), a critical assessment of numerical chromosome abnormalities in cancer cells became possible for the first time. The identification of the first specific cancer associated chromosome change soon followed, with the discovery of an abnormally small karyotypic marker in patients with chronic myeloid leukaemia, the Philadelphia chromosome (Nowell and Hungerford, 1960). A confusing richness of chromosomal abnormalities in other malignancies initially defied further analysis, although patterns of association, in which particular chromosome types increased or decreased were discerned. (Levan, 1966; van Steenis, 1966). The invention of chromosome banding (Caspersson et al, 1970) provided a powerful tool for a more discriminatory quantification of karyotypic change. It was now possible to accurately identify individual chromosomes and therefore to detect and characterise structural rearrangements.
The Philadelphia chromosome was revealed as an exchange of material (a translocation) between chromosomes 9 and 22. (Rowley, 1973) and allowed the detection of other rearrangements specific to particular malignancies, for example the t(8;14) which is found in the majority of Burkitt lymphomas (Zech et al, 1976).

1.1.2) The Nature of chromosome change

It had been clear to workers in the field that a fundamental genetic instability underlay the karyotypic changes they were observing in cancer cells. However, whether these anomalies were the result (and therefore random) or the cause (and therefore specific) of the malignancy was uncertain. As data has accumulated, increasing numbers of associations between particular cytogenetic changes and specific malignancies have been found (Sandberg, 1980; Yunis, 1983). Chromosomal abnormalities have now been detected in all tumour types (Heim and Mitelman, 1987a) and the conclusion that these changes are non-random is inescapable.

Although many specific associations between karyotype and cancer are now catalogued, the overall pattern of change remains very heterogeneous. A further classification, that may well reflect the biological reality, into primary and secondary aberrations, has been proposed (Heim and Mitelman, 1987a). Primary changes often occur as sole abnormalities and are frequently associated with particular tumour types. Secondary changes do not occur alone, are found more commonly in more advanced disease and may appear in a wider range of malignancies. It is noteworthy that these secondary abnormalities also appear to show a non random distribution (Heim and Mitelman, 1986). It may be inferred that secondary aberrations provide a pool of genetic changes in the context of which cells are tested for "fitness" in terms of their proliferative capacity within a particular environment, thus leading to tumour progression. The selective
pressures will vary, being dependent on the primary changes, the particular malignancy and the type of tissue involved.

An already highly complex pattern of cytogenetic change is further exacerbated by a geographic heterogeneity affecting a range of neoplasia-associated chromosome abnormalities. In a recent study (Johansson et al, 1991), the authors found highly significant differences in the frequency of characteristic aberrations in consecutive series of patients with the same diagnosis. Most of the results were for haematological malignancies, for example, in cases of chronic myeloid leukaemia an additional Philadelphia chromosome was found at a frequency of 9% in Eastern Europe but at 43% in Japan. Some differences were also noted in the frequency of some solid tumour associated cytogenetic changes, monosomy 22 in meningioma being one example. Some of this variation may be due to differences in technique, as has been shown when direct preparations were compared to short term culture for example (Atkin and Baker, 1990). However, the external environment and variations in genetic background must also be considered.

1.1.3) Chromosomes and genes

Evidently, chromosomes are structures that carry genes. A natural corollary to the study of chromosome abnormalities in cancer is an investigation of those molecular changes mediated by large scale cytogenetic alterations. Different categories of chromosome abnormality will effect a variety of events at the molecular level. Numerical changes, involving whole chromosomes, lead to a change of gene dosage, either positive or negative, at all gene loci present on the chromosomes involved. Structural changes can involve exchange of material at particular loci through translocations or inversions, a loss of specific genetic material through deletion or a gain of material through duplication. Small circular extra-chromosomal DNAs, termed double minutes and chromosome structures not amenable to normal banding procedures (homogeneously staining regions)
have also been observed in human tumour cell lines and direct preparations (Barker, 1982). Further examination has revealed these structures as sites of amplification of specific genes, such as MYCN in neuroblastoma (Schwab et al, 1983).

Intensive investigation of the molecular genetics of cancer has revealed several classes of oncogenes (cancer causing genes). Although originally discovered by retroviral transduction (Stehelin et al, 1976), these genes are not indigenous to viral genomes, but are present in the cells of mammals (and many other phyla) playing important roles in the control of cellular growth and proliferation. A first class, proto- or cellular oncogenes, typically gain in function when genetically damaged by point mutation, amplification or translocation (Bishop, 1991). This group appears to act in a dominant fashion, in that these genes may directly contribute to malignant transformation. A second class, that of tumour suppressor genes (TSG), were discovered following the proposal by Knudson of a "two hit" hypothesis as an explanation of the different aetiological patterns of retinoblastoma, an inherited childhood cancer (Knudson, 1971). In the familial form of the disease, one defective copy of a gene was inherited and only a single somatic mutation of the other allele was required to engender malignancy. In the sporadic form, which has a later age of onset, two separate inactivating events are needed. After the discovery of occasional interstitial deletions at chromosome 13q14 in retinoblastoma cells, the RB 1 gene was found to be the target for this dual inactivation (Cavenee et al, 1983). TSG regulate cell proliferation by actuating temporary withdrawal from the cell cycle, differentiation or apoptosis (programmed cell death). Genetic lesions that inactivate these genes liberate the cell from the constraints they impose on growth.

The non-random nature of chromosome changes in human malignancy has been further emphasised by the realisation that many of the oncogenes so far identified are situated at cancer specific chromosomal breakpoints. (Heim and Mitelman, 1987b). Furthermore, of those aberrations found as the sole anomalies in at least
two neoplasms of the same morphology, only 83 bands, one fourth of the standard human karyotype, were involved (Mitelman, 1986). This intimate relationship, between cytogenetic and molecular levels of genetic change in cancer, provides an additional justification for the continuation of cytogenetic studies in neoplastic disease. A fuller discussion of the significance of specific chromosome changes and associated oncogenes will be found in section 1.3 below.

1.2) MALIGNANT LYMPHOMAS

1.2.1) Introduction

Lymphocytes are those immunologically competent cells which assist the phagocytes both in the defence of the body against infection and other foreign invasion and add specificity to the attack. In lymphoid malignancy, an uncontrolled proliferation of lymphocytes or their precursor cells arises. When this (usually) monoclonal population of abnormal cells infiltrates or replaces normal lymphoid tissue the resulting disease is usually diagnosed as a lymphoma. However, involvement of the bone marrow and consequently of the peripheral blood, may also occur. This latter condition is considered diagnostic of leukaemia and therefore uncertainty may arise as to the exact definition of the disease.

Although a very heterogeneous group of cancers, the lymphomas may be broadly divided, firstly into Hodgkin's disease, which is characterised by the presence of Reed-Sternberg cells and secondly into non-Hodgkin's lymphomas (NHL). This latter category embraces a spectrum of disease, presenting with diverse histopathology and described by several systems of classification. The remainder of this section explores this complexity in greater detail.
1.2.2) Lymphocyte ontogeny

A vital feature of cells of the immune system is their ability to recognise foreign antigens through their possession of antigen receptor genes. In the case of B-lymphocytes, derived from the bone marrow, which mediate humoral (circulatory) immunity, the immunoglobulins (lg) fulfil this function. The cellular component is provided by T-lymphocytes, derived from the thymus, which utilise T-cell receptors (TCR). Each of these cellular compartments consists of precursor cells and a variety of mature cell types which subserve different functions. The antigen receptor genes have evolved mechanisms whereby, through the differential assembly of variable and constant components, each cell possesses receptors containing unique variable regions (Wall and Kuel, 1983; Staudt and Lenardo, 1991). Thus each receptor molecule has the ability to react specifically with one antigen amongst the huge number to which the cell may be exposed. It is noteworthy that the genes that code for the constant and variable regions of the receptors are separated in the genome and therefore, during their assembly, are particularly likely to become involved in chromosome translocations. This has implications for the activation of certain oncogenes implicated in the genesis of malignant lymphoma and is discussed further below.

This process of rearrangement takes place in a series of discrete steps and thereby allows the developmental stage of a cell to be identified by antibodies to specific cell surface markers. Until antigenic activation, lymphoid cells are maintained in a resting state and rearrangements occurring up to this point are known as antigen independent. After encountering the specific antigen with which they react, lymphocytes undergo proliferation, during which further identifiable stages of differentiation, which are antigen dependant, commence. An outline of this development in B cells is shown below in table 1.1, indicating the relationship of different phenotypes to lymphoid malignancy.
1.2.3) Classification of non-Hodgkin's lymphomas

NHLs range from highly proliferative, rapidly fatal diseases to indolent and well tolerated malignancies. Practically all follicular and most diffuse lymphomas are derived from B-lymphocytes, with less than 10% carrying membrane features of T cells. NHL apparently retain the characteristics of their normal counterpart cells and so attempts have been made to classify them on that basis. Changes in the cyto-architecture of the lymph node are also seen and have

Table 1.1 Relationship of B-Cell Development to Lymphoid Malignancy

<table>
<thead>
<tr>
<th>Leukaemia or NHL subtype</th>
<th>B-cell counterpart</th>
<th>B-cell differentiation pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL, Low grade lymphocytic NHL</td>
<td>Mature immunocompetent B-cell</td>
<td>Primary, antigen independent</td>
</tr>
<tr>
<td>CLL, Low grade lymphocytic NHL</td>
<td>Memory B-cell</td>
<td>Secondary, antigen independent</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>Pre B-cell</td>
<td>Primary, antigen independent</td>
</tr>
<tr>
<td>Low grade follicular NHL and High grade diffuse counterparts</td>
<td>Follicular centre cells: centroblasts and centrocyts</td>
<td>Secondary, antigen dependent</td>
</tr>
<tr>
<td>High grade immunoblastic NHL</td>
<td>Immunoblasts</td>
<td>Secondary, antigen dependent</td>
</tr>
<tr>
<td>High grade lymphoblastic NHL</td>
<td>Lymphoblasts</td>
<td>Secondary, antigen dependent</td>
</tr>
<tr>
<td>High grade lymphoblastic NHL</td>
<td>Pre B-cell</td>
<td>Primary, antigen dependent</td>
</tr>
</tbody>
</table>

great importance in some systems. Among widely employed schemes is the Rappaport classification, which divides NHL by pattern (nodular vs diffuse) and
by cytologic cell type (Nathwani et al, 1976). Other major systems are the Lukes and Collins (Lukes and Collins, 1974) and Kiel (Stansfeld et al, 1988) classifications, which attempt to predict immunologic phenotype and function from morphology.

In 1980, an attempt was made to unify the six major classifications in use at the time and to provide a means of translation between systems. This Working Formulation (The Non-Hodgkin's Lymphoma Pathologic Classification Project, 1982) has since become a classification scheme in its own right, although this was not the original intention and may now be used to compare data compiled under the various classification systems. The updated Kiel classification (Stansfeld et al, 1988) was used to describe the cases discussed in this thesis. Very recently, proposals were made by an international group of pathologists to completely revise the classification of lymphoid neoplasms (Harris et al, 1994). It is too soon to predict how far this new classification will supplant existing systems.

1.2.4) Incidence

NHL account for approximately 3-5% of the deaths from all malignant neoplasms in the developed world, but a more variable percentage elsewhere (Magrath, 1990). However, because of the many available classification systems for NHL there is great difficulty in comparing the frequency or incidence of different subtypes. This problem notwithstanding, there appears to have been a dramatic and ongoing increase in the incidence of NHL, that cannot be attributed to changes in diagnostic criteria (Banks, 1992). One study from the USA estimated that there has been an increase in risk of 10.3% for females and 9.2 % for males every five years since 1965 (Holford et al, 1992). In Britain, the incidence rate of NHL has increased annually in recent years by approximately 4% (Cartwright, 1992). At present no clear explanation is available for this phenomenon.
1.3) GENETICS OF NON-HODGKIN'S LYMPHOMA

1.3.1) Introduction
Genetic studies of NHL have produced a picture of complex and often contradictory changes. Data has been gathered from a large number of different centres (see below), which have employed a variety of classification systems when codifying their results. The situation is further exasperated by the finding of chromosome abnormalities in reactive lymph nodes (Grace et al, 1989; Zhand et al, 1993). This has in turn made assessing the relationship of genetic changes to histological grade and the value of such changes as prognostic indicators a matter of some difficulty. Large numbers of samples must be studied, in order that the variation in local methods and reporting procedures has a minimal effect on the analysis of the data as a whole. Cytogenetic studies will be discussed first, followed by a consideration of specific molecular changes in NHL, which in this disease are often intimately related to chromosome abnormalities.

1.3.2) Cytogenetics of non-Hodgkin's lymphomas
The majority of information on chromosome changes in NHL has been derived from the short term culture of lymph node biopsies, although some data has been obtained from fine needle aspirates (Kristoffersson, 1985). The fourth edition of the "Catalog of chromosome aberrations in cancer" (Mitelman, 1991) described some 1500 cytogenetically abnormal cases and the literature to date contains over 2000 cases. The majority of these are of B-cell immunophenotype, by far the most common form of NHL in the UK, and most of the information considered in this section was gained from the study of B-cell disease.

Although many different chromosome changes have been observed in NHL, only a limited number have been consistently found at an incidence of greater than 2%. Even in these cases, the rate of occurrence has varied widely from study to study. Table 1.2 (adapted from Offit and Chaganti, 1991) compares the incidence
rates of the most commonly occurring abnormalities in a range of large series. These recurring abnormalities fall into three groups. Aneuploidy of whole chromosomes, structural abnormalities of a single chromosome, such as deletions and inversions and finally translocations.

The significance of aneuploidy in malignancy is difficult to determine for several reasons. It seems unlikely that every gene on an extra chromosome is aberrantly active; it is also uncertain whether these are primary or secondary changes, playing either an active role in the genesis of NHL, by conferring a selective advantage on cells in which they occur, or resulting from a general genetic instability in the tumour. It may be that these abnormalities fulfil both these roles at different times in neoplastic development, since additional chromosomes 3, 7 and 12 have each been found as the sole aberration in small numbers of NHL (Mitelman et al, 1991b).

Structural abnormalities of single chromosomes, particularly deletions, may point to the possible presence of tumour suppressor genes (Jonveaux and Berger, 1990), whilst a chromosomal region consistently involved in rearrangements may be the site of an oncogene. Chromosome 6 in particular has been closely investigated in NHL (Barletta et al, 1987; Offit et al, 1993; Menasce et al, 1994) and data from these studies are considered below.

The t(8;14)(q24;q32) was the first recurring translocation to be associated with a lymphoproliferative disease (Manolov and Manolova, 1972). This translocation or one of its variant forms, the t(8;22)(q24;q11) or t(2;8)(p12;q24), has been found in nearly 100% of Burkitt lymphomas (Heim and Mitelman, 1987a) and rearrangements such as these have provided fundamental insights into the role that chromosome translocations play in malignant transformation. B (and T) cell tumours have large numbers of well characterised chromosome abnormalities that involve the "rearranging" genes (immunoglobulins and T cell receptors) and there is general agreement that errors in normal V-D-J joining result in the formation of this type of aberrant arrangement (Rabbits and Boehm, 1991).
Table 1.2 Incidence of recurring abnormalities in large series of NHL

<table>
<thead>
<tr>
<th>Series Reference</th>
<th>Number of Cases</th>
<th>t(14;18)</th>
<th>t(8;14)</th>
<th>t(11;14)</th>
<th>t(3;22)</th>
<th>6q</th>
<th>1q</th>
<th>1p</th>
<th>Structural</th>
<th>+7</th>
<th>+3</th>
<th>+12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fifth International Workshop, 1987</td>
<td>253</td>
<td>22</td>
<td>7</td>
<td>0.4</td>
<td>NR</td>
<td>17</td>
<td>5</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Offit et al, 1991a</td>
<td>278</td>
<td>29</td>
<td>15</td>
<td>4</td>
<td>3</td>
<td>19</td>
<td>29</td>
<td>19</td>
<td>5</td>
<td>12</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Bloomfield et al, 1983</td>
<td>94</td>
<td>23</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>31</td>
<td>20</td>
<td>24</td>
<td>19</td>
<td>12</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>Schouten et al, 1990a</td>
<td>104</td>
<td>26</td>
<td>2</td>
<td>5</td>
<td>NR</td>
<td>14</td>
<td>9</td>
<td>-</td>
<td>19</td>
<td>14</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Yunis et al, 1982 and 1984</td>
<td>82</td>
<td>34</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>17</td>
<td>30</td>
<td>18</td>
<td>18</td>
<td>9</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Juneja et al, 1990</td>
<td>147</td>
<td>47</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>29</td>
<td>19</td>
<td>18</td>
<td>11</td>
<td>19</td>
<td>16</td>
<td>13</td>
</tr>
</tbody>
</table>

NR = not recorded
The recombinase enzyme involved in class switching recognises 8 or 9 base signal sequences and it has been established that in some cases these sequences are involved on the chromosome not carrying the rearranging gene. However, in other examples, alternative mechanisms appear to be involved (Wyatt et al, 1992; Seite et al, 1993). Whatever the mechanism, a model in which chromosomal rearrangement at one of the rearranging genes erroneously involves a second chromosome, carrying a proto-oncogene at the breakage junction and which latter is activated to become an oncogene, seems generally valid. Chromosome studies of NHL have revealed a variety of examples, which are shown in table 1.3

**Table 1.3 Translocations which activate oncogenes in B-cell NHL**

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Oncogene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;14)(q24;q32)</td>
<td>MYC</td>
<td>Dalla-Favera et al, 1982</td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>BCL-2</td>
<td>Tsujimoto et al, 1985</td>
</tr>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>CCN-D1</td>
<td>Motakura et al, 1991</td>
</tr>
<tr>
<td></td>
<td>(BCL-1)</td>
<td></td>
</tr>
<tr>
<td>t(10;14)(q24;q32)</td>
<td>LYT-10</td>
<td>Neri et al, 1991</td>
</tr>
<tr>
<td>t(14;19)(q32;q24)</td>
<td>BCL-3</td>
<td>McKeithan et al, 1987</td>
</tr>
<tr>
<td>t(3;22)(q27;q11)</td>
<td>BCL-5</td>
<td>Miki et al, 1994a</td>
</tr>
<tr>
<td>t(3;14)(q27;q32)</td>
<td>BCL-6</td>
<td>Ye et al, 1993</td>
</tr>
</tbody>
</table>

Although most of these oncogenes are activated by being positioned adjacent to the immunoglobulin heavy chain locus (IgH) on chromosome 14q32, variant arrangements are also found, as described above for Burkitt lymphoma. The BCL-2 gene, for example, has also been detected in rearrangements with both the immunoglobulin kappa and lambda light chain loci, at 2p12 and 22q11 respectively (Hillion et al, 1991; Leroux et al, 1990). It should also be noted that BCL-3 is only rarely found rearranged in NHL (van Krieken, 1990).
1.3.3) Molecular biology of non-Hodgkin's Lymphoma

Those genes activated in the translocation events described above, have been studied, to determine what part their protein products may play in malignant transformation. The myc protein has several functional domains, including a binding region for DNA and two protein dimerization motifs. It functions in the context of a transcriptional network involving at least three other proteins and must dimerize with the max protein to become transcriptionally active. When this occurs, target genes downstream are activated (Rabbits, 1994). It is currently thought that MYC mediates the transfer of cells from G0 into either G1 or programmed cell death (apoptosis) (Wyllie et al, 1987; Askew et al, 1991; Evan et al, 1992).

BCL-2 had no sequence homology to any other known gene when first cloned (Tsujimoto, 1984a), but two related genes, BAX and BCL-X have since been identified (Oltvai et al, 1993; Boise et al, 1993). The gene product is mostly located on the inner mitochondrial membrane and acts to prevent the passage of cells into apoptosis, including that induced by MYC. It can thus act synergistically with MYC to prevent cell death and allow continuous cell cycling to take place (Vaux et al, 1988; Bissonnette et al, 1992). In NHL, high levels of the BCL-2 protein are normally found only in neoplastic germinal centre cells (Hockenberry et al, 1990).

Although the BCL-1 region was first cloned in 1984 (Tsujimoto et al, 1984b), there has been considerable debate about which particular gene or genes were activated, since none were found to be close to the translocation breakpoint. However, it now seems likely that PRAD-1, a gene identical to human cyclin D1, is the one activated by the rearrangement (Withers et al, 1991). Functionally, it appears to be involved in the G1 to S phase transition in the cell cycle (Motokura and Arnold, 1993).

The LYT-10 gene, now named NFkB-2 (Chang et al, 1994), is a member of a family of transcription factors implicated in the transcriptional control of a wide range of genes, including those controlling B and T cell activation. The BCL-3
gene shares some sequence homology to $NKFB-2$ (Nolan et al, 1993) and probably also acts as a transcription factor.

The final two genes in Table 1.3, $BCL-5$ and $BCL-6$, probably represent the independent discovery of the same entity. Cloned only recently, molecular analysis has revealed an identical structure, including an homology with zinc finger transcription factors. Expression of the gene has been found in 33% of diffuse large cell lymphomas (Ye et al, 1993) and in Burkitt lymphoma cell lines (Miki et al, 1994b).

Deletions and other alterations of chromosome 6q and chromosome 17p are common in NHL and have provoked interest in the possible involvement of oncogenes mapped to these locations. The $MYB$ proto-oncogene has now been localised to 6q23 (See section 5.3.2.2) and the $P53$ tumour suppressor gene maps to 17p13 (McBride et al, 1986). However, there is limited evidence to support the involvement of these genes (Barletta et al, 1987; Rodriguez et al, 1991).

1.3.4) Histological associations with karyotype.

In spite of considerable efforts from different centres, very few cytogenetic abnormalities have proved to be associated with a particular histologic sub-group of NHL. In one recent study, although 16 associations between karyotypic aberrations and a particular histologic subtype NHL were found, in none of the cases was the abnormality found in more than 30% of cases (Juneja et al, 1990). Even the t(14;18), which has a universally close association with follicular lymphoma, is found in different frequencies from study to study (see Table 1.2).

Furthermore, it has also been found in other sub-types at quite high rates; 18% in diffuse large cell lymphomas (Offit et al, 1991b) and in 54% of reactive lymph nodes by a sensitive molecular method (Limpens et al, 1991).

Possibly the only translocation with an invariable association with only one disease type in NHL is the t(2;5)(p23;q35), which occurs in most anaplastic large cell lymphomas arising from activated T lymphocytes (Ki-1 lymphoma) and is
not found elsewhere (Kaneko et al, 1989). Interestingly, a novel putative oncogene (ALK), has recently been identified at 2p23 in this rearrangement. (Morris et al, 1994)

1.3.5) Clinical implications of genetic abnormalities in NHL

A major aim in studying the genetics of malignancy has always been the hope of identifying changes that have prognostic significance for the course of the disease. In NHL for example, the ability to predict which patients with low grade disease were likely to transform to high grade, would have important implications for treatment. Table 1.4 summarises those correlations which have been found between chromosome abnormalities and prognosis in those centres at which adequate follow up data was available. In five out of the eight studies documented, abnormalities of chromosome 17 carried a poor prognosis. Four studies found changes in 1p carried a worse prognosis.

Three studies have analysed serial changes in the observed karyotypes with time (Sanger et al, 1987; Levine et al, 1990; Whang-Peng et al, 1995). In one case, the acquisition of trisomy 7 accompanied transformation of histologic grade. When taken together with the observation that trisomy 7 is found significantly more frequently in t(14;18)-bearing intermediate and high grade NHL when compared to low grade NHL (Yunis et al, 1987), the possibility that acquisition of trisomy 7 is associated with transformation to a higher grade must be considered.

1.4) MOLECULAR CYTOGENETICS

1.4.1) Introduction

Even when high resolution banding techniques visualising up to 1000 bands per human genome are employed, there is a huge disparity in resolution between cytogenetic and molecular methodology. Although the study of chromosomes can provide information on numerical abnormalities, structural aberrations are only
<table>
<thead>
<tr>
<th>Reference</th>
<th>Number</th>
<th>Histologic Type</th>
<th>Cytogenetic Abnormalities</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kristofferson et al, 1987</td>
<td>106</td>
<td>NS</td>
<td>&gt;10 clonal abnormalities, 1p+, +7</td>
<td>poor</td>
</tr>
<tr>
<td>Levine et al, 1988</td>
<td>69</td>
<td>NS</td>
<td>breaks at 17p, 17q, 1p32</td>
<td>poor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2p breaks</td>
<td>favourable</td>
</tr>
<tr>
<td>Cabanillas et al, 1989</td>
<td>102</td>
<td>NS</td>
<td>breaks at 17p or 7p, -17 or -7</td>
<td>poor</td>
</tr>
<tr>
<td>Schouten et al, 1990a</td>
<td>104</td>
<td>NS</td>
<td>+5, +6, +18, breaks at 17p or 17q, 5p or 5q, 14q11</td>
<td>poor</td>
</tr>
<tr>
<td>Yunis et al, 1987</td>
<td>71</td>
<td>follicular</td>
<td>+2, duplication 2p, breaks at 13q32</td>
<td>poor</td>
</tr>
<tr>
<td>Yunis et al, 1989</td>
<td>104</td>
<td>large cell</td>
<td>+2 or duplication 2p, -17 or deletion 17q, 18q21 rearrangement</td>
<td>poor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+3 or duplication 3</td>
<td>favourable</td>
</tr>
<tr>
<td>Offit et al, 1991b</td>
<td>104</td>
<td>DLCL</td>
<td>1q21-23 break or karyotypic complexity</td>
<td>poor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>low grade</td>
<td>breaks at 1p32-36, +7 or +12</td>
<td>poor</td>
</tr>
<tr>
<td>Tilly et al, 1994</td>
<td>66</td>
<td>follicular</td>
<td>breaks at 1p21-22, 6q23-26 or 17p, karyotypic complexity</td>
<td>poor</td>
</tr>
</tbody>
</table>

NS = not specified; DLCL = diffuse large cell lymphoma.
detectable down to a limit of approximately 5 megabases of DNA. The advantages of being able to investigate both genetic changes in the whole cell simultaneously and gathering data on the degree of heterogeneity in the population of cells under investigation maybe be out-weighed by the necessity of obtaining good quality mitotic figures, which can often be difficult in the study of neoplastic cells.

Molecular techniques such as Southern blotting (Southern, 1975) and polymerase chain reaction amplification (PCR) (Saiki et al, 1988) provide sensitive and specific methods for the investigation of defined structural and numerical genetic changes. However, it is not possible either to differentiate between the normal and malignant cells in a population or to derive specific relationships between phenotype and genotype.

In-situ hybridisation techniques, since they involve the annealing of nucleic acid probes to target sequences in which the morphology is preserved, provide a bridge between cytogenetic and molecular genetic methods. It is possible to apply this approach both to interphase nuclei and metaphase chromosomes, giving rise to the general description of molecular cytogenetics. A review of in situ technology and development is given in the following sections, accompanied by a consideration of ways in which the technique may be applied.

1.4.2) The origins of in-situ hybridisation

If the two strands of the DNA double helix are broken apart by treatment with heat or alkali, it is possible, under appropriate conditions, to anneal another complementary single DNA strand to a homologous sequence. Although this technique was developed by studying nucleic acids in solution, it has proved possible to hybridise DNA in solution (the probe) to DNA fixed on a supportive matrix (the target). When the hybridisation is performed in-situ, the target remains mostly within the tissue of origin. This is in contrast to a technique such
as Southern blotting, in which DNA is extracted from the tissue before hybridisation.

The early workers in this field labelled probes isotopically and detected the results with autoradiography (Pardue and Gall, 1969; John et al, 1969; Buongiorno-Nardelli and Amaldi, 1969). Although sensitive, this system has several disadvantages. Experiments are time consuming, mainly due to the long autoradiographic exposures required. Resolution is poor, since isotopic particles may travel in any direction through the photographic emulsion coating the slides before encountering a silver grain and the level of background is generally high, necessitating a lengthy statistical analysis of the results.

Since the early 1980's, novel methods of probe labelling, detection and imaging have been developed and a huge expansion in the use of non-radioactive in situ hybridisation has followed.

1.4.3) Probes and vectors

1.4.3.1) Single copy probes

Unique sequences are available as probes cloned into a variety of different vectors. Plasmids carry inserts of up to about 10 kb. Bacteriophage vectors take up to 24 kb and cosmids 35-45 kb although in the case of the former, vector sequences may make up the greatest part of the construct. Yeast artificial chromosomes (YACs) may contain between 200 - 1000 kb of cloned human DNA.

Although all these vectors may be used to carry probes for in-situ hybridisation, those with larger inserts are more likely to contain interspersed repetitive sequences (IRS) which are found throughout the human genome (Korenberg and Rykowski, 1988). During in-situ hybridisation these are likely to anneal non-specifically, obscuring the signal at the target sequence. The introduction of chromosome in-situ suppression hybridisation (CISSH) has allowed the
localisation of unique sequences contained within phage, cosmid and YAC vectors as well as in whole chromosome libraries (Landgent et al, 1987; Pinkel et al, 1988; Lichter et al, 1988). The technique involves the annealing of the labelled probe to unlabelled competitor DNA before hybridisation. The competitor is either total human DNA, sheared or sonicated to 50 - 500 bp in length, or human Cot 1 DNA. The latter is obtained by denaturing and then reannealing total human DNA under conditions which enrich highly repetitive sequences. Pre-annealing with either competitor results in the blocking of IRS within the probe and also, since excess unlabelled competitor is added to the reaction, within the target material.

1.4.3.2) Chromosome-specific repetitive probes
Chromosome-specific repetitive probes are now available for each human chromosome. DNA at or near the centromere of human chromosomes consists of one or more families of satellite DNA, of which of the alpha satellite DNA is the most common (Willard and Waye, 1987). These sequences consist of tandem arrays of repeated DNA motifs and therefore a probe for a particular consensus sequence will detect a multiplicity of hybridisation sites. When hybridised at the appropriate stringency, these centromere-specific probes produce discrete hybridisation signals either on chromosome homologues in metaphase preparations, or in interphase nuclei. They may therefore be used both as an aid to the identification of a specific chromosome and also to establish chromosome ploidy.

1.4.3.3) Chromosome painting probes
Complex mixtures of probes specific to a single chromosome type can be used to delineate that chromosome in metaphase and interphase. This has given rise to the term "chromosome painting". Collections of such probes have been derived from both somatic cell hybrids containing a single human chromosome and from flow

1.4.4 Probe labelling and detection

Probes may be detected either by direct labelling with a reporter molecule or indirectly, by labelling with a low molecular weight compound (a hapten), which is then detected using a variety of reporter molecules.

Although the earliest direct chemical coupling of fluorochromes to nucleic acids was achieved some 15 years ago (Bauman et al, 1980), this approach has not been easily employed until more recently, when fluorochrome-conjugated nucleotides became commercially available. Probes may now be labelled directly with fluorochromes and although this does not give such sensitivity as indirect methods, there are gains in speed, lower background and the potential of accurately quantifying the signal. Recent studies have reported the localisation of directly labelled probes of 50-100 kb (Wiegant et al, 1991; 1993).

For indirect techniques, the most commonly used haptens are biotin (Langer et al, 1980) and digoxigenin (Heiles et al, 1988). The latter has the advantage of being derived from plant material (Digitalis sp) and is therefore not indigenous to animal tissues. Both these haptens are available covalently linked to dNTPs via a flexible linker arm and may be incorporated into the probe by established enzymatic labelling methods such as nick translation, oligonucleotide primed synthesis or PCR. Nick translation is particularly appropriate for in-situ hybridisation, since the size of the labelled fragment may be controlled by the amount of DNase1 used in the reaction. Probe size has been found to be a critical factor in successful in-situ hybridisation, with an average of 300 bp being optimal (Lawrence and Singer, 1985). Other haptens have been incorporated into probe DNA by chemical modification. Examples are acetyaminofluorene (Landgent et al, 1984), sulphone (Viscidi et al, 1986) and mercury (Hopman et al, 1986).
However, since the advent of fluorochrome-conjugated nucleotides these techniques are rarely used.

A variety of reagents have been employed for the detection of haptenised probes. For biotin, either avidin or streptavidin (both of which possess a high affinity for biotin) have been used, conjugated either to enzymes such as peroxidase or alkaline phosphatase (Garson et al, 1987; Habeebu et al, 1990), or to fluorochromes (Pinkel et al, 1986). The enzyme conjugates are visualised by chemical reactions which generate a coloured precipitate; a brown diaminobenzidine product with peroxidase and a blue indole-formazan product with alkaline phosphatase. Both products are detected with conventional light microscopy. Fluorescent detection of biotin labelled probes is by the use of sequential layers of avidin-fluorochrome, biotinylated anti-avidin antibodies and avidin-fluorochrome (Pinkel et al, 1986). Digoxigenin is detected by specific antisera followed by a layer of fluorescently conjugated anti-immunoglobulins against the primary antibody (Lichter et al, 1990) The most widely employed fluorochromes are fluorescein isothiocyanate (FITC) emitting in the green, tetramethylrhodamine isothiocyanate (TRITC) and texas red (TR) both emitting in the red and aminomethylcoumarin acetic acid (AMCA) which emits in the blue. Newly available fluorophores include Cy 5 which, although emitting in the infra red, is visible to a CCD camera equipped with the appropriate filters (see below). As fluorescent detection systems have become more widely employed, the acronym FISH (fluorescence in-situ hybridisation) has become synonymous with the use of the technique.

1.4.5) FISH imaging

For many uses of FISH, an epifluorescence microscope equipped with appropriate filter sets is adequate. Modern instruments may be equipped with double and triple filter blocks, which enable more than one fluorochrome to be viewed simultaneously. Where only single filters are available, it is necessary to
make multiple exposures, employing each filter in turn, thus overlaying the
different images onto the same section of film. This may introduce registration
problems as a consequence of altering the optical pathway, since the dichroic
mirror in each filter block will not be at exactly the same angle to the camera light
beam. Errors in localising the signal may thus be produced.

More recently, confocal laser scanning microscopes and charge coupled device
(CCD) cameras have been used for detecting FISH signals.

The principal of confocal microscopy is the point illumination of the specimen by
a focused laser beam, combined with point detection using an aperture the size of
the illuminated spot. By then scanning across the specimen in a controlled
pattern, a high resolution image of a thin layer (an optical section) can be
recorded (Brakenhoff et al, 1985). Originally designed to facilitate the acquisition
of a three dimensional image of a specimen, confocal microscopy has been used
to successfully map single copy probes to chromosome bands (Albertson et al,
1991). The confocal microscope is about as sensitive as a conventional
epifluorescence microscope, but the ability to detect signal present at different
levels within a specimen may provide an enhanced capacity to detect low level
fluorescence. Accurate registration of different fluorescent images from the same
specimen is achieved by the simultaneous detection of signal from two separate
filter channels. The two images are exactly merged by computer software and
stored in memory until required for further analysis, providing a considerable
advantage over conventional photography.

The cooled CCD camera is many times more sensitive than the two instruments
described above. Originally devised for astronomical use, it is extremely sensitive
to photons over a wide range of wavelengths (400-1000 nm), using an array of
light sensitive CCDs to collect the fluorescent image (Hiraoka et al, 1989).
Photons striking the array are proportionately transduced to electrons for as long
as the shutter remains open and the resulting image information stored by
computer in digital form. An image for each fluorophore is collected separately.
via the appropriate filter and a complete pseudocolour picture is produced by software. Registration problems may be avoided by mounting the excitation filters on a wheel between the lamp and the microscope, eliminating the need to move filters through the light path of the camera.

1.5) THE APPLICATION OF FISH TO THE STUDY OF MALIGNANCY

1.5.1) Introduction

FISH technology is now widely employed to investigate many aspects of tumour genetics. In the context of cytogenetic studies perhaps the most important are:- the characterisation of derived (der) chromosomes, in which only part of the material is identifiable; the mapping and ordering of cloned DNA sequences; the detection of numerical and structural chromosome abnormalities; and the analysis of non-dividing cells, now termed interphase cytogenetics (Cremer et al, 1986). In many tumours, including NHL, this latter technique is proving very valuable, since it is often difficult to obtain metaphase preparations. Numerical and structural abnormalities are detectable and it is now possible to correlate cytogenetic abnormalities with cell phenotype in some cases (Price et al, 1992). The application of these technologies is discussed below.

1.5.2) FISH Mapping Techniques

1.5.2.1) Metaphase mapping

Previously, hybridised probes were localised by comparing their position either with G- or R-bands generated before or after the hybridisation experiment, (Cannizzaro and Emanuel, 1984) or with a standard karyogram (Lichter et al, 1990). DNA sequences can now be mapped directly onto metaphase chromosomes using simultaneous fluorescent banding techniques. Several methods for producing fluorescent banding are available, most of which involve
the incorporation of 5-bromodeoxyuridine (BrdU) into lymphocyte cultures before subsequent treatment with other chemicals (Albertson et al, 1991; Fan et al, 1990). Human DNA amplified with primers for the ALU or LINES repetitive sequences, labelled and used as a probe, results in R- or G-banding respectively (Baldini and Ward, 1991). More recently, the use of image processing software has allowed the production of G-bands from simple DAPI staining (Carter, 1994). The resolution of metaphase mapping appears to be limited to sequences separated by more than 1 Mb (Trask et al, 1991; Lawrence et al, 1990; 1992).

1.5.2.2) Interphase mapping
The use of chromosomes in interphase has increased the sensitivity of mapping and ordering probes. Hybridisation to G0 interphase nuclei from fibroblast cell lines has increased the resolution to 50-1000 kb. Sequences from the large and well characterised dystrophin gene have been used to demonstrate that there is a strong correlation between the interphase distance of pairs of hybridised probes and the linear distance within the 100-1000 kb range (Lawrence et al, 1990). Other recent work, using 13 probes from a 4-megabase pair region of the Huntington disease locus, supports these findings (van den Engh et al, 1992).

Other methods have been used to obtain finer resolution for interphase mapping. In one technique, drugs or alkaline treatment are utilised to release strands of chromatin from nuclei (Heng et al, 1992). A resolution of 21 kb was obtained using five cosmids from the cystic fibrosis transmembrane regulator gene and gave a good correlation with the physical map. Another approach employs detergent and high salt extraction to create nuclear halos. Hybridisation signals appeared as "beads on a string" and it was possible to detect overlaps between cosmid clones within the range 10-200 kb (Wiegant et al, 1992). The two most recent techniques are less complex to carry out. In one method, detergent was used to produce long strands of linear DNA down a tilted microscope slide (Parra and Windle, 1993). In the other method, either alkali or formamide were applied
to previously fixed material and the DNA was linearized by pulling a coverslip along the slide (Fidlerova et al, 1994). In both cases a good correlation with the expected physical distances was obtained.

1.5.3) Identification of Derived Chromosomes

1.5.3.1) Chromosome painting

FISH with whole chromosome paint probes allows the accurate identification of structural chromosome abnormalities in metaphase cells. Several methods of constructing such libraries are now available. Those originally used for painting were flow sorted from hybrid cell lines containing small numbers of human chromosomes. Each paint consisted of many sub-clones representing a specific sorted chromosome (Fuscoe et al, 1989; Collins, 1991). Chromosome paints derived in this way are now commercially available for each human chromosome and have made the use of the technique widely available. Many groups of workers have combined G-banding and chromosome painting to characterise derived chromosomes in a variety of tumour material. Some recent applications have been in prostatic cancer (Brothman and Patel, 1992), NHL (Bajalica et al, 1993) and ovarian carcinoma (Xu and Wang, 1994).

1.5.3.2) Ratio-labelling and reverse chromosome painting

The application of chromosome paints can be laborious and time consuming because of the numerous combinations of probes required. However, recent developments in ratio-labelling, in which probes are labelled with varying ratios of different haptens and detected using combinations of fluorescent affinity reagents, may make this less tedious in future. One group used this approach to simultaneously paint half the human chromosome complement in twelve different colours (Dauwerse et al, 1992).
An alternative strategy has been to flow sort the abnormal chromosome, which is then amplified by PCR and hybridised to normal metaphase spreads. Degenerate oligonucleotide primed (DOP) PCR (Telenius et al, 1992a) is used for the amplification step. This method randomly amplifies any DNA and therefore requires relatively few chromosomes (200-500) to produce a whole chromosome paint which is representative of the abnormal chromosome. When this is labelled and painted back onto normal human chromosomes, the cytogenetic origins of the abnormal chromosome will be revealed. This method is thus "reverse" chromosome painting (Carter et al, 1992). The technique requires both the availability of a cell line containing the abnormal chromosome and skilled flow sorting. Reverse painting has been used to characterise translocations involving the locus for multiple endocrine neoplasia type 2A (Telenius et al, 1992b) and for the analysis of constitutional karyotypes (Carter et al, 1992; Rack et al, 1993).

1.5.3.3) Micro-FISH

Although multicolour FISH analysis has made significant advances, simultaneous detection of 24 colours, required to delineate the whole human karyotype is still not possible. Additionally, painting probes give no information with regard to which specific region of a particular chromosome is involved. A recent development makes it possible to obtain region-specific probes for any part of the human genome.

The technique combines microdissection of human chromosomes with direct enzymatic amplification of the micro-dissected DNA fragments (Meltzer et al, 1992). Although other groups have used micro-dissection to generate chromosome specific libraries (Lengauer et al, 1991; Trautman et al, 1991), they directly cloned the fragments using a difficult microcloning method (Ludecke et al, 1989). The micro-FISH procedure of Meltzer and colleagues is simpler and faster. 25-30 micro-dissected fragments are cut from the region of interest and DOP-PCR amplification is carried out. After purification, the PCR products are
labelled with biotin and hybridised to normal human metaphases, revealing the chromosome location(s) of the region of interest. The technique has been used to identify a translocation and deletion chromosome in a melanoma cell line (Meltzer et al, 1992) and more recently to demonstrate that terminal deletions of 6q are telomeric translocations (Meltzer et al, 1993) and to detect variant Philadelphia translocations in chronic myeloid leukaemia (Zhang et al, 1993).

1.5.4) Interphase cytogenetics

1.5.4.1) Detection of structural abnormalities in interphase cells

Although the detection of structural abnormalities in interphase nuclei is possible by chromosome painting, problems of interpretation are possible due to the overlap of extended chromosome domains (Pinkel et al, 1988). Detection of specific chromosome translocations in interphase cells is more accurately achieved by the use of locus specific probes, but this is only possible in cases where the translocation has been characterised at a molecular level.

Dual colour FISH, using probes which flank specific translocation breakpoints, has been successfully employed in the interphase cells of haematological malignancies to detect the Philadelphia translocation in chronic myeloid leukaemia (CML) (Arnoldus et al, 1990; Tkachuk et al, 1990) and to investigate rearrangements of the CCND1, BCL-2 and BCL-3 genes in CLL (Amiel et al, 1994). Translocations were detected by the presence of a coalesced "orange" (red + green) signal.

In the solid tumour field, several groups have examined the breakpoint of the t(11;22)(q24;q11.2) associated with Ewing's sarcoma (Giovanni et al, 1992; Taylor et al, 1992). Giovanni and colleagues employed single colour FISH with cosmids from either side of the 11q24 breakpoint. When the translocation was present, the hybridisation pattern showed two signals widely separated and two signals very close together. Taylor and co-workers used two colour FISH with
cosmids that flanked the 22q11 breakpoint. Although dual colour is theoretically more accurate, since it ensures that the distance between two different probes is measured, both groups found approximately 50% of nuclei to be evaluable, with no false positives.

1.5.4.2) Detection of numerical chromosome abnormalities
Labelled chromosome-specific centromere probes are now available for all human chromosomes. Since individual chromosomes occupy discrete and relatively compact domains within interphase nuclei (Haaf and Schmid, 1991), these alphoid satellite sequences generate strong and discrete hybridisation signals within interphase nuclei. Such probes have now been used successfully to enumerate chromosome copy number in a range of solid tumours and haematological malignancies. Those solid tumours investigated include breast tumours (Devilee et al, 1988), ovarian carcinoma (Nederlof et al, 1989) and brain tumours (Arnoldus et al, 1991). Amongst the haematological malignancies that have been studied, chronic lymphocytic leukaemia (CLL) was investigated for the presence of trisomy 12 (Anastasi et al, 1992). The abnormality was detected in 30% of cases, including those which were unsuccessful or had shown a normal karyotype by conventional cytogenetics.

1.5.4.3) Correlation of chromosome abnormalities with cell type
It has recently proved possible to identify the specific cell type in which particular chromosome abnormalities are occurring. One approach has used previously stained and morphologically classified peripheral blood or bone marrow cells. After photography, the cells were subjected to FISH, relocated and re-photographed (Anastasi et al, 1991).

In many cases, however, immunophenotyping is necessary to classify the cells of interest. A new method for the simultaneous detection of immunophenotype and karyotypic abnormalities employed the finding that the alkaline phosphatase anti
alkaline phosphatase (APAP) technique is resistant to the harsh pretreatment required for FISH. When Fast Red was used as a chromogen in the APAP procedure, both immunophenotype and chromosome copy number were detectable in the same cell (Price et al, 1992). Using this method to study polycythaemia vera, Price and colleagues established that myeloid and erythroid cells were trisomy 8 positive but not B- and T-lymphocytes. More recently, this approach has been applied to studies of acute myeloid leukaemia (AML) (Baurmann et al, 1993) and CLL (Garcia-Marco et al, 1994).

1.5.5) Comparative Genomic Hybridisation (CGH)

A completely novel approach to the global identification of chromosome copy number has recently been described, which requires no previous knowledge of the tumour karyotype. The CGH technique (Kallioniemi et al, 1992; du Manoir et al, 1993) is carried out with DNA extracted from tumour cells and normal reference DNA. Tumour DNA is labelled with biotin and normal DNA with digoxigenin; after mixing with unlabelled Cot I DNA, hybridisation is carried out to normal human metaphase cells. Biotinylated probe is detected with avidin-FITC (green) and digoxigeninylated probe with anti-digoxigenin-rhodamine (red). Gene amplification in the tumour generates a relative increase in the green to red ratio at the appropriate locus on the target metaphase. Similarly, a deletion in the tumour will be indicated by an increase in the red to green ratio, since each DNA has an equal chance of hybridising to each locus of the target chromosomes. The signals are quantitated by a digital image analysis system, which generates a fluorescence ratio profile for each target chromosome examined.

At present, the system will detect a change in copy number of DNA from 10-20 Mb in length, about the size of a chromosome band (Kallioniemi et al, 1994). Contamination of the tumour DNA by normal tissue is a potential problem, because if this exceeds 50% the results cannot be considered reliable (Kallioniemi et al, 1994). It is also noteworthy that balanced translocations are not detectable.
by this method, since this does not lead to a relative difference in DNA content between the tumour and the normal reference.

Several solid tumours have now been evaluated by CGH. These include liposarcoma (Suijkerbuijk et al, 1994), small cell lung cancer (Reid et al, 1994), malignant glioma (Schröck et al, 1994), prostate cancer (Cher et al, 1994) and uveal melanoma (Speicher et al, 1994). Extrachromosomal gene amplification in AML has also been characterised by CGH (Mohamed et al, 1993). This technique also has potential for the analysis of archival material, since it has proved possible to use material from paraffin embedded solid tumours for CGH experiments, in which DNA was extracted from tissue sections and amplified using DOP-PCR (Speicher et al, 1993).

1.6) AIMS OF THE STUDY

The primary task of the project was to analyse the malignant cell chromosomes in a series of NHL lymph node biopsy samples from the United Kingdom. Initially, this was carried out by conventional G-banding. By collating data on numerical and structural chromosome changes, it was hoped to establish a relationship between these changes and the genetic aetiology of NHL.

It was envisaged, however, that conventional cytogenetic analysis would not be adequate for the complete characterisation of all chromosome changes detected in the series. The FISH technique was therefore adopted for the further analysis of cytogenetic aberrations, which involved the optimisation of the technique for application to NHL material.

Numerical aberrations were studied by interphase cytogenetics, using chromosome specific centromere probes. Structural abnormalities were investigated by the application of both unique sequence and chromosome painting probes. Dual colour hybridisation was carried out whenever possible, in order to increase the information obtained from individual NHL cells.
CHAPTER TWO

MATERIALS AND METHODS
HEALTH AND SAFETY

All biological specimens were processed in safety cabinets. Laboratory coats and gloves were worn both when handling biological specimens and during all molecular biology procedures.

All practical work followed the health and safety requirements for the laboratory recommended by COSHH (Control of Substances Hazardous to Health).

Before conducting any procedure involving recombinant DNA, a genetic manipulation medical was attended.
2.1) MATERIALS

2.1.1 Clinical samples

2.1.1.1) Lymph node biopsies

Samples from lymph node biopsies were made available from both Weston Park Hospital (WPH) and the Royal Hallamshire Hospital (RHH). After a pathologist had made an initial assessment of the biopsy, a piece of tissue was placed in culture medium and kept at 4°C until taken to the Centre for Human Genetics for processing, which took place within two hours of surgery. Details of diagnosis were obtained after the samples were processed. A pathologist at either WPH or RHH examined stained paraffin sections of the biopsy microscopically, in order to determine the lymph node architecture and the nature of the cells involved. In some 10-15% of cases, immunohistochemistry was also applied, particularly to distinguish between B and T-cell lymphomas. Those samples classified as NHL were analysed cytogenetically and the patient details obtained for each case. This information is summarised in Table 2.1.

2.1.1.2) Peripheral blood

Samples of peripheral blood from volunteers were collected into lithium-heparin tubes and processed as described below.

2.1.2) Tissue culture

Solutions were stored at 4°C unless otherwise stated.

2.1.2.1) Culture media

Both TC199 and HAM-F10 media were from GIBCO Life Technologies Ltd. Supplements were added to the media and these are listed below.
<table>
<thead>
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<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Staging at presentation</th>
<th>Treatment before biopsy</th>
<th>Diagnosis</th>
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Abbreviations: CC—intravenous administration of combination chemotherapy, ND—not determined, NT—no treatment, OC—oral administration of chlorambucil, OCP—oral administration of chlorambucil and prednisolone, RT—radiotherapy.
Human serum (Blood Transfusion Service; see section 2.1.2.3)
Penicillin (Glaxo)
Streptomycin (Glaxo)
L-Glutamine (Sigma)
25mM Hepes buffer (Gibco)

2.1.2.2) Phytohaemagglutinin (PHA)
PHA from Gibco was prepared by adding 10ml sterile distilled water to the
lyophilised M form of the chemical.

2.1.2.3) Human serum
Pooled human serum was obtained from Sheffield blood donation centre and
sterilised with a 0.2μ filter. 20ml aliquots were stored at -20°C.

2.1.2.4) Thymidine
Thymidine was prepared by dissolving 300mg in 10ml sterile distilled water with
occasional stirring and was then filter sterilised.

2.1.2.5) Phosphate buffered saline (PBS)
One PBS tablet (Oxoid Ltd) was dissolved in 100ml distilled water, aliquoted and
sterilised by autoclaving.

2.1.2.6) Deoxycytidine
A solution of 22.7mg/ml in sterile PBS was stored in 1ml aliquots at -20°C.
2.1.3) Cytogenetics

Stock solutions were stored at 4°C unless otherwise stated.

2.1.3.1) Colcemid

A stock solution of colcemid (Gibco) was prepared by reconstituting 100 μg of sterile lyophilised powder with 10 ml sterile distilled water.

2.1.3.2) Hypotonic solutions

Stock hypotonic solutions were prepared by adding 5.59 g or 3.19 g KCl each to 1 l distilled water to give 0.075 M and 0.0375 M solutions respectively. The former was used in lymph node culture and the latter in blood cell culture.

2.1.3.3) Fixative

Methanol and glacial acetic acid were freshly mixed 3:1 when required.

2.1.3.4) Slides

One side double frosted slides (Horwell Ltd) were soaked overnight in Decon at 4°C and then washed under hot and cold tap water. Once clean, slides were kept at 4°C in distilled water until required, which was usually the same day.

2.1.3.5) Blotting paper

Fibre free Postlip papers (Hollingsworth and Vose) were used.

2.1.3.6) Gurrs buffer

One Gurrs buffer tablet pH6.8 (BDH) was dissolved in 100 ml sterile deionised water with constant stirring and stored at room temperature.
2.1.3.7) Sorensens buffer
9.47g disodium hydrogen phosphate and 9.08g potassium dihydrogen phosphate were dissolved in 11 deionised distilled water, and stored at room temperature.

2.1.3.8) Leishmans stain
Stock solutions were prepared by adding 2.4g Leishmans powder (BDH) to 1.61 methanol. The mixture was stirred constantly on a hot plate until boiling, then removed from the heat and stirred for a further two hours at room temperature. The solution was then filtered through Whatman No. 1 filter paper into a glass beaker and stored at room temperature until use. For staining, the stock solution was diluted 1:4 with Gurrs Buffer.

2.1.3.9) Trypsin solution
Trypsin for G-banding was prepared by dissolving 0.6g Trypsin 1:250 powder (Difco) in 500ml Sorensens buffer with constant stirring. The stock solution was dispensed into 10ml aliquots and stored at -20°C.

2.1.3.10) Lipsol solution
Lipsol liquid concentrate (Lip Ltd) was diluted to 1% with Normal Saline (4.38g NaCl in 500ml distilled water) when required.

2.1.3.11) Borate buffer
3.55g 50mM Na2SO4 and 0.48g Na2B4O7 were dissolved in distilled, deionised water and the pH adjusted to 9.2. The stock was stored at room temperature.

2.1.3.12) Wrights giemsa stain
Wrights Stain was obtained ready prepared from Sigma and stored at room temperature in the dark. Immediately before use, the stock solution was diluted 1:2 in Borate Buffer.
2.1.3.13) Immersion lens oil
Lenzol immersion oil was obtained from BDH

2.1.4) Molecular biology

2.1.4.1) Strains of Escherichia Coli
E. coli JM109 (Yanisch-Perron et al, 1985) was used for plasmid work. E. coli LE392 (Murray et al, 1977) was used with Lambda phage. Glycerol stocks of the strains (Promega) were stored at -20°C.

2.1.4.2) DNA clones
Details of the DNA clones used are given in Table 2.2 below. All inserts were of genomic DNA.

2.1.4.3) Gene libraries
In addition to those libraries detailed in Table 2.2, commercially available chromosome paints were obtained (Cambio, Cambridge) for chromosomes X, 3, 6, 8, 10 and 11. These consisted of PCR products with biotin label already incorporated. A pan-centromere probe also labelled with biotin was obtained from Alpha Lab.

2.1.4.4 Antibiotics
Ampicillin stock (50mg/ml) was made up in deionised water and filter sterilised. Tetracycllin stock (5mg/ml) was made up in ethanol. Both antibiotics were obtained from Glaxo. Stocks were stored in light-proof containers at -20°C.
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</tr>
</tbody>
</table>

ATCC: American Type Culture Collection
2.1.4.5) Luria-Bertani (LB) medium
For one litre, 10g Bacto-Tryptone (Difco), 5g Bacto Yeast extract (Difco) and 5g NaCl were added to 950ml deionised water. The pH was then adjusted to 7.0 and the solution was made up to 1L with deionised water before finally being autoclaved. If solid media was required, 15g Agar (Difco) was added before autoclaving.

2.1.4.6) Calcium Chloride solution (For preparation of competent cells)
4.41g CaCl₂, 1.51g PIPES and 75ml glycerol were made up to 500ml with deionised H₂O and the pH was adjusted to 7.0. The solution was filter sterilised and then stored at room temperature.

2.1.4.7) Glycerol solution (For preparation of E.Coli stocks)
0.2g MgSO₄ and 0.3g Tris were dissolved in 100ml 65ml Glycerol/35ml deionised H₂O. The solution was filter sterilised and stored at room temperature.

2.1.4.8) STET buffer
5.8g NaCl, 1.21g Tris and 0.34g EDTA were added to 995ml distilled H₂O and the solution was autoclaved. 5ml Triton X-100 was added after the other constituents had cooled from autoclaving.

2.1.4.9) 1M Tris stock
121.1g Tris base was dissolved in 800ml H₂O, adjusted to pH 8.0 with concentrated HCl and the volume made up to 1 litre with H₂O. The solution was autoclaved and stored at room temperature.
2.1.4.10) 0.5M EDTA stock
186.1g disodium ethylenediaminetetraacetate.2H2O (EDTA) was added to 800ml H2O. While stirring on a magnetic stirrer the pH was adjusted to 8.0 with NaOH. When all the EDTA was dissolved, the solution was autoclaved and stored at room temperature.

2.1.4.11) TE buffer
10ml of 1M Tris stock and 2ml 0.5M EDTA stock were added to 988ml dH2O

2.1.4.12) Lysozyme
A stock solution was prepared at a concentration of 50 mg/ml in sterile deionised H2O, dispensed into aliquots and stored at -20°C. Aliquots were discarded after use.

2.1.4.13) DNAase free RNAase
Pancreatic RNAase (RNAase A) was dissolved to a concentration of 10mg/ml in 10mM Tris (pH 7.5), 15mM NaCl, heated at 100°C for 15 minutes and allowed to cool slowly to room temperature. 100μl aliquots were stored at -20°C.

2.1.4.14) Materials for Maxi preparations of plasmid DNA
For large scale preparation of plasmid DNA, a Qiagen-tip 500 column was used with the buffers supplied in the Qiagen kit. The composition of these buffers is shown below.

Buffer P1 :- 100μg/ml RNAase A, 50mM Tris-Cl, 10mM EDTA pH 8.0, stored at 4°C.

Buffer P2 :- 200mM NaOH, 1%SDS.
Buffer P3: - 2.55M potassium acetate pH 4.8

Buffer QBT: - 750mM NaCl, 50mM MOPS, 15% ethanol pH 7.0, 0.15% Triton X-100.

Buffer QC: - 1.0M NaCl, 50mM MOPS, 15% ethanol pH 7.0.

Buffer QF: - 1.25M NaCl, 50mM MOPS, 15% ethanol pH 8.2.

2.1.4.15) Phenol: chloroform: isoamyl alcohol (25:24:1)
Liquefied phenol was stored at -20°C. For equilibration, the solution was allowed to warm to room temperature and then melted at 65°C. An equal volume of 0.5M Tris was added, the solution stirred for 15 minutes and the phases allowed to separate. The upper (aqueous) phase was removed and the procedure repeated with 0.1M Tris. The equilibrated phenol was then mixed with an equal volume of chloroform: isoamyl alcohol (24:1) and stored under 0.1M Tris in a light proof bottle at 4°C.

2.1.4.16) Restriction enzymes and molecular size markers
Enzymes, lambda ladder and kilobase ladder were supplied by Gibco-BRL and stored at -20°C. Restriction enzymes were used in the recommended manufacturers buffers.

2.1.4.17) 5x Tris-borate (TBE)
54g Tris base, 27.5g boric acid and 20ml 0.5M EDTA (pH 8.0) were added to 800 ml distilled water, stirred well and made up to one litre with distilled water.
2.1.4.18) 10x Loading buffer
20% Ficoll 400, 0.1M Na2EDTA, 1% SDS and 0.25% bromophenol blue. 10ml was made up and stored in 1ml aliquots at room temperature.

2.1.4.19) Electrophoresis tanks and power packs
Standard and midi tanks were from BRL. Mini tanks and power packs were from Bio-rad.

2.1.4.20) Ethidium Bromide (EtBr)
A stock solution of 1mg/ml was prepared by adding one EtBr tablet (Bio-rad) to 11 ml distilled H2O and was then stored at 4°C.

2.1.4.21) DNA purification kit
A Prep-A-Gene kit from Bio-Rad was used to purify DNA from agarose gel electrophoresis. In addition to the binding matrix, the contents of which were unspecified by the manufacturers, the kit contained the following buffers, described below.

Binding buffer: - 50mM Tris, 1mM EDTA, 6M NaClO4, pH 7.5

Wash buffer: - 40mM Tris, 4mM EDTA, 0.8M NaCl, pH 7.4

Elution buffer: - 10mM Tris, 1mM EDTA, pH 8.0

2.1.4.22) Sephadex G-50
10g Sephadex beads were slowly added to 160ml distilled H2O. The swollen resin was then washed twice with fresh water and then equilibrated in TE (pH 7.6), autoclaved and stored at room temperature.
2.1.4.23) Gel photography

Electrophoresis gels were photographed with a Polaroid MP4 Land Camera using 667 ASA 300 film (Polaroid)

2.1.5) In-situ hybridisation

2.1.5.1) Nick translation

Two kits, both supplied by BRL, were used to label DNA probes with haptens. For biotin labelling, the "Bionick" kit was employed. For labelling with digoxigenin the Nick Translation System (8160SB) was used. Appropriate components of each kit are listed below.

8160SB

*Solution A4* (10x nucleotide mix) 0.2mM dATP, dCTP and dGTP, 500mM Tris (pH 7.8), 50mM MgCl₂, 100mM 2-mercaptoethanol and 100 µg/ml nuclease free BSA.

*Solution C* (10x enzyme mix) 0.4 U/µl DNA Polymerase I, 40 pg/µl DNAse I, 50mM Tris (pH 7.5), 5mM magnesium-acetate. 1mM 2-mercaptoethanol, 0.1mM phenylmethylsulfonyl fluoride, 50% (v/v) glycerol and 100 µg/ml nuclease free BSA.

*Solution D* (stop buffer) 300mM Na₂EDTA (pH 8.0)

Bionick

10x dNTP mix 0.2mM dCTP, dGTP and dTTP, 0.1mM dATP, 0.1mM biotin-14-dATP, 500mM Tris (pH 7.8), 50mM MgCl₂, 100mM 2-mercaptoethanol and 100 µg/ml nuclease free BSA.
10x enzyme mix 0.5 U/μl DNA Polymerase I, 0.0075 U/μl DNAse I, 50mM Tris (pH 7.5), 5mM magnesium-acetate. 1mM 2-mercaptoethanol, 0.1mM phenylmethylsulfonyl fluoride, 50% (v/v) glycerol and 100 μg/ml nuclease free BSA.

Stop buffer 300mM Na₂EDTA (pH 8.0)

20x Digoxigenin mix
For labelling with digoxigenin, a 50μl stock, containing 0.1mM digoxigenin-11 -dUTP (5μl of a 1mM stock, Boehringer) and 0.3mM dTTP (1.5μl of a 10mM stock, Sigma) was made in sterile distilled water and stored at -20°C.

2.1.5.2) Hybridisation mix
One gram dextran sulphate was dissolved in a solution which comprised 1ml 20x SSC, 5ml formamide, 1ml 10% Tween 20 (Pierce) and 3ml H₂O. All solutions were filter sterilised before mixing. The pH was adjusted to 7.0 and 100μl aliquots were stored at -20°C.

2.1.5.3) Rubber solution
Cover slips were sealed during hybridisation with Neoprene (Phillips, Manchester).

2.1.5.4) 20x SSC stock solution
175.3g NaCl and 88.2g sodium citrate made up to 1L with distilled H₂O and the pH was adjusted to 7.0. The solution was then autoclaved.

2.1.5.5) SSCT
4x SSC containing 0.05% Tween 20, adjusted to pH 7.0.
2.1.5.6) SSCTM
SSCT containing 5% non-fat dried milk (Marvel, Cadburys).

2.1.5.7) Detection reagents
The following chemicals were used to visualise hybridisations. Fluorescein-labelled Avidin DCS and Biotinylated anti-Avidin D (both from Vector) and Anti-digoxigenin-rhodamine Fab fragments (Boehringer Mannheim).

2.1.5.8) Slide mountant
Citifluor AFI (Citifluor, London) was used, with either propidium iodide (0.5 μg/ml) or DAPI (0.1 μg/ml) (both from Sigma) as a counter stain.

2.1.5.9) Borate buffer
A 500 ml batch of buffer containing 50mM Na₂SO₄ (3.55g) and 2.5mM Na₂B₄O₇ (0.48g) was made with distilled water, adjusted to pH 9.2, and stored at room temperature.

2.1.5.10) Competitor DNA
Human placental DNA sonicated to ~500bp in length or human COT-1 DNA sonicated to between 70 and 300bp (GibcoBRL) were used.

2.1.5.11) Pepsin stock solution
One gram of pepsin (Sigma) was dissolved in 10ml dH₂O and stored at -20°C in 100μl aliquots.

2.1.5.12) Transfer RNA
25mg E.Coli transfer RNA (Sigma) were dissolved in 2ml distilled H₂O and 50μl aliquots stored at -20°C.
2.1.5.13) Salmon sperm DNA
50μl aliquots of sonicated salmon sperm DNA at 10mg/ml were stored at -20°C.

2.1.5.14) Dot blot assays
A BluGENE non radioactive nucleic acid detection system (BRL) was used. The kit contained the following reagents.

**SAAP conjugate** 1mg/ml streptavidin-alkaline phosphatase in 3M NaCl, 1mM MgCl₂, 0.1mM ZnCl₂, 30mM triethanolamine (pH 7.6)

**NBT** 75mg/ml nitroblue tetrazolium in 70% dimethylformamide

**BCIP** 50mg/ml 5-bromo-4-chloro-3-indolylphosphate in dimethylformamide

**Biotinylated DNA** 0.2μg/μl biotinylated lambda DNA, 0.2μg/μl sheared herring sperm DNA in 6x SSC.

**DNA dilution buffer** 0.1μg/μl sheared herring sperm DNA in 6x SSC.

2.1.6) General chemicals

2.1.6.1) Water
All aqueous solutions were prepared using either deionised or distilled water. Deionised water was prepared by passing tap water through two PWT Permutit 250 deionisers. Double distilled water was from an Aquatron Bibby still.

2.1.6.2) Reagents
All chemicals were of at least analar grade. Unless otherwise stated, reagents were supplied by Sigma Chemical Company, BDH Chemicals Ltd or Fisons Scientific. Storage followed the manufacturers recommendations.
2.1.7) General laboratory consumables

2.1.7.3) Automatic pipettes and tips
Gilson "Pipetman" pipettes and Treff tips were used.

2.1.7.4) Disposable plastics
Syringes and hypodermic needles were from Becton-Dickinson, capped test-tubes and universals were from Falcon Plastics. Petri dishes and plastic pasteur pipettes were from Sterilin and microfuge tubes were from Sarstedt.

2.1.7.5) Glassware
Pyrex glassware was washed with detergent, rinsed twice with tap water, once with deionised water and then dried in a hot air cabinet. Glassware required for microbiological work was then autoclaved.

2.1.8) Equipment

2.1.8.1) Centrifuges
An MSE Centaur 2 was used for tissue culture and cytogenetics procedures. For molecular biology techniques either an IEC Centra or a Beckman J2 were used. Microcentrifugation was carried out with an Eppendorf microcentrifuge.

2.1.8.2) Heating equipment
Heating blocks and water baths were from Techne. The shaking incubator was from New Brunswick Scientific Co., and the incubator from Harvard/LTE. A Memmert high temperature oven and a Tricity microwave were also used.
2.1.8.3 Sterilising equipment
For autoclaving, a Rodwell series 32 was used for 20 mins at 121°C, 15 psi. For filter sterilisation, antipyrogenic 0.22 micron disposable syringe filters (Millipore) were used.

2.1.8.4) Surgical scissors
Curved scissors from Gallencamp were sealed in aluminium foil, autoclaved and stored wrapped until required.

2.1.8.5) Measurement of absorbance
A Jenway 6050 colorimeter was used for monitoring the growth of E. Coli cultures.
A Cecil series 2 spectrophotometer was used for DNA estimation.

2.1.8.6) Transilluminator
A model TM20 was used, obtained from UVP inc, Cambridge, UK.

2.1.9) Microscopy

2.1.9.1) Photomicroscopy
For standard cytogenetics, an Olympus BH2 microscope and automatic camera system was used, with Agfa Copex PAN AHU 35mm film (Agfa Gevaert). For fluorescence in-situ hybridisation (FISH) a Leitz Laborlux 12 equipped for epifluorescence and fitted with filters for FITC, rhodamine and DAPI was utilised. Photographs were taken with an MPS 46/52 camera system, using 640T colour slide film (Scotch). Film was stored at 4°C until use and developed within 48 hours of exposure.
2.1.9.2) Digital imaging microscopy

A KAF 1400 cooled CCD camera (Photometrics) attached to an Axioskop microscope (Zeiss) equipped for epifluorescence and fitted with both a multi-bandpass transmission filter and an external filter wheel carrying excitation filters for FITC, Rhodamine and DAPI was used. Images were captured and recorded using Smartcapture software (Digital Scientific Instruments, Cambridge, UK) running on a Quadra 840AV computer (Apple Macintosh).

2.1.9.3) Photographic papers

Kodakrome II RC monochrome F1M, F2M, F3M and F4M papers (Eastman Kodak), sizes 5x8 in. and 8x10 in., were used.

2.1.9.4) Processing

Developers. For films, G141C (Agfa Gevaert) was stored at 4°C and diluted 1:4 in warm tap water for use. For papers, Dektol powder (Kodak) was dissolved in 800ml water at 38°C with constant stirring and made up to 1 litre. In use, the solution was diluted 1:2 in water at 20°C.

Fixer. Kodak Unifix solution was diluted with water 1:4 for film and 1:9 for papers.

Colour film was processed commercially (Propix, Sheffield, UK).

2.1.9.5) Enlarger

An Autofocus Focomate V35 (Leitz) was used for exposing negatives to paper.
2.2) METHODS

2.2.1) Tissue culture

2.2.1.1) Peripheral blood

A 0.5 ml aliquot of heparinized venous blood was added to 10ml TC199 medium, (which contained 2% PHA and 20% Human serum and was supplemented with L-glutamine, sodium carbonate, penicillin and streptomycin) and incubated at 37°C. After 48 hours, thymidine was added to a final concentration of 0.3mg/ml and this block released 18 hours later by the addition of deoxycytidine to a final concentration of 0.3µg/ml. 4 hours later, colcemid was added to a final concentration of 0.1µg/ml and after 20 mins the culture harvested. The cell suspension was centrifuged at 1000 rpm at room temperature. The supernatant was discarded and the pellet resuspended in 0.075M KCl, prewarmed to 37°C and added dropwise, whilst continuously shaking the tube. The suspension immediately centrifuged and the supernatant removed. The cells were fixed by the dropwise addition of 5ml 3:1 methanol:glacial acetic acid whilst the tube was agitated. The suspension was pelleted and the procedure repeated until the fixative remained clear. After a final fixation the cell suspension was stored at -20°C until slides were prepared.

2.2.1.2) Lymph node

Biopsy material was transported to the laboratory in culture medium and four 10ml aliquots of F10 medium (containing 10% human serum and supplemented with 25mM Hepes buffer and L-glutamine) in universals were prewarmed at 37°C. The tissue sample was removed from the transport medium, placed in the lid of a petrie dish and moistened with pre-warmed F10. Curved scissors were used to cut up the specimen, which was then transferred with a pasteur pipette to 10ml F10 at 37°C.
If the sample was small, the transport medium was spun down, any pelleted cells were resuspended in F10 medium and added to the main suspension. If the specimen was large (greater than 1cm³), part of the material was removed with a scalpel and stored at -20°C before setting up the remainder.

An aliquot of the main suspension was used to set up three cultures (1,2 and 3). Material sufficient to just produce a hazy appearance was added to each of the remaining universals. Culture 1 had colcemid added to a final concentration of 0.1µg/ml and was then incubated at 37°C for 30 min before harvesting. Culture 2 had colcemid added to a final concentration of 0.01µg/ml and was then incubated at 37°C overnight. Culture 3 was incubated overnight and then had colcemid added to a final concentration of 0.1µg/ml for 1 hour, at which time cultures 1 and 2 were harvested.

The cell suspension was centrifuged at 1200 rpm for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in 0.075M KCl, prewarmed to 37°C and added dropwise, whilst continuously shaking the tube. After a 10 min incubation at 37°C the suspension was spun down for 5 min, the supernatant removed and the cells fixed by the dropwise addition of 5ml 3:1 methanol/glacial acetic acid as the tube was agitated. The suspension was pelleted and the procedure repeated twice. The cell suspension was then stored at -20°C until slides were prepared.

2.2.2) Cytogenetics

2.2.2.1) Slide making

Each cell suspension was centrifuged at 1200 rpm for 10 min and then resuspended in a small quantity of fixative until it was slightly opaque. Using a pasteur pipette, one drop of suspension was dropped onto the centre of cold wet grease free slides. Excess fixative was blotted off and the slides allowed to dry at room temperature.
2.2.2.2) G-banding

Slides were stained with freshly made Leishmans stain for 90 seconds, rinsed with Gurr's buffer and blotted dry. They were then examined under the microscope to assess the number and quality of mitotic figures present. Slides were then aged by leaving them at room temperature for at least two days or by incubating them at 80°C for two hours. Immersion lens oil was removed with ethanol, the slides were destained with fixative and air-dried.

A modified version of Seabright's method (Seabright 1971) was used for chromosome banding. Trypsin solution was spread over the slides for 5-30 seconds and then rinsed off with Sorensen's buffer. Leishman's stain was then applied for 90 seconds, the slides rinsed with Gurr's buffer and then blotted dry. Banded metaphases were examined under the microscope and their karyotypes recorded according to the International System for Cytogenetic Nomenclature (ISCN) (ISCN, 1991). Cells were then photographed and representative photo-karyotypes constructed for each specimen.

2.2.3. Molecular biology

Gloves were worn for all laboratory work involving nucleic acids. All solutions and disposable items were sterilised before use.

2.2.3.1) Preparation of competent cells

A single colony of E. Coli JM109 was inoculated into 50ml LB medium and grown overnight with moderate shaking (250 rpm). 4ml of the culture was inoculated into 400ml LB medium in a 2-litre flask and grown at 37°C with shaking to an OD590 of 0.375. The culture was then aliquoted into 16 pre-chilled polypropylene tubes which were left on ice for 10 min. The cells were kept cold.
for all subsequent steps. The tubes were centrifuged at 2500rpm for 7 min at 4°C in the IEC centrifuge. The supernatant was poured off and each pellet was resuspended in 5ml ice-cold CaCl₂ solution. The contents of each pair of tubes were pooled into one and then centrifuged at 2500rpm for 5 min at 4°C. The supernatant was discarded, each pellet was resuspended in 10ml cold CaCl₂ and kept on ice for 30 min. The cells were again centrifuged at 2500rpm for 5 min at 4°C and finally resuspended in 2ml cold CaCl₂. 250μl aliquots were dispensed into chilled microfuge tubes and frozen immediately at -70°C (Ausubel et al, 1991).

2.2.3.2) Transformation of competent cells

Where clones were provided as plasmid stocks, it was necessary to use them to transform E. coli cells in order to replicate a sufficient quantity for further use. Aliquots of 10ng DNA in a volume of 10-25μl were added to a round-bottom 15ml test tube and placed on ice. A tube of competent cells was warmed rapidly between the hands and 100μl dispensed immediately into the tubes containing the DNA. The contents were mixed gently and placed on ice for 10 min. The cells were then heat shocked by placing the tubes in a water bath at 42°C for 2 min. 1ml LB medium was added to each tube and they were then shaken at 250 rpm for 1 hr at 37°C (Ausubel et al, 1991). Aliquots were placed on LB agar plates containing the appropriate antibiotic (ampicillin at 50-100μg/ml or tetracyclin at 10μg/ml). The remainder of the mixture was stored at 4°C for subsequent platings.

2.2.3.3) Growth of clones in plasmid vectors

A single colony of E.Coli JM109 containing the DNA clone of interest was inoculated into 50ml LB in a 250ml flask containing the appropriate antibiotic (see above) and grown overnight at 37°C with moderate shaking (250 rpm). The culture was left at room temperature the following day and was then added to
500ml LB (also containing antibiotic) in a 2 litre flask and incubated overnight at 37°C with moderate shaking (250 rpm). Plasmid DNA was prepared from the culture the next day.

If required, glycerol stocks were also prepared at this stage by mixing 500μl of culture with 500μl glycerol solution and storing in a sealed microfuge tube at -20°C.

2.2.3.4) Preparation of plasmid DNA

Extraction and purification of plasmid DNA was by alkaline lysis (Birnboim, 1983) using the Qiagen kit for maxi preparations. The composition of the buffers is given in section 2.1.4.14.

**Alkaline lysis.** The 500ml culture from 2.2.3.3 above was centrifuged at 4000 rpm for 10 min at 4°C in two 250ml bottles. The pellets were resuspended in a total of 10ml buffer P1 and transferred to a high speed centrifuge tube. 10ml buffer P2 was then added, mixed in by tube inversion and incubated at room temperature for 5 min. 10ml Buffer P3 was added, mixed thoroughly by inverting the tube 6 times and the suspension was centrifuged at >20,000 g for 30 min at 4°C. The supernatant was removed to a fresh tube as carefully as possible and re-centrifuged at >20,000 g for 10 min at 4°C (Birnboim, 1983).

**Column purification.** A Qiagen tip-500 column was equilibrated with 10ml buffer QBT and allowed to empty completely by gravity flow. The supernatant was then applied and allowed to enter the column, which was then washed three times with buffer QC. The purified DNA was eluted with 15ml buffer QF into a clean tube, allowing the column to drain completely.

**Precipitation.** The DNA was precipitated with 0.7 vol (10.5ml) isopropanol at room temperature for 10 min and then centrifuged at high speed for 30 min at
4°C. The supernatant was carefully removed and the pellet was washed with 70% ethanol. The DNA was re-centrifuged for 15 min, dried briefly but not completely and resuspended in 200μl TE.

2.2.3.5) Quantification of DNA.

Purified DNA was measured to estimate the quality and quantity obtained. An aliquot was diluted 100 times in TE and absorbance readings were taken at 260 nm and 280 nm. The $OD_{260} \times 50 \times 10^2$ equals the approximate concentration of DNA in μg /ml. The ratio between the readings at 260 nm and 280 nm ($OD_{260}/OD_{280}$) gives an estimate of purity. (Pure preparations of DNA have an $OD_{260}/OD_{280}$ of 1.8.) If this ratio is significantly lower than 1.8 (less than 1.4) the sample is contaminated and accurate quantitation will not be possible.

2.2.3.6) Digestion of DNA by restriction enzymes.

DNA from purified vectors was cut with appropriate enzymes in order to confirm their identity and to recover cloned inserts. To a microfuge tube, the below were added sequentially to a total volume of 20μl:

- x μl DNA solution (usually approximately 1μg)
- 2 μl appropriate enzyme buffer
- y μl sterile distilled water to 20 μl
- z μl enzyme (approximately 1 unit/μg)

The tube was incubated in a water bath at 37°C for 2 hours, after which the digested DNA was subjected to gel electrophoresis.

2.2.3.7) Gel electrophoresis of DNA.

0.5g or 1g agarose was added to 50ml (minigel) or 100ml (midigel) 1x TBE in a conical flask and melted in a microwave oven. A gel casting platform was sealed at both ends with tape and a gel comb inserted. The melted agarose was then poured into the platform and allowed to set at room temperature. When
completely set the comb was carefully removed from the gel and the platform placed in an electrophoresis tank. The gel was covered to a depth of 1 mm with 1x TBE, containing ethidium bromide at a final concentration of 1 μg/ml. 2 μl of loading buffer was added to each sample, which were then loaded into individual slots. 5 μl size marker was loaded into a free slot after mixing with 2 μl loading buffer. The lid of the tank was fitted and the power pack connected, making certain the DNA would migrate into the gel toward the anode. Voltage was applied at between 1-10 V/cm until the dye from the loading buffer had migrated a distance judged suitable for the separation of the appropriate DNA fragments. The apparatus was disconnected and the platform containing the gel was positioned over a UV transilluminator for examination and photography.

2.2.3.8) Recovery of DNA from electrophoresis gels

A Prep-A-Gene kit (see section 2.1.4.21) was used to extract DNA from agarose gels. The piece of gel containing the DNA of interest was excised with a sterile scalpel and cut into 2 mm pieces which were added to a microcentrifuge tube. The tube was spun briefly and the volume of the contents estimated by measuring water into a similar tube and comparing their appearance. Three volumes of binding buffer were added to the agarose pieces, the tube was vortexed and then incubated at 50°C, vortexing intermittently. For each μg of DNA thought to be present, 5 μl of matrix was added. After mixing, the tube was incubated at room temperature for 10 minutes and then microfuged for 30 seconds. The pellet was then resuspended in 50 matrix volumes of binding buffer and spun down for 30 seconds. This step was repeated once. The pellet was then resuspended in 50 matrix volumes of wash buffer, spun down for 30 seconds and the step repeated twice more. The resulting pellet was resuspended in one matrix volume of elution buffer and incubated at 50°C for 5 minutes. The suspension was spun down for 2 minutes to obtain a solid pellet and the supernatant, which contained the DNA, was carefully removed. The elution step was repeated once more and the
supernatants were pooled. The total supernatant was microfuged briefly to remove any trace of matrix and transferred to a fresh tube for storage at -20°C.

2.2.4. Photography

2.2.4.1) Development of PAN AHU film

In absolute darkness, film was removed from the cassette, wound onto a spool and placed in a developing canister. Under safety light, developer was added to the canister, which was constantly shaken for 2-3 minutes. After a brief wash with tap water, fixer was added to the canister, followed by constant shaking for 10 minutes. The film was washed with running tap water for 10 minutes, removed from the spool and hung up to dry.

2.2.4.2) Printing from PAN AHU film

Under safety light, film was exposed onto photographic paper with the enlarger. The paper was developed for 1-4 minutes and then stopped with tap water, slightly acidified with acetic acid. After fixing for 5 minutes, prints were washed under running tap water for 10-20 minutes before air drying. Adjustments to the exposure time, aperture and choice of paper were necessary to obtain good prints.

2.2.4.3) Photography of electrophoresis gels

Gels were photographed in the tray, whilst excited by the transilluminator. A red filter, an aperture of f5.6 and an exposure time of 1/30 second were used.

2.2.5) Fluorescence in-situ hybridisation

This technique and associated methods underwent a considerable degree of modification and development in the course of this work. The methodology is therefore presented separately in the following chapter.
CHAPTER THREE

TECHNICAL DEVELOPMENT OF FLUORESCENCE IN-SITU HYBRIDISATION
3.1) INTRODUCTION

In-situ hybridisation is the annealing of a nucleic acid probe to a target in which the morphology is preserved. Early workers in this field labelled probes isotopically (Pardue and Gall, 1969), but since the 1980s non-isotopic labelling has been used with increasing frequency (Langer et al, 1981). There are a number of reasons for this: experiments take less time to perform, the techniques are safer to carry out, there are improvements in resolution and with the use of multiple fluorescence detection systems (Nederlof et al, 1989), it is possible to visualise several target regions simultaneously in the same specimen (Reid et al, 1992).

In the work presented here, FISH was applied to human metaphase chromosomes and interphase nuclei. In each case, the same series of steps were undertaken: slide preparation, probe labelling, denaturation of probe and target, hybridisation, detection and photography. Optimisation of each step was attempted in order to maximise the success of individual experiments. The number of cells in which signals were observed (the hybridisation efficiency), the intensity of those signals and the signal to noise ratio, were taken into account when comparing differences between protocols. The effect of different treatments on target morphology was also noted. In the following sections, individual steps will be considered and the methods found to give the best results will be described.

3.2) SLIDE PREPARATION

3.2.1 Slide making

Cell suspensions of material for FISH were dropped onto slides as described above (Methods 2.2.1). If the suspensions had been stored for more than a few weeks, at least two re-fixations were required in order to produce good preparations. Quality was most easily determined by inspection with phase
contrast microscopy. A phase dark appearance and lack of cytoplasm were the best indicators of a good preparation (Donlon, 1986).

3.2.2 Ageing and storage of preparations

Slides aged at room temperature for one to two weeks gave the best results. If the slides were then stored in 70% ethanol at 4°C, good FISH results could be obtained for about another two months.

3.2.3 Pre-treatment of preparations

Slides were subjected to the following protocol prior to FISH.

Those slides previously stored in 70% ethanol at 4°C were air dried and equilibrated in 2xSSC for 5 minutes. Slides at room temperature were used directly.

An incubation with 100μg RNaseA/ml in 2xSSC (1:100 dilution of the stock solution) was carried out at 37°C for one hour. 150μl were applied to a 22x50 mm coverslip. A slide was touched to the coverslip and hung upside down in a slide holder placed on its side in a humid chamber (a plastic sandwich box containing paper towels moistened with water). The coverslip was pulled off and the slide washed 3 x 5 min in 2xSSC at room temperature, in a Coplin jar on a shaking platform.

A 0.01M solution of HCl was made by adding 1ml 1N HCl to 99ml distilled H2O. After warming to 37°C, 50μl pepsin stock was added and the slide then incubated at 37°C for 10 min. After 2 x 5min washes in 1xPBS and 1 x 5min wash in 1xPBS, 50mM M902 in a Coplin jar on a shaking platform at room temperature, the slide was post-fixed.

A 1% formaldehyde solution was freshly made by adding 2.7ml formaldehyde (37%) to 97.3ml 1xPBS, 50mM MgCl2 in a Coplin jar on a shaking platform at room temperature, the slide was post-fixed.

A 1% formaldehyde solution was freshly made by adding 2.7ml formaldehyde (37%) to 97.3ml 1xPBS, 50mM MgCl2. The slide was incubated for 10 min at room temperature and then washed for 5 min in 1xPBS at room temperature, in a
Coplin jar on a shaking platform. After dehydration in a 70%, 95% and 100% ethanol series, the slide was air dried.

3.2.4 Evaluation

At the inception of the FISH work, no pre-treatment other than dehydration was employed. Subsequent additions to the protocol were evaluated individually. Treatment with RNase A was found to reduce background signal; a factor of major importance when working with small single copy probes (Figure 3.1). Pepsin digestion increased hybridisation efficiency and signal strength but chromosome morphology was adversely affected (Figure 3.2). Digestion with Proteinase K was also tested (0.4μg/ml in 20mM Tris-HCl, 2mM CaCl₂; 5-10 min, 37°C). This gave a similar but less effective result to Pepsin (data not shown). The addition of a post-fixation step maintained target integrity, whilst allowing the benefits of protease digestion to be maintained.

3.3) PROBE LABELLING

3.3.1 Introduction

Probes were labelled with one of two reporter molecules in the course of this work. Nucleotides, modified with either biotin or digoxigenin, were incorporated into probe DNA by a nick translation reaction (Langer et al, 1981). This was considered preferable to random priming (Feinberg and Vogelstein, 1984), since the size of the labelled probe fragments has been found to be important for effective FISH (Lawrence and Singer, 1985). This optimum range (100 - 500 bp) can be obtained for a probe of any size by adjusting the DNAse concentration in the nick translation reaction.
Figure 3.1

Metaphase spread from peripheral blood lymphocytes, hybridised with probes for the X centromere and the MYB proto-oncogene.

Pre-treatment with RNase was not applied and a high level of background signal is visible.
Figure 3.2

Metaphase spreads from peripheral blood lymphocytes. Pre-treatment with RNase was applied in each case.

a) Male metaphase hybridised with probes for the X-chromosome centromere and the *MYB* proto-oncogene. No pepsin pre-treatment was applied. No signals from the *MYB* probe are visible.

b) Metaphase hybridised with probes for the chromosome 6 centromere and the *MYB* proto-oncogene. Pepsin pre-treatment was applied. Clear signals from the *MYB* probe are visible on one chromosome 6, indicated by the arrow.
3.3.2 Labelling protocol

For labelling with biotin, the Bionick kit (Section 2.1.5.1) was used. 1µg probe DNA, 5µl dNTP mix and sterile distilled water were added to an Eppendorf tube, to a total volume of 45µl. Following the addition of 5µl Bionick enzyme mix, the tube was incubated at 15°C for 1 hour. The tube was then transferred to an ice bath and 5µl stop buffer added.

During the incubation period, a separation column was prepared. A pasteur pipette was plugged with glass wool and filled to within 1cm of the top with Sephadex G50. The incubation mixture was loaded onto the column and then eluted with TE (pH7.0). The second 600µl of eluate, which contained the probe, was collected.

50µg E.Coli tRNA (4µl of the stock) and 50µg salmon sperm DNA (5µl of the stock) were added as carrier. After the addition of 0.1 volumes (60µl) 3M Sodium acetate pH5.6, the mixture was vortexed briefly and split equally into two tubes. Two volumes ethanol (660µl) were added and the tubes placed at -70°C for 1 hour. The precipitated probe was pelleted by microfugation at full speed for 15 minutes, dried completely in a speedvac and resuspended in 50µl TE. Storage was at -20°C.

A similar procedure was followed for labelling with digoxigenin. However, the Nick Translation System (Section 2.1.5.1) was used and the reaction mixture consisted of 1µg DNA, 5µl solution A4, 2.5µl digoxigenin mix, sterile distilled water to a final volume of 45µl and 5µl solution C (the enzyme mix). The incubation was for 90 minutes at 15°C.

3.3.3 Dot blot assay of probe labelling

When necessary, a colourimetric assay was employed to assess the quality of the hapten labelling. This was indicated when, for example, a newly labelled probe failed to give an adequate result. A Blu-gene kit (Section 2.1.5.14) was used to perform the assay.
The following buffers were made up.

Buffer 1 containing 0.1M Tris-HCl (pH7.5), 0.15M NaCl
Buffer 2 containing 3% bovine serum albumin (fraction V) in buffer 1
Buffer 3 containing 0.1M Tris-HCl (pH9.5), 0.1 NaCl, 50mM MgCl2
Stop buffer containing 20mM Tris (pH7.5), 0.5mM Na2EDTA.

All solutions were then filtered through 0.45μm filters.

Serial dilutions were made in the kit DNA dilution buffer from both the labelled probes under test and from the labelled control DNA provided with the kit, such that 5μl aliquots would contain 20, 10, 5, 2 or 0 pg. These were spotted onto a nylon membrane which was then dried at 80°C for 1.5 hours.

The filter was rehydrated for 1 minute in sufficient buffer 1 to cover it and then incubated in sufficient buffer 2 to cover for 1 hour at 65°C. Immediately prior to use, the SAAP conjugate was diluted to 1μg/ml by adding 4μl of stock to 4ml buffer 1. The filter was then incubated in diluted SAAP conjugate for 10 minutes with gentle agitation, occasionally pipetting the solution over the filter. The conjugate was decanted off and the filter washed 2x10 minutes with buffer 1, using 20-40x the volume of conjugate previously employed. The filter was then washed in buffer 3 for 10 minutes and approximately 7.5ml dye solution was prepared by adding 33μl NBT solution to buffer 3, mixing gently and then adding 25μl BCIP solution followed by gentle mixing. The filter was then incubated in the dye solution within a sealed hybridisation bag for 30 minutes to 3 hours in the dark, to allow colour development. The reaction was terminated by washing in stop buffer and the filter was dried between sheets of blotting paper at 80°C for 2 minutes.

When probe labelling had been successful, a coloured spot was visible at the site of the appropriate 2pg aliquot, of approximately the same intensity as that seen in the 2pg spot of the control DNA.
3.4) DENATURATION AND HYBRIDISATION

3.4.1 Introduction

The strategy that was followed in bringing target and probe together depended both on the size and copy number of the target and on the nature of the probe. For example, a small target that was present in a high copy number could be successfully detected with less probe than a larger but unique target. Some of the probes used in this work contained interspersed repetitive sequences (IRS) that are scattered throughout the human genome (Korenberg and Rykowski, 1988). If such probes were hybridised directly, non-specific signal would be detected. To overcome this problem, probes were pre-hybridised with unlabelled human DNA. This approach (Lichter et al, 1988) is termed chromosomal in situ suppression (CISS). Figure 3.3 shows an example of this technique in practice. The following sections describe the general protocols used for denaturation and hybridisation and the variations required for different probe/target combinations.

3.4.2 Probe mixtures

Each hybridisation event occupied half a slide and took place in an 11µl volume. Amounts of probe appropriate to each type are given below.

- Chromosome specific repeat: 10-20ng
- Single copy: 50-100ng
- Total DNA from an individual human chromosome (chromosome paint): 150-500ng

Labelled probes were added to an Eppendorf tube as required. When a chromosome paint was part of the probe mixture, a 10-25x excess of unlabelled human DNA was included. A 2x volume of ethanol was added to the tube, mixed well and then lyophilised. The pellet was resuspended in 11µl hybridisation mix.
Figure 3.3

Male metaphase spread from peripheral blood lymphocytes, hybridised with a painting probe for the X chromosome. Signal is visible both on the whole of the X chromosome (arrow) and on the pseudo-autosomal region of the Y chromosome (arrow head).
3.4.3) Denaturation and hybridisation methods

If no IRS were present in the probe(s) used, it was possible to denature probe and target simultaneously. The hybridisation mixture was pipetted onto the target area of the slide and covered with a 22x22mm coverslip. The slide was placed in an oven at 80°C for 3-5 minutes and then transferred to an incubator at 37°C in a humid chamber where hybridisation took place overnight.

When denaturation of probe and target took place separately, the tube containing the hybridisation mixture was heated at 75°C for 5 minutes in a heating block. At the end of that time, if IRS were present, the tube was transferred to a 37°C water bath for at least 30 minutes. If no pre-annealing was required, the tube was placed on ice immediately after denaturation.

Target slides were pre-warmed to approximately 50°C and then denatured in 70% formamide, 2xSSC, pH 7.0 at 70°C for 2 minutes. They were then immediately dehydrated in an ice cold ethanol series of 70%, 95% and 100% and air dried. The denatured hybridisation mixture was pipetted onto the target area of the slide and covered with a 22x22mm coverslip which was then sealed with rubber solution. Hybridisation then took place overnight in a humid chamber at 37°C.

It was noted that separate denaturation appeared to give more consistent results and this method was adopted for all subsequent experiments.

3.5) DETECTION

3.5.1) Post hybridisation washes

After hybridisation, usually overnight, the rubber solution was carefully removed with tweezers and the coverslip gently pulled off. Slides were then washed 3x5 minutes in 50% formamide, 2x SSC, pH 7.0 at 42°C. This temperature was used for most experiments, but occasional variations were employed to either lower the stringency (37°C) or to raise it (60°C). Slides were then washed 3x5 minutes in 2x SSC, pH 7.0 at 42°C.
3.5.2) Immuno-cytochemistry

Biotin labelled probes were detected with a layer of FITC-conjugated avidin. An amplification step was usually applied, consisting of an additional layer of biotinylated anti-avidin followed by another layer of FITC-conjugated avidin (Pinkel et al, 1986). If necessary, further layers could be added, but this often resulted in a poor signal to noise ratio (Figure 3.4).

Immediately following the post hybridisation washes, slides were washed for 3 minutes in SSCT at room temperature. A blocking incubation with SSCTM was then carried out. 1ml SSCTM was centrifuged for 1.5 minutes to remove solids and 100μl applied to a 22x50mm coverslip. A slide was touched to the liquid and hung upside down in a slide holder placed on its side in a humid chamber at room temperature. (Subsequent incubations were carried out in the same manner). After 10 minutes the coverslips were allowed to slip off and the slides washed in SSCT for 3 minutes.

2μl FITC-avidin stock were mixed with 1ml SSCTM and 100μl loaded onto each slide as above. A 20 minute incubation in a humid chamber was then carried out in the dark at room temperature. 3x3 minute washes with SSCT were followed by a 20 minute incubation with biotinylated anti-avidin. 10μl stock were dissolved in 1ml SSCTM, the solids were spun out and 100μl applied to each slide. After a further 3x3 minute washes with SSCT, a final incubation with FITC-avidin was carried out exactly as above.

A final wash in SSCT for 3 minutes was followed by 2x5 minute washes in PBS. Slides were then dehydrated through a series of 70%, 95% and 100% ethanol, allowed to air dry and mounted in 25μl AF1 containing 0.5μg/ml propidium iodide counterstain.

When digoxigenin labelled probes were to be simultaneously detected, the following changes were made to the protocol described above. 12μl of antidigoxigenin-rhodamine stock were added to the solution for the second
Figure 3.4

Metaphase spreads from peripheral blood lymphocytes, hybridised with a centromere probe specific for the X chromosome and a probe for the MYB proto-oncogene.

a) Preparation with one layer of amplification. The signals on the X centromere are indicated by arrows; each pair of MYB signals is indicated by an arrow head.

b) Preparation with two layers of amplification, hybridised under identical conditions to a). Although the signals are stronger, the increase in background makes interpretation practically impossible.
Figure 3.5

Example of pre- and post-banding on metaphase spreads from peripheral blood lymphocytes.

a) Metaphase pre-banded with a low trypsin concentration. (Smit et al, 1990).

b) Metaphase banded after FISH with the borate buffer method. (Cannizzaro and Emanuel, 1984).
preparations from peripheral blood lymphocytes. Successful hybridisations were photographed and their co-ordinates were recorded.

If all single signals had been taken into account, large numbers of cells would have had to be assessed, in order to determine the target site statistically, since some background signals were invariably present. To avoid this, only those signals occurring as symmetrical pairs, one on each chromatid, were considered as true hits. The probability of paired signals occurring by chance at the same chromosome locus in more than one or two cells was extremely low.

Following FISH, slides were post-banded as described above. Cells that had been successfully hybridised were located and re-photographed. Photographic slides were projected onto a screen to enable measurements to be taken.

The distance from the centromere to the probe and from the centromere to the telomere were measured. The ratio of cen-probe/cen-telomere was calculated to give the fractional length (FL) relative to the distance from centromere to telomere on the target chromosome arm (Lichter et al, 1990). Some workers have applied the FL to standard karyograms (ISCN, 1985) to determine a corresponding band location (Williams et al, 1991). This might lead to inaccuracies, since such diagrams cannot reflect differing states of chromosomal condensation. In this protocol, the FL was applied to the corresponding post-banded chromosome. The cen-telomere distance was determined and multiplied by the FL. This measurement was then used to determine a band location on the post-banded chromosome (Figure 3.6).

3.8) PHOTOGRAPHY

Microscopic inspection was carried out in a darkened room. If only FITC detection and propidium iodide counterstain had been used, slides were scanned with the rhodamine filter to reduce photo-bleaching of the signal. Photographs were taken with the FITC filter, which allowed co-visualisation of the counterstain. The camera controller was set to darkfield and the automatic
Figure 3.6

a) A metaphase spread from peripheral blood lymphocytes, hybridised with probes for the chromosome 6 centromere (arrow) and the *MYB* proto-oncogene (arrow head). In this case the hybridisation of MYB could only be seen on one of the chromosomes 6.

b) the same metaphase after post-banding.
exposure control was set one or two stops faster than the film rating, in order to reduce exposure times.

When both FITC and rhodamine were used, the material was counterstained with DAPI. Slides were then scanned with the DAPI filter and photographed by exposing them independently through the rhodamine, FITC and DAPI filters, in that order, without activating the film transport. The camera controller was set up as above. This produced a single multiple exposure, in which all three colours were represented.

In the latter part of this work, a CCD camera and associated software were used to capture digitised images of the preparations. The slide was automatically exposed through the rhodamine, FITC and DAPI filters and a composite pseudocolour image was stored in the computer memory. The stored images could then be manipulated and printed as required.
CHAPTER FOUR

RESULTS OF CYTOGENETIC STUDIES
4.1) INTRODUCTION
A total of 43 lymph node biopsies from 39 cases were cultured and metaphase chromosomes analysed by Giemsa banding. (See Chapter 2, sections 2.2.1.2 and 2.2.2.) The quality of the karyotypes varied. This was due in part to differences in the amount of original biopsy material available, which in turn depended on the size of the excised node and the requirements of the pathologist. The other major source of variation was the degree to which individual samples grew in culture. Overall, successful cytogenetic analyses were obtained from 40/43 samples. The results of these analyses are shown in table 4.1. Information on individual cases may be found in table 2.1, (section 2.1).

4.2) KARYOTYPES OF INDIVIDUAL BIOPSIES
L001- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 25 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.1.

L002- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 29 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.2.

L003- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 34 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.3.

L004- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 40 cells, of which 75% exhibited an abnormal karyotype, shown in figure 4.4.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Numbers of cells examined</th>
<th>Percentage normal</th>
<th>Karyotype</th>
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<td>0</td>
<td>47, XY, dup(1)(q11q44), del(2)(q21), +3, del(9)(q21), add(11)(q23)</td>
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<tr>
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<td>29</td>
<td>0</td>
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<tr>
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<td>34</td>
<td>0</td>
<td>46, XY, +X, add(3)(q13), add(6)(q13), -8, del(14)(q22q24), add(17)(p13)</td>
</tr>
<tr>
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<td>0</td>
<td>48, XX, +3, del(6)(p21), +del(6)(q21), t(10;13)(q22;q12), del(14)(q31), del(17)(p11), add(22)(q13)</td>
</tr>
<tr>
<td>L009</td>
<td>24</td>
<td>12</td>
<td>46, XX</td>
</tr>
<tr>
<td>L010A</td>
<td>25</td>
<td>0</td>
<td>89, XXXX, +X, add(1)(p36), +del(3)(p21) x2, del(6)(q15q21) x3, del(9)(p22)t(9;13)(q21;q12), der(9)del(9)(p22)t(9;13)(q21;q12), dup(12)(q13q22) x2, -13, -13, t(14;18)(q32;q21), -15, -16, add(16)(q12), -der(18)t(14;18)(q32;q21), add(19)(p11)</td>
</tr>
<tr>
<td>L010B</td>
<td>18</td>
<td>0</td>
<td>48, XX, +add(X)(p22), add(1)(p36), add(3)(q21), del(6)(q15q21), +7, del(9)(p22)t(9;13)(q21;q12), dup(12)(q13q22), t(14;18)(q32;q21), add(19)(q13.4)</td>
</tr>
</tbody>
</table>
| L011 | 28 | 18 | 46, XY  
49, XY, +8, +9, add(14)(q32), +18 [4]  
49, idem, -9, +i(9)(q10) [17]  
49, idem, ins(1)(q21q25;?) [2]  
{add(14)(q32) revised to der(14)t(3;14)(?:q32) following FISH} |
| L012 | 28 | 18 | 46, XY  
48, XY, add(1)(p32), +7, -11, del(14)(q24), del(15)(q24), del(20)(q13),  
+mar1 x2 |
| L013 | 22 | 0  | 45, X, -X, +12, -14 |
| L014 | 22 | 18 | 46, XX  
46, XX, t(6;8)(p21;q24), t(14;18)(q32;q21)  
47, idem, +8 |
| L015 | 23 | 0  | 48, XX, add(10)(q22), add(11)(p11), t(14;18)(q32;q21), +18,  
+der(18)t(14;18)(q32;q21), del(22)(q23) |
| L016 | 24 | 4  | 46, XX  
47, XX, +12, del(14)(q22) |
| L017 | 31 | 7 | 46, XY  
|      |    |   | 47, XY, +X, add(6)(q23), ins(7;?)p13;?, t(9;17)(q11;p11), +12, -13, t(14;18)(q32;q21), -15, +18 |
| L018 | 32 | 100 | 46, XY |
| L019 | 35 | 6 | 46, XY  
|      |    |   | 46, XY, -5, -9, add(10)(p11), add(11)(q21), der(16)t(1;16)(q21;p11), +2mar |
| L020A | 27 | 4 | 46, XX  
|      |    |   | 46, XX, add(11)(q13) |
| L020B | 17 | 0 | 46, XX  
|      |    |   | 46, XX, add(11)(q13) |
| L021 | 27 | 15 | 46, XY  
|      |    |   | 47, XY, add(1)(q21), add(5)(p13), -8, -9, -10, -11, del(13)(q31), add(14)(q24), +add(17)(p12), +22, +4mar  
|      |    |   | {add(14)(q24) revised to der(14)t(11;14) and one marker revised to der(11) following FISH} |
| L022 | 24 | 0 | 50, XX, +X, der(1)t(1;9)(p32;q12), add(5)(q13) x2, +5 der(6)t(6;?:5)(q13;?:q13), -8, +12, add(14)(q32), add(14)(q32), der(15)t(1;15)(q21;q22), +18, +mar {one add(14)(q32) revised to der(14)t(14;18)(q32;?) following FISH} |
| L023 | 29 | 14 | 46, XX 52-54, XX, +X, dup(1)(q21q32), add(1)(p32), add(2)(p21), +3, +5, i(6)(p10), +7, +add(8)(p11), +11, t(13;15)(q10;q10), t(14;18)(q32;q21), +del(18)(q21), +21, +2r [cp25] {del(18)(q21) revised to der(18)t(14;18)(q32;q21) following FISH} |
| L024 | 20 | 0 | 46, XY, add(1)(q42), del(2)(q23), t(8;14)(q24;q32) |
| L025 | 23 | 22 | 46, XX 51, XX, dup(1)(q11q25), add(2)(p23), +3, +3, add(22)(q13), +3mar |
| L026A | 25 | 0 | 46, XY, +12, -13, del(14)(q24), add(17)(q25), add(17)(q21) |
| L026B | 21 | 0 | 46, XY, +12, -13, del(14)(q24), add(17)(q25), add(17)(q21) |
| L027 | 19 | 11 | 46, XX 48, XX, +5, i(6)(p10), +12, t(14;18)(q32;q21), -18, +mar |
L001

B-cell high-grade centroblastic

47, XY, dup(1)(q11q44), del(2)(q21), +3, del(9)(q21), add(11)(q23)
Figure 4.1  Representative karyotype from case L001
L002

B-cell low-grade follicular

46, XX, inv(9)(p13q22), t(14;18)(q32;q21), add(16)(p13.3)
Figure 4.2  Representative karyotype from case L002
L003

B-cell high-grade centroblastic

46, XY, +X, add(3)(q13), add(6)(q13), -8, del(14)(q22q24), add(17)(p13)
Figure 4.3 Representative karyotype from case L003
L004

B-cell low-grade follicular

46, XY, del(5)(p14), add(12)(p11), add(15)(q22)
Figure 4.4 Representative karyotype from case L004
L005- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 49 cells, of which 73% exhibited an abnormal karyotype. The most common clone is shown in figure 4.5. Another clone (represented by 5 cells) did not show a trisomy X.

L006- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 22 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.6.

L007- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 28 cells, of which 82% exhibited an abnormal karyotype, shown in figure 4.7.

L008- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 28 cells, of which 82% exhibited an abnormal karyotype, shown in figure 4.8.

L009- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 24 cells, of which 88% exhibited an abnormal karyotype, shown in figure 4.9.

L010- This case was initially diagnosed as a B-cell low-grade follicular lymphoma. Cytogenetic analysis of the first sample (L010A), was carried out on 25 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.10. A second biopsy was obtained two years after the first. This sample (L010B) was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 18 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.11.
L005

B-cell low-grade follicular

49, XX, +8, +12, +X
Figure 4.5  Representative karyotype from case L005
L006

B-cell high-grade centroblastic

46, XX, add(14)(q32), del(14)(q31), t(20;22)(q13.3;q12)

{add(14q)(q32) revised to der(14)t(3;14)(?:q32), following FISH}
Figure 4.6 Representative karyotype from case L006
L007

B-cell low-grade follicular

91-94, XXYY, +X, +X, add(1)(p13), add(2)(p21) x2, add(3)(p13).-4,
del(6)(q16q22), -7, +12, +12, t(14;18)(q32;q21) x2, -17, -22 [cp18]
Figure 4.7 Representative karyotype from case L007
L008

B-cell low-grade follicular

48, XX, +3, del(6)(p21), +del(6)(q21), t(10;13)(q22;q12), del(14)(q31),
del(17)(p11), add(22)(q13)
Figure 4.8 Representative karyotype from case L008
L009

B-cell low-grade follicular

62, XX, +X, +add(1)(p11), +2, +ins(3;?) (q21;?) x2, +del(5)(q31), del(7)(q32),
+del(7)(q32), +8, +9, del(10)(q22), +add(10)(q23), +11, +12, +13,
t(14;18)(q32;q21), +add(14)(q32), +19, +20, +21
{add(14)(q32) revised to der(14)t(14;18)(q32;?) and both abnormal chromosome
10s revised to der(10)t(10;18)?, following FISH}
Figure 4.9 Representative karyotype from case L009
L010A

B-cell low-grade follicular

89, XXXX, +X, add(1)(p36), +del(3)(p21) x2, del(6)(q15q21) x3,
del(9)(p22)t(9;13)(q21;q12), der(9)del(9)(p22)t(9;13)(q21;q12), dup(12)(q13q22) x2, -13, -13, t(14;18)(q32;q21), -15, -16, add(16)(q12),
-der(18)t(14;18)(q32;q21), add(19)(p11)
Figure 4.10 Representative karyotype from case L010A
LO10B

B-cell high-grade centroblastic

48, XX, +add(X)(p22), add(1)(p36), add(3)(q21), del(6)(q15q21), +7,
del(9)(p22)t(9;13)(q21;q12), dup(12)(q13q22), t(14;18)(q32;q21), add(19)(q13.4)
**Figure 4.11** Representative karyotype from case L010B
L011- This case was diagnosed as B-cell low-grade centrocytic lymphoma. Cytogenetic analysis was carried out on 28 cells, of which 82% exhibited an abnormal karyotype. The most common clone is shown in figure 4.12. Other clones showed either an additional abnormality of chromosome 1 (represented by 2 cells) or a normal extra copy of chromosome 9 (represented by 4 cells).

L012- This case was diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 28 cells, of which 82% exhibited an abnormal karyotype, shown in figure 4.13.

L013- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 22 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.14.

L014- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 22 cells, of which 82% exhibited an abnormal karyotype. The most common clone is shown in figure 4.15. Another clone (represented by 4 cells) did not show trisomy of chromosome 8.

L015- This case was diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 23 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.16.

L016- This case was diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 24 cells, of which 96% exhibited an abnormal karyotype, shown in figure 4.17.
L011

B-cell low-grade centrocytic

49, XY, +8, +i(9)(q10), add(14)(q32), +18
Figure 4.12 Representative karyotype from case L011
L012
B-cell low-grade lymphocytic
48, XY, add(1)(p32), +7, -11, del(14)(q24), del(15)(q24), del(20)(q13), +mar1 x2
Figure 4.13  Representative karyotype from case L012
L013

B-cell low-grade follicular

45, X, -X, +12, -14
Figure 4.14 Representative karyotype from case L013
L014

B-cell low-grade follicular

47, XX, t(6;8)(p21;q24), +8, t(14;18)(q32;q21)
Figure 4.15 Representative karyotype from case L014
L015

B-cell low-grade lymphocytic

48, XX, add(10)(q22), add(11)(p11), t(14;18)(q32;q21), +18,
+der(18)t(14;18)(q32;q21), del(22)(q23)
Figure 4.16 Representative karyotype from case L015
L016

B-cell low-grade lymphocytic

47, XX, +12, del(14)(q22)
Figure 4.17 Representative karyotype from case L.016
L017- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 31 cells, of which 93% exhibited an abnormal karyotype, shown in figure 4.18.

L018- This case was diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 32 cells, all of which exhibited a normal karyotype (data not shown).

L019- This case was diagnosed as T-cell low-grade T-zone lymphoma. Cytogenetic analysis was carried out on 35 cells, of which 94% exhibited an abnormal karyotype, shown in figure 4.19.

L020- This case was initially diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis of the first sample received (L020A) was carried out on 27 cells, of which 96% exhibited an abnormal karyotype, shown in figure 4.20. When two years later a second biopsy was obtained (L020B) This diagnosis was again B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 17 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.21.

L021- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 27 cells, of which 85% exhibited an abnormal karyotype, shown in figure 4.22.

L022- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 24 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.23.
L017

B-cell high-grade centroblastic

47, XY, +X, add(6)(q23), ins(7;?) (p13;?), t(9;17)(q11;p11), +12, -13,
t(14;18)(q32;q21), -15, +18
Figure 4.18 Representative karyotype from case L017
L019

T-cell low-grade T-zone

46, XY, -5, -9, add(10)(p11), add(11)(q21), der(16)t(1;16)(q21;p11), +2mar
Figure 4.19 Representative karyotype from case L019
L020A
B-cell low-grade lymphocytic
46, XX, add(11)(q13)
Figure 4.20 Representative karyotype from case L020A
L020B

B-cell low-grade lymphocytic

46, XX, add(11)(q13)
Figure 4.21 Representative karyotype from case L020B
L021

B-cell high-grade centroblastic

47, XY, add(1)(q21), add(5)(p13), 8, -9, -10, -11, del(13)(q31), add(14)(q24),
+add(17)(p12), +22, +4mar

{add(14)(q24) revised to der(14)t(11;14) and one marker revised to der(11)
following FISH}
Figure 4.22 Representative karyotype from case L021
L022

B-cell low-grade follicular

50, XX, +X, der(1)t(1;9)(p32;q12), add(5)(q13) x2, +5der(6)t(6;7q13;7q13),
-8, +12, add(14)(q32), add(14)(q32), der(15)t(1;15)(q21;q22). +18, +mar

{one add(14)(q32) revised to der(14)t(14;18)(q32;?) following FISH}
Figure 4.23  Representative karyotype from case L022
L023- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 29 cells, of which 86% exhibited an abnormal karyotype, shown in figure 4.24.

L024- This case was diagnosed as B-cell high-grade immunoblastic lymphoma. Cytogenetic analysis was carried out on 20 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.25.

L025- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 23 cells, of which 78% exhibited an abnormal karyotype, shown in figure 4.26.

L026- This case initially was diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis of the first sample received (L026A) was carried out on 25 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.27. Three years later a second biopsy was obtained (L026B) and again diagnosed as B-cell low-grade lymphocytic lymphoma. Cytogenetic analysis was carried out on 21 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.28.

L027- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 19 cells, of which 89% exhibited an abnormal karyotype, shown in figure 4.29.

L028- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 29 cells, of which 86% exhibited an abnormal karyotype, shown in figure 4.30.
L023

B-cell low-grade follicular

52-54, XX, +X, dup(1)(q21q32), add(1)(p32), add(2)(p21), +3, +5, i(6)(p10), +7,
+add(8)(p11), +11, t(13;15)(q10;q10), t(14;18)(q32;q21), +del(18)(q21), +21, +2r

[cp25]

{del(18)(q21) revised to der(18)t(14;18)(q32;q21) following FISH}
Figure 4.24  Representative karyotype from case L023
L024

B-cell high-grade immunoblastic

46, XY, add(1)(q42), del(2)(q23), t(8;14)(q24;q32)
Figure 4.25  Representative karyotype from case L024
L025

B-cell high-grade centroblastic

51, XX, dup(1)(q11q25), add(2)(p23), +3, +3, add(22)(q13), +3mar
Figure 4.26 Representative karyotype from case L025
L026A

B-cell low-grade lymphocytic

46, XY, +12, -13, del(14)(q24), add(17)(q25), add(17)(q21)
Figure 4.27 Representative karyotype from case L026A
L026B

B-cell low-grade lymphocytic

46, XY, +12, -13, del(14)(q24), add(17)(q25), add(17)(q21)
Figure 4.28  Representative karyotype from case L026B
L027

B-cell low-grade follicular

48, XX, +5, i(6)(p10), +12, t(14;18)(q32;q21), -18, +mar
Figure 4.29  Representative karyotype from case L027
L028

B-cell high-grade centroblastic

43-46, X, add(X)(p22), add(1)(p32), +3, add(4)(q21), -6, add(7)(p21),
add(8)(p21), -10, -13, add(16)(p12), -17, add(18)(q23),
der(20)t(X;20;?) (q13;q13;?), -22, +2mar [cp25]
Figure 4.30  Representative karyotype from case L028
L029- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 35 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.31.

L030- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 23 cells, of which 100% exhibited an abnormal karyotype, shown in figure 4.32.

L031- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 36 cells, of which 83% exhibited an abnormal karyotype. The most common clone (represented by 10 cells) is shown in figure 4.33. Three other clones were identified. One (7 cells) showed only an add(14)(q32), another (6 cells) displayed an additional add(3)(q27) and the third (2 cells) revealed additional abnormalities of chromosomes X, 2, and 20.

L032- This case was diagnosed as B-cell high-grade centroblastic lymphoma. Cytogenetic analysis was carried out on 32 cells, of which 50% exhibited an abnormal karyotype, shown in figure 4.34.

L033- This case was diagnosed as T-cell high-grade immunoblastic lymphoma. Cytogenetic analysis was carried out on 59 cells, of which 3% exhibited an abnormal karyotype (data not shown). However, since none of the aberrant cells were found to be clonal, they were not included in the overall analysis of the series.

L034- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 24 cells, of which 87% exhibited an abnormal karyotype, shown in figure 4.35.
L029

B-cell high-grade centroblastic

87, XXY, -5, del(6)(q23q26), -9, -10, -13, t(14;18)(q32;q21) x2, -16,
del(16)(q22), -19, -20, +2mar
**Figure 4.31** Representative karyotype from case L029
L030

B-cell high-grade centroblastic

49, XY, +X, +12, +mar
Figure 4.32  Representative karyotype from case L030
L031

B-cell low-grade follicular

46, XX, add(14)(q32), del(17)(p13)
Figure 4.33 Representative karyotype from case L031
L032

B-cell low-grade follicular

82-85, XXY, -Y, i(1)(q10), add(1)(p36) x2, add(2)(p13), +i(3)(p10) x2,
add(3)(p13), -4, del(4)(q12q25), +del(6)(q22q23), del(7)(q22) x2, -8, add(8)(p11),
-9, add(9)(q34), -11,-11, -11, -12, add(12)(p13), -13, +add(14)(q22), -15, -15,
add(16)(q11) x2, +inv(16)(q11p12), -17, -17, -18, -18, -19, -21, +3mar [cp16]
{add(14) revised to mar and two mar revised to der(18)? following FISH}
Figure 4.34  Representative karyotype from case L032
L034

B-cell low-grade follicular

49, XX, +X, +7, +8, add(6)(q13), add(6)(q23), t(14;18)(q32;q21), add(17)(q21)
Figure 4.35 Representative karyotype from case L034
L036

B-cell low-grade follicular

72-74, XX, +X, +X, +add(1)(p13), +2, +add(3)(q27), +4, +5, +5,
+6, +add(6)(q23), +7, +7, +8, +9, +add(9)(q34),
+add(11)(q23), +13, +add(14)(q32), t(14; 18)(q32;q21) x2, +16, -18, -19, +20,
+21, +del(22)(q11), +11mar [cp30]
Figure 4.36 Representative karyotype from case L036
L037

B-cell low-grade, follicular

46, XX, t(14;18)(q32;q21)
Figure 4.37 Representative karyotype from case L037
L035- This case was diagnosed as B-cell high-grade immunoblastic lymphoma. Cytogenetic analysis was carried out on 24 cells, none of which exhibited an abnormal karyotype (data not shown).

L036- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 33 cells, of which 91% exhibited an abnormal karyotype, shown in figure 4.36.

L037- This case was diagnosed as B-cell low-grade follicular lymphoma. Cytogenetic analysis was carried out on 21 cells, of which 81% exhibited an abnormal karyotype. The most common clone is shown in figure 4.37. Another clone (represented by 7 cells) showed an additional translocation between chromosomes 9 and 19.

4.3) OVERALL ANALYSIS OF THE SERIES

4.3.1) Introduction

In order to identify consistent data, the results were pooled. Three of the samples were re-biopsies (cases L010B, L020B and L026B) and information from them was not included in the analyses described below, because this might bias the frequencies determined when considering the overall data.

The majority of cases showed a pseudo- or hyper-diploid karyotype. Additionally, two samples were near triploid and four were near tetraploid. Only three cases (L018, L033 and L035) had no clonal abnormalities, although in about half the samples a mixture of normal and abnormal cells was observed.

4.3.2) Numerical analysis

The X chromosome was gained most frequently (32% of cases), which was observed in both males (25%) and females (33%). This was followed in frequency
by trisomy of chromosomes 12 (27%), 3 (24%), 7 (16%) and 8 (16%). Chromosome loss was far less common, most frequently involving chromosome 13 (13.5%). With the exception of L028 and L032, which both exhibited highly complex karyotypes with several marker chromosomes, loss of a chromosome 11, 13 or 17 appeared to be mutually exclusive. As a result, a quarter of the samples studied showed loss of one of these chromosomes. Figure 4.38 shows the percentage of cases in which a gain or loss of each of the chromosomes was observed.

**Figure 4.38  Numerical Chromosome Changes in NHL**

![Graph showing numerical chromosome changes in NHL](image)

The series contained both high and low grade lymphomas (13 and 24 cases respectively), allowing comparison between the karyotypes of these two classes of NHL. Cases of loss of chromosomes were relatively few and have therefore not been considered; however, numerical gains are summarised in Figure 4.39, which shows an interesting difference between high and low grade NHL, since most of the chromosome gains appeared to be concentrated in the low-grade samples,
although additional chromosomes 3 and X occurred at similar frequencies in both groups.

Figure 4.39 Chromosome Gains in High and Low Grade NHL

The series could also be split into two approximately equal sized groups on the basis of treatment before biopsy. Again there were few chromosome losses to analyse, but numerical gains are summarised in Figure 4.40. The samples from patients who had previously been treated exhibited an increased frequency of gains of all chromosomes, with the exception of chromosome 12 and to a much lesser extent chromosomes 21 and 22.
4.3.3) Structural analysis

Figure 4.41 summarises the percentage of cases in which structural abnormalities were observed. All chromosomes, with the exception of 21 and Y, were involved in structural changes. The most frequently affected chromosome was 14 (70%), which in approximately half the samples was involved in the t(14;18)(q32;q21). However, 11% of cases contained an add(14)(q32) with no identifiable donor chromosome. Chromosome 1 was affected in 40.5% of cases, with bands p32-36 and q21 most frequently involved. Abnormal chromosomes 6 were observed in 35% of cases and most exhibited an altered long arm. When translocation breakpoints were included, 27% of all cases exhibited deletion or rearrangements of 6q21-25. Other commonly rearranged chromosomes were 9 (22%) with breakpoints at p22, q12 and q21 each occurring in two cases, 17 (22%) with 17.5% of cases involving the p arm and 3 (19%), with bands p13, q13, q21 and
q27 each involved in two cases. A majority of the analysed samples contained chromosomes that could not be fully identified.

**Figure 4.41 Structural Chromosome Changes in NHL**

When the structural rearrangements seen in high and low-grade NHL were compared, a similar pattern of changes was revealed (Figure 4.42). However, a slightly increased frequency of structural abnormalities was seen in the low-grade samples.

When the structural rearrangements observed in those cases that had received treatment was contrasted with untreated samples a broadly similar pattern of occurrence was seen (Figure 4.43), although they appeared to be more common in the treated group. Low-grade samples in particular showed more structural abnormalities after treatment than the high-grade group.
Figure 4.42 Structural Chromosome Changes in High and Low Grade NHL

![Bar chart showing chromosome changes in high and low grade NHL.

- Percentage of cases for each chromosome.
- Bars for high grade NHL.
- Bars for low grade NHL.

Figure 4.43 Structural Chromosome Changes in Treated and Untreated NHL

![Bar chart showing chromosome changes in treated and untreated NHL.

- Percentage of cases for each chromosome.
- Bars for treated NHL.
- Bars for untreated NHL.
CHAPTER FIVE

RESULTS OF FISH STUDIES
5.1) INTRODUCTION

In order to gain further information from the cytogenetic analysis of the NHL karyotypes, the FISH technique was used. Amongst the numerical changes observed, a significant percentage of cases had shown additional copies of the X chromosome, but, since the activation status of those extra chromosomes was not known, it was difficult to assess their importance in terms of potential oncogene activation. It had been noted that a proportion of the karyotypes contained add(14)(q32), in which the additional material could not be identified cytogenetically. This was of particular interest, since several oncogenes are known to be activated by translocation to the immunoglobulin heavy chain locus at 14q32. Structural abnormalities of chromosome 6, particularly of the long arm, were very common in the series and this frequently rearranged area was known to be the location of several oncogenes. Unusually, one case of NHL was found to contain double minutes and a FISH approach was used to characterise these extra chromosomal bodies in more detail. Interphase FISH was also applied, both to cases from the series and to bone marrow smears from cases of childhood acute lymphoblastic leukaemia to investigate the possible uses of the technique on archival material.

5.2 NUMERICAL CHANGES

5.2.1) Investigation of X activation

Additional copies of the X-chromosome were observed in almost one third of the series. This high frequency was observed in both male and female patients, and in both low and high grade disease. It was therefore possible that the presence of extra X-chromosomes could be an important causative event in the evolution of NHL. Individuals with sex-chromosome syndromes, such as Klinefelter males and super-females, have additional X-chromosomes in all of their cells, but do not appear to show any significantly increased predisposition to NHL (Sandberg,
In the latter cases, however, the additional X-chromosomes are present in the inactive state (Lyon, 1972). It was therefore of interest to determine whether the activation state of the X-chromosomes in the NHL cases was different to that observed in sex-chromosome syndrome individuals.

The inactive state of the X-chromosome had previously been identified in cells with a structure called the Barr body. Unfortunately, it was not possible to identify Barr bodies with certainty amongst the clumped heterochromatin in interphase lymphoid cells, and therefore two alternative strategies were adopted. The first of these involved FISH analysis of the binding of a X-chromosome centromere probe to interphase nuclei to reveal the degree of decondensation of the X-chromosome centromeric region. This method had previously been shown to distinguish active from inactive X-chromosomes in fibroblasts, and had also allowed for the identification of X-chromosomes that had been reactivated by 5-azacytidine (Dyer et al., 1989). The second approach involved an analysis of the methylation state of the X-chromosomes. It was known that the inactive state was generally associated with a high degree of methylation but, in this study, the M27β probe (Fraser et al., 1989) was used, which was a probe for the DXS255 locus (mapped to Xp11.22) that was methylated in active X-chromosomes (Boyd et al., 1990; Gale et al., 1992). Southern blot analysis of the methylation status of this locus in the NHL DNA samples could therefore allow for the inactive and active X-chromosomes to be distinguished.

In order to determine whether the first approach was possible in material from lymphomas, the technique was first used on cultures of peripheral blood lymphocytes. An attempt was made to enrich cultures for different stages of the cell cycle, since it was expected that a difference would only be detectable between active and inactive X chromosomes at G0/G1. During S-phase, both X chromosomes would be in a similar decondensed conformation to allow DNA replication to take place and during G2/M, both X chromosomes would be highly condensed, prior to cell division. Differences were noted between cell cycle
stages and figure 5.1 illustrates typical G1, S and G2 nuclei. G1 cells showed one compact and one diffuse signal, S-phase cells showed two diffuse signals and G2 cells showed two compact signals.

Signal categorisation was subjective, since differences in both signal size and the degree of condensation from cell to cell made direct measurement impracticable. Furthermore, the orientation of the centromeres was often not parallel to the plane of view which made quantitation of their lengths impossible.

These findings were then confirmed, by studying interphase nuclei of peripheral blood lymphocytes from both a super female with three constitutive X chromosomes and from a Kleinfelter male with a sex chromosome complement of XXY. As expected, cells from the XXX sample revealed one diffuse and two compact signals and the XXY nuclei showed one compact and one diffuse signal. (figure 5.2)

The technique was then applied to NHLs from the series that had been previously shown to have an aberrant copy number of the X chromosome. The results are shown below as table 5.1.

Examples of three cases that appeared to contain more than one activated X-chromosome are shown in figure 5.3. In sample L022 there were three X-chromosomes, and FISH analysis revealed one discrete signal with two elongated signals (fig. 5.3a). L036 had four X-chromosomes, and FISH analysis revealed one discrete and three elongated signals (fig. 5.3b). In L010A there were five X-chromosomes, and analysis of the interphase cells revealed one discrete signal but four elongated signals (fig. 5.3c). Interestingly, all of these samples had been diagnosed as low grade, follicular, NHL. On the basis of the FISH data, the other samples in the series appeared to contain additional X-chromosomes that were present in the inactive state.
Figure 5.1  Interphase nuclei of normal peripheral blood lymphocytes, hybridised with a centromeric probe for the X-chromosome.

a) Typical G1 nucleus, showing one compact and one diffuse signal.
b) Typical S-phase nucleus, showing two diffuse signals.
e) Typical G2 nucleus, showing two compact signals.

Compact signals are indicated by arrows and diffuse signals by arrow heads.
Figure 5.2  Centromeric probe for the X-chromosome hybridised to interphase nuclei from peripheral blood lymphocytes.

a) Nuclei from a super female (XXX), showing one diffuse and two compact signals.

b) Nuclei from a Kleinfelter male (XXY), showing one compact and one diffuse signal.

Compact signals are indicated by arrows and diffuse signals by arrow heads.
Figure 5.3  FISH analysis of X-chromosome centromeres in interphase nuclei from a series of NHL cells. The cells were karyotyped and found to contain a) 3 X-chromosomes (case L022), b) 4 X-chromosomes (case L036), c) 5 X-chromosomes (case L010A). The arrow indicates the compact signal in the G1/S nucleus that represents the inactive X-chromosome.
### Table 5.1 Analysis of X Activation in NHL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex chromosomes</th>
<th>FISH analysis</th>
<th>Methylation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Active X</td>
<td>Inactive X</td>
</tr>
<tr>
<td>L005</td>
<td>XXX</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L010A</td>
<td>XXXXX</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>L010B</td>
<td>XXX</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L022</td>
<td>XXX</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>L023</td>
<td>XXX</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L032</td>
<td>XXY</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L034</td>
<td>XXX</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L036</td>
<td>XXXX</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>L109</td>
<td>X(X)</td>
<td>1/2</td>
<td>1/0</td>
</tr>
</tbody>
</table>

NA - tissue not available for analysis; Methylation data taken from Grierson et al, 1994.

Methylation of the X-chromosomes was studied by hybridising the M27β probe to Southern blots of Msp I / Kpn I, and Hpa II / Kpn I, digested NHL DNA samples. This work was carried out by another member of the laboratory (Grierson et al, 1994). The hybridisation pattern obtained after Msp I digestion of the DNA revealed fragments of approximately 7-10 kb in size. The size differences were due to the presence of a VNTR which was highly polymorphic. Therefore in some samples the two alleles were readily resolved (L022), but in others they coincided (L009). Hpa II digestion of the DNA samples demonstrated that one of the alleles was heavily methylated and could not be cut at the Msp I / Hpa II site, and therefore produced a Kpn I fragment of over 12 kb. The latter represented the active X-chromosomes in the sample. The hybridisation patterns were scanned by a densitometer to quantify the ratios of the alleles and to assess the proportion of X-chromosomes in the active state (table 5.1). This indicated that in L010A there was more than one X-chromosome present in the active state,
which had already been suggested by the FISH analysis. The methylation study did not, however, support the observation of the FISH analysis that additional X-chromosomes were present in the active state in patient L022 (Fig. 5.3a). It was also noteworthy that in some cases (L010B, L034) the densitometer ratios implied that there were many more inactive X-chromosomes present than had actually been observed cytogenetically.

When patient L109 was examined for methylation of the DXS255 locus, there appeared to be a significant increase in the hybridisation signal representing the activated X-chromosomes. This sample was further analysed for LOH of the DXS14 locus, which was located close to, and proximal to, the DXS255 locus. The region deleted from the abnormal X-chromosome was distal to the positions of these loci. These data revealed evidence for loss of one of the X-chromosomes in a substantial number of cells in the biopsy sample, as the 2.5kb fragment was far more prominent in the NHL biopsy sample than in peripheral blood leukocyte DNA from the same patient. Cytogenetic analysis had not previously been carried out on case L109. When the chromosomes in this biopsy sample were examined, although there were a number of normal cells present, a second clone appeared to show monosomy of the X-chromosome in addition to other abnormalities that included a marker chromosome and double minutes (figure 5.4).

However, FISH analysis of this sample had revealed two signals of differing degrees of decondensation in each cell (table 5.1). This anomaly was resolved by demonstrating, with chromosome painting, that the unidentified marker chromosome was in fact a derivative X-chromosome that had lost most of its p and q arms (Section 5.2.2). It was not clear, however, whether the one active / one inactive X-chromosome combination of signals referred only to the normal cells, and whether the combination of two active X-chromosomes represented the malignant cells.
**Figure 5.4** Apparent karyotype of case L109: 49, X, -X, +3, i(6p), +8, t(14;18)(q32;q21), +add(18)(q21), +mar

**5.2.2) Investigation of monosomy X**

Chromosome painting was used to examine abnormal chromosomes whose origins were unclear. In one case (L109), an apparent monosomy of the X chromosome was investigated (see section 5.2.1). Although loss of the X chromosome had been observed in over a third of T-cell leukaemias and lymphomas (Pui et al, 1988), it was uncommon in B-cell NHL (Mitelman et al, 1991a). An X chromosome paint was therefore used to identify the presence of X chromosome sequences on other chromosomes. We found that the paint hybridised to both the single X chromosome and also to a marker chromosome
(fig. 5.5). The latter was apparently a second X chromosome that had lost parts of both its p and q arms.

5.2.3) Interphase analysis

5.2.3.1) Interphase analysis of NHL
When studying the cytogenetics of malignancy, there is often difficulty in obtaining a sufficient number of metaphases suitable for analysis. In order to investigate the possibilities for analysing non-dividing material, FISH was used to identify numerical chromosome abnormalities in interphase NHL cells. A series of experiments were performed, involving the hybridisation of an alphoid repeat sequence, which hybridised to the centromeric region of the X chromosome. In lymphocytes from a normal female donor, both of the X chromosomes were clearly indicated in both metaphase spreads and interphase nuclei (fig. 5.6), as were the three X chromosomes in lymphocytes from a superfemale donor (fig. 5.2a). This approach was used to identify the X chromosomes in interphase nuclei from NHL cells containing three (fig. 5.3a), four (fig. 5.3b) and five (fig. 5.3c) X chromosomes.

5.2.3.2) Interphase analysis of archival material
In order to explore the potential use of interphase FISH in archival material, a series of experiments was performed on bone marrow smears from cases of childhood acute lymphoblastic leukaemia (ALL). To evaluate the reliability of FISH on aged bone marrow slides, a probe for the centromeric region of the X chromosome was applied to a series of bone marrow smears from ALL patients in remission, which had been stored from 6 months to 20 years. Centromere probes for chromosomes X, 6 and 18 were also hybridised to smears obtained at diagnosis, from cases found to have an abnormal karyotype by conventional cytogenetics, to assess the degree of concordance between results obtained by
Figure 5.5 Metaphase spread from case L109 that had apparently exhibited monosomy of the X chromosome, hybridised with a painting probe specific for the X-chromosome. Signal is apparent on both the X chromosome (arrow head) and on an unidentified marker chromosome (arrow).
FISH and routine chromosome analysis. This work was carried out in collaboration with the Section of Paediatric Haematology at the Sheffield Children's Hospital and also the Centre for Human Genetics in Sheffield, where the chromosome analysis was performed.

Table 5.2 gives patient information, smear type and age and the percentages of nuclei with different numbers of X centromere hybridisation signals for the series of patients in remission. The hybridisation efficiency ranged from 51-99% with an average of 76.7%. The oldest sample showed the lowest efficiency and the most recent the highest. A small percentage of nuclei demonstrated extra X centromere signals (two in males and three in females), with a mean of 1.86±1.21. Figure 5.7 illustrates hybridisation of the X centromere to typical interphase nuclei from smears stored for 10 and 15 years.

Details of the hybridisation of probes specific for the centromeric regions of chromosomes X, 6 and 18 to bone marrow smears from children with ALL are given in table 5.3. In this series of samples a hybridisation efficiency of between 76.5 and 100% was obtained, with the unfixed frozen material showing very few nuclei without signals. A trisomic clone was defined as being present in an ALL bone marrow smear when the percentage of nuclei displaying three signals was greater than the mean % ± twice the standard deviation of cells showing an extra signal in the remission samples (Anastasi et al, 1991). When this criterion was applied to the ALL smears, all cases clearly demonstrated the presence of extra copies of those chromosomes previously identified by conventional cytogenetics as aneusomic. Results from the unfixed material showed a closer correspondence.
Figure 5.6  FISH analysis of the X chromosome in peripheral blood lymphocytes by the use of a centromeric alphoid repeat probe in metaphase and interphase cells.
**Figure 5.7** Hybridisation of X centromere probes to interphase nuclei from Romanowsky stained bone marrow smears.

a) 10 year old bone marrow smear from a female patient with ALL in remission. Two signals are clearly visible in most cells.

b) 15 year old bone marrow smear from a male patient with ALL in remission. One signal is present in a number of cells.
Table 5.2 FISH with an X Centromere Probe applied to Stored Bone Marrow Smears taken from Patients with ALL in Remission

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age (Y) /Sex</th>
<th>Age of smear (M)</th>
<th>Type of smear</th>
<th>% of cells with number of signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/M</td>
<td>6</td>
<td>RM</td>
<td>26  72  2  0</td>
</tr>
<tr>
<td>2</td>
<td>8/M</td>
<td>6</td>
<td>RM</td>
<td>49  49.5  1.5  0</td>
</tr>
<tr>
<td>3</td>
<td>8/F</td>
<td>6</td>
<td>RM</td>
<td>22.5  11  65  1.5</td>
</tr>
<tr>
<td>4</td>
<td>4/F</td>
<td>12</td>
<td>RM</td>
<td>5  14  81  0</td>
</tr>
<tr>
<td>5</td>
<td>2/F</td>
<td>36</td>
<td>RM</td>
<td>23.5  18  56  2.5</td>
</tr>
<tr>
<td>6</td>
<td>9/F</td>
<td>240</td>
<td>RM</td>
<td>49  15  36  0</td>
</tr>
<tr>
<td>7</td>
<td>15/F</td>
<td>120</td>
<td>RM</td>
<td>16  16  66  2</td>
</tr>
<tr>
<td>8</td>
<td>3/M</td>
<td>60</td>
<td>RM</td>
<td>21  74.5  4.5  0</td>
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<td>9</td>
<td>5/F</td>
<td>1</td>
<td>RM</td>
<td>1  6  90  3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>3  10  85  2</td>
</tr>
<tr>
<td>10</td>
<td>6/M</td>
<td>180</td>
<td>RM</td>
<td>40.5  58  1.5  0</td>
</tr>
</tbody>
</table>

RM: - Romanowsky stained, UF: - unfixed

To the proportion of abnormal cells found cytogenetically, than did results from Romanowsky stained slides.

In case 15, the data confirmed the identity of a marker chromosome thought to be derived from chromosome 6 and revealed the possible presence of a small clone containing three chromosome 6 centromeres.

5.3) STRUCTURAL CHANGES

5.3.1) Investigation of add(14)(q) chromosomes

Although the long arm of chromosome 14 was the most common region of rearrangements in the series, it was not always possible to determine the origin of
<table>
<thead>
<tr>
<th>Case No</th>
<th>Age/Sex</th>
<th>Age of Smear (m)</th>
<th>Type of Smear</th>
<th>Probe Specificity</th>
<th>Percentage of Cells with Number of Signals</th>
<th>Cytogenetic Abnormality</th>
<th>Percentage Abnormal Mitoses</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>4/F</td>
<td>13</td>
<td>RM</td>
<td>X</td>
<td>4</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>12</td>
<td>2/F</td>
<td>42</td>
<td>UF</td>
<td>X</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>13</td>
<td>4/M</td>
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<td>X</td>
<td>2</td>
<td>18</td>
<td>78</td>
</tr>
<tr>
<td>14</td>
<td>1/F</td>
<td>75</td>
<td>RM</td>
<td>X</td>
<td>0</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>9/F</td>
<td>11</td>
<td>RM</td>
<td>X</td>
<td>6</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>16</td>
<td>8/M</td>
<td>52</td>
<td>UF</td>
<td>18</td>
<td>2</td>
<td>10</td>
<td>73</td>
</tr>
<tr>
<td>17</td>
<td>1/F</td>
<td>76</td>
<td>RM</td>
<td>18</td>
<td>26.5</td>
<td>44.5</td>
<td>18.5</td>
</tr>
<tr>
<td>18</td>
<td>8/M</td>
<td>76</td>
<td>RM</td>
<td>18</td>
<td>16.5</td>
<td>20.5</td>
<td>34.5</td>
</tr>
</tbody>
</table>

RM: Romanowsky stained, UF: unfixed.
material translocated onto chromosome 14. These cases were initially investigated using chromosomes 14 and 18 paints since chromosome 18 is the most common translocation partner for chromosome 14 in NHL. If this first approach proved uninformative, paints for chromosome 14 and either chromosome 3, 8, 10, 11 or 19 were then applied because, as discussed above (Section 1.3.2), all of these chromosomes carry oncogenes which are activated in translocations with chromosome 14.

**Patient L023.** An example of a typical t(14;18)(q32;q21) was observed in the malignant cells of the biopsy sample (fig. 5.8), which had been diagnosed as low grade follicular NHL. The chromosome 14 paint hybridised to the normal chromosome 14 and to the der(14), however, the region of chromosome 14 that was translocated to form the der(18) was too small to detect by painting. The hybridisation of the chromosome 18 paint to these cells clearly demonstrated the presence of three chromosomes 18, two of which were der(18q), as well as chromosome 18 material that had been translocated to the q arm of the der(14) chromosome (fig. 5.9a).

**Patient L009.** The karyotype was pseudotriploid and in addition to a t(14;18)(q32;q21) also contained a normal chromosome 14, a der(14) (fig. 5.8) and two chromosomes 18. This biopsy had been diagnosed as low grade follicular NHL. The chromosome 14 paint hybridised to all three chromosomes 14, but the chromosome 18 paint hybridised to both der(14), the chromosomes 18 and to two der(10) chromosomes (fig. 5.9b).

**Patient L022.** This karyotype contained two der(14)t(14;?) (q32;?) (fig. 5.8) with three apparently normal chromosomes 18. The biopsy was diagnosed as low grade follicular NHL. Dual painting with chromosome 14 and 18 paints demonstrated that both der(14) hybridised to the chromosome 14 paint, but only
Figure 5.8 Partial karyotypes of non-Hodgkin's lymphomas showing the normal and derivative chromosomes 14. Giemsa-banding of the malignant cell metaphases was carried out as described in the Materials and Methods. The normal chromosomes 14 are shown on the left in each case and the apparent translocation breakpoints on the der(14) indicated by arrowheads.

a) L023; b) L009; L022; d) L031; e) L011; f) L006 - the del(14q) is on the left and the der(14) on the right; g) L021; h) L032.
**Figure 5.9** Analysis of non-Hodgkin's lymphoma metaphases by dual chromosome painting. In all experiments the chromosome 14 paint is shown in red and the other paint in green. The chromosomes were counterstained using DAPI (blue).

a) L023 - chromosomes 14 and 18 paints were used. The der(14) is indicated by the arrow.

b) L009 - chromosomes 14 and 18 paints were used. The der(14) are indicated by the white arrows and the der(10) chromosomes by the yellow arrows.

c) L022 - chromosomes 14 and 18 paints were used. The der(14) which hybridized to the chromosome 18 paint is indicated by the white arrow and the der(14) that did not hybridize with the chromosome 18 paint is indicated by the yellow arrow.

d) L031 - chromosomes 14 and 3 paints were used. The der(14) chromosome is indicated by the arrow.

e) L011 - chromosomes 14 and 3 paints were used. The der(14) is indicated by the white arrow.

f) L006 - chromosomes 14 and 3 paints were used. The der(14) is indicated by the white arrow.

g) L021 - chromosomes 14 and 11 paints were used. The der(14) is indicated by the white arrow and the marker by the yellow arrow.

h) L032 - chromosomes 14 and 18 paints were used. The four normal chromosomes 14 are in red, the two normal chromosomes 18 in green and two of the markers also in green.
one der(14) hybridised with the chromosome 18 paint; which also hybridised to the three chromosomes 18 (fig. 5.9c). The unknown material that had been translocated to the q arm of the other der(14) did not hybridise with paints for either chromosome 3 or 19 and cytogenetically did not resemble chromosome 11 material. No further analysis was possible on this sample, which had become depleted.

**Patient L031.** Several different clones of cells were identified in this sample, which had been diagnosed as low grade follicular NHL. The only common abnormality in all clones was the presence of a der(14)t(14;?)(q32;?), which was not typical of the product of the t(14;18) (fig. 5.8). The paints for chromosomes 14 and 18 only hybridised to their respective chromosomes. Therefore paints from chromosomes 14 and 3 were used, as a der(3)t(3;?)(q27;?) had been observed in one of the clones. In this case the chromosome 14 paint hybridised only to the normal and der(14) chromosomes and the chromosome 3 paint only to the normal and der(3) chromosomes (fig. 5.9d). Similar results were obtained by dual painting with chromosome paints for 14 and 8, 14 and 10, 14 and 11 and 14 and 19 where in each case the paints hybridised only to their respective chromosomes. As a result, the origins of the material that had been translocated to the q arm of this der(14) were not identified.

**Patient L011.** Several malignant cell clones were identified in this biopsy, which had been diagnosed as low grade centrocytic NHL (though within a few months of this biopsy the patient relapsed with high grade centroblastic NHL). The only consistent abnormalities observed in all of the clones was a der(14), which was not typical of the product of the t(14;18) (fig. 5.8), and extra chromosomes 8 and 18, which were apparently normal. Dual painting with chromosome 14 and 18 paints, demonstrated that they hybridised to their respective chromosomes. Chromosome 14 and 3 paints were then used and this time the material
translocated to the q arm of the der(14) was shown to be from chromosome 3. The two chromosomes 3 appeared to be cytogenetically normal and hybridised with the chromosome 3 paint, but not with the chromosome 14 paint (fig. 5.9e).

**Patient L006.** Two abnormal chromosomes 14 were observed, one of which was a del(14q) and the other a der(14) (fig. 5.8) similar to the one observed in L011. The diagnosis for this biopsy was high grade centroblastic NHL. The chromosome 14 and 18 paints hybridised only with their respective chromosomes. Therefore chromosome 14 and 3 paints were used and this time the material that had been translocated to the q arm of the der(14) was identified as originating from chromosome 3 (fig. 5.9f).

**Patient L021.** A der(14)t(14;?)q24;?) (fig. 5.8) and a number of marker chromosomes were observed in this biopsy, which had been diagnosed as high grade centroblastic NHL. The paints for chromosomes 14 and 18 hybridised with their respective chromosomes. Similarly paints for chromosomes 14 and 3, 14 and 8, and 14 and 19 all hybridised with their respective chromosomes. However, when the chromosomes were dual painted with chromosome 14 and 11 paints, the chromosome 11 paint hybridised to a region that had apparently been inserted into the q arm of the der(14), in a manner which was not suspected from the Giemsa banding. This paint also hybridised to the normal chromosome 11 and to a marker chromosome (fig. 5.9g).

**Patient L032.** The karyotype was pseudotetraploid and contained four normal chromosomes 14 and an apparent der(14)t(14;?)q22;?) (fig. 5.8) with two chromosomes 18 and three marker chromosomes. The diagnosis for this biopsy was low grade follicular NHL. Dual painting with chromosome 14 and 18 paints demonstrated that the paints hybridised to their respective chromosomes, but the chromosome 18 paint also hybridised to two of the markers (fig. 5.9h). The
surprising result was that only the four normal chromosomes 14 hybridised with the chromosome 14 paint and the larger abnormal chromosome that had been identified as a der(14) by Giemsa-banding did not in fact contain chromosome 14 material. This observation was consistent in all ten cells analysed.

5.3.2) Investigation of abnormalities of chromosome 6.
Abnormalities of chromosome 6 were studied by two methods. In the first approach, a chromosome 6 paint was hybridised to NHL metaphases obtained from cases displaying an aberrant chromosome 6, in order to reveal either any translocated 6 material or the presence of non 6 chromatin (which would be visible as counterstain) in rearrangements of chromosome 6 detected by conventional cytogenetics. The second strategy involved refining the previously mapped position of the MYB proto-oncogene on the long arm of chromosome 6, using metaphase spreads from normal PBLs. The MYB probe was then hybridised to NHL metaphases to determine whether the locus of the gene was involved in any sub-microscopic rearrangements.

5.3.2.1) Painting of chromosome 6
Chromosome painting of cases L007, L010A, L017, L022, L023, L032, L034 and L036 failed to reveal any unexpected absence of chromosome 6 material from previously noted abnormal chromosomes 6. Furthermore, in only one case (L036), did any other chromosome display signal from the 6 paint, which in this instance was considered to indicate the presence of 6 material in one of the marker chromosomes revealed by conventional cytogenetics. Figure 5.10 shows examples of chromosome painting in cases L036, L034 and L032.

5.3.2.2) FISH analysis of the MYB proto-oncogene
The MYB probe was mapped to metaphase spreads according to the methods described in Section 3.7. Four separate experiments provided 13 metaphases, in which both FISH and post banding results were of sufficient quality to derive
Figure 5.10 Analysis of NHL metaphases with chromosome 6 painting.

a) L036; arrows indicate apparently normal chromosomes 6. A marker containing chromosome 6 material is indicated by an arrowhead.

b) L034; arrowheads indicate add(6) chromosomes.

c) L032; arrows indicate apparently normal chromosomes 6. An arrowhead indicates a del(6) chromosome.
clear mapping data. Table 5.4 displays the raw data (in millimetres) obtained from projected slides of the preparations, the Fractional Length (derived from cen-pter/probe-qter on the FISH preparations), the measurement (derived with the FL) for probe-qter on the post banded chromosomes and the specific band identified by application of the measurement.

Table 5.4 Mapping of the MYB Proto-oncogene onto Metaphase Chromosomes

<table>
<thead>
<tr>
<th>FISH cen-qter</th>
<th>probe-qter</th>
<th>FL</th>
<th>POST BANDING cen-qter</th>
<th>probe-qter</th>
<th>band</th>
</tr>
</thead>
<tbody>
<tr>
<td>157</td>
<td>48</td>
<td>3.3</td>
<td>146</td>
<td>45</td>
<td>6q23.2</td>
</tr>
<tr>
<td>113</td>
<td>38</td>
<td>3.0</td>
<td>102</td>
<td>34</td>
<td>6q23.1</td>
</tr>
<tr>
<td>149</td>
<td>47</td>
<td>3.2</td>
<td>147</td>
<td>46</td>
<td>6q23.2</td>
</tr>
<tr>
<td>68</td>
<td>22</td>
<td>3.1</td>
<td>66</td>
<td>21</td>
<td>6q23.3</td>
</tr>
<tr>
<td>94</td>
<td>29</td>
<td>3.2</td>
<td>93</td>
<td>29</td>
<td>6q23.2</td>
</tr>
<tr>
<td>67</td>
<td>23</td>
<td>2.9</td>
<td>67</td>
<td>23</td>
<td>6q23.2</td>
</tr>
<tr>
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<td>27</td>
<td>3.2</td>
<td>84</td>
<td>26</td>
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</tr>
<tr>
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<td>18</td>
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<td>60</td>
<td>18</td>
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</tr>
<tr>
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</tr>
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<td>26</td>
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</tr>
<tr>
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<td>125</td>
<td>46</td>
<td>6q23.2</td>
</tr>
<tr>
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<td>30</td>
<td>3.2</td>
<td>96</td>
<td>30</td>
<td>6q23.3</td>
</tr>
</tbody>
</table>

Figure 5.11 illustrates probe hybridisation and post banding in a typical mapping metaphase. In all scored cells, the MYB probe was mapped to band 6q23, with over 50% of the metaphases showing hybridisation to 6q23.2.

The MYB probe was then applied to metaphases obtained from NHLs, in order to investigate any occult rearrangements involving the 6q23 locus. Acceptable FISH results were obtained in two cases (LO10A and L017). Metaphases from these cases, hybridised with both the MYB probe and the p308 chromosome 6 centromere probe, are shown in figures 5.12. L010A showed three pairs of signals on at least three of the four chromosomes 6 present, implying that submicroscopic
Figure 5.11 FISH analysis of the MYB gene on normal and NHL metaphases. a) Mapping of the MYB proto-oncogene to chromosome 6q23. FISH localization of the MYB proto-oncogene on a metaphase spread from normal human lymphocytes (indicated by the arrow). A chromosome 6 centromeric probe was also used to aid identification.

b) Post-banding of the metaphase spread shown in a).
Figure 5.12 FISH analysis of the MYB gene on NHL metaphase spreads. The arrows indicate the positions of the MYB hybridization signals on the visible parts of the chromosomes 6. A chromosome 6 centromeric probe was also used to aid identification.

a) Case L010A  b) Case L017
amplification and subsequent reintegration of the 6q23 region had taken place. In contrast, L017 revealed only a single pair of signals on each of the two chromosomes 6. However, on one chromosome, the signals were present at an aberrant, more proximal locus on the long arm. This suggested a previously unsuspected chromosomal rearrangement involving the 6q23 region had occurred.

5.3.4) Analysis of double minutes in NHL

Double minutes are rarely found in NHL and none were observed in the series of samples reported in Chapter four. However, when more than 30 abnormal cells were examined from an additional node biopsy from a case (L109) with a diagnosis of follicular low grade NHL (see Section 5.2.1), unexpectedly, all were found to contain double minutes (dmin). The number of dmin ranged between 5-20, with a median of 10 dmin per cell. The identification of these extrachromosomal bodies as dmin was based on their morphology (fig. 5.13a) and the fact that they were negative by C-banding (fig. 5.13b). As part of the characterisation of abnormalities in these cells, an X chromosome paint had been hybridised to the metaphases (see Section 5.2.2). This unexpectedly revealed that the dmin were composed of X chromosome material (fig. 5.13e). In parallel studies an X chromosome centromere probe was observed to hybridise to both the X chromosome centromeres and to all of the dmin (fig. 5.13d). As a negative control, a centromere probe for another chromosome (in this case chromosome 6) was used and it was observed to hybridise only to the chromosomes 6 and not to the dmin. It was thus demonstrated that the dmin in these cells were derived from an X chromosome and that they contained sequences that were specific for the centromeric region.

Further FISH analysis of the case demonstrated that a pan-centromere probe hybridised not only to the centromeres of all chromosomes, but to each of the dmin (fig. 5.14a). The hybridisation of this probe was therefore examined in cells from a neuroblastoma biopsy sample which contained many (usually >20) dmin.
Figure 5.13 Cytogenetic and FISH analysis of cells from a NHL node biopsy. a) G-banded metaphase, b) C-banded metaphase.
Cells hybridized with an X chromosome paint c) or an X chromosome centromere probe d). Examples of dmin are indicated by arrowheads.
Figure 5.14  FISH analysis of the presence of centromere-associated DNA sequences in dmin in malignant cells. NHL cell hybridized with the pan-centromere probe (a). Neuroblastoma cell stained with propidium iodide (b) and hybridized with the pan-centromere probe (c). Soft tissue sarcoma cell stained with propidium iodide (d) and hybridized with the pan-centromere probe (e). Acute myeloid leukaemia cells from patients AML-6528 and AML-5926 stained with propidium iodide (f and h, respectively) and hybridized with the pan-centromere probe (g and i, respectively). Examples of dmin are indicated by arrowheads.
per cell (fig. 5.14b). In this case there was no evidence of hybridisation to the
dmin (fig. 5.14c). As it was known that the dmin in these tumour cells are
associated with amplification of the N-MYC gene from chromosome 2 (Kohl et
al, 1983), a chromosome 2-specific centromere probe was also hybridised to the
metaphase spreads. Even under conditions of low stringency there was still no
evidence of hybridisation of this centromere probe to the dmin. Cells from a soft
tissue sarcoma biopsy sample, which contained approximately 20 dmin per cell
(fig. 5.14d), were also hybridised with the pan-centromere probe and similarly
failed to reveal hybridisation to the dmin (fig. 5.14e). Acute myeloid leukaemia
cells from two patients were also examined. These cells were observed to contain
a variable number of dmin, but in both cases there were always <20 dmin per cell
(fig. 5.13f, h). It was found that approximately 25% of the dmin in these cells
hybridised with the pan-centromere probe (fig. 5.14g, i). Although this might
indicate that the dmin were not homogeneous in the AML cells, the failure to
detect signal in some of the dmin might also have been due to the generally
fainter hybridisation signals observed in these cells. In both of these figures,
examples of dmin are indicated by five arrows, two of which indicate dmin that
contained centromere-associated DNA and three arrows that indicate dmin which
did not contain centromere-associated DNA. The hybridisation data thus
demonstrated the existence of dmin containing centromere-associated DNA
sequences in the malignant cells of lymphoma / leukaemias, but not in cells from
those solid tumours examined.
CHAPTER SIX

DISCUSSION
6.1) CYTOGENETICS

6.1.1) General considerations

Cytogenetic analysis was performed on a series of 40 NHL node biopsies obtained from U. K. patients. This group was composed of both low- and high-grade NHL samples and approximately equal numbers of untreated and previously treated patients.

A considerable number of series detailing NHL karyotypes have been published over the last decade (Kaneko et al, 1983; Kristofferson et al, 1987; Yunis et al, 1987; Levine et al, 1988; Cabanillas et al, 1989; Yunis et al, 1989; Schouten et al, 1990a; Offit et al, 1991b; Tilly et al, 1994). These studies have shown wide variation, both in terms of the percentage of successful analyses achieved and the number of analysed cases which showed only a normal karyotype.

The 40 karyotypes reported in this thesis were the result of culturing forty three lymph node biopsies, a success rate of 93%. This compares very favourably with other studies in the literature, which report success rates from 65% (Kaneko et al, 1983) to 84% (Juneja et al, 1990). Several aspects of the culture system (Section 2.2.2.1) may well contribute to the high percentage of analysable metaphases. Firstly, three separate cultures were set up for each specimen, increasing the chances of overall success, since it was not unusual for one of the cultures to fail. Further, no attempt was made to produce pro-metaphase chromosomes by manipulating the culture conditions. Lymph node tissue was disaggregated in pre-warmed media by a combined cutting and pressing action, using curved scissors. This technique generated a suspension containing low numbers of cells from the capsule and infra-structure of the node. Finally, the culture media contained human serum, in contrast to the foetal calf serum used in other studies (Koduru et al, 1987; Juneja et al, 1990).

Only three out of 40 samples (7.5%) in this series revealed an exclusively normal karyotype. Other studies have reported findings of 14% (Kristofferson et al,
1987), 20% (Offit et al, 1991a) and 24% (Koduru et al, 1987) cytogenetically normal cases. It is of course possible that normal karyotypes apparently obtained from tumour cells contain sub-microscopic or other abnormalities, which are not detectable by conventional cytogenetics. Alternatively, normal rather than tumour cells may have cultured. The lower incidence of normal karyotypes in this series may be a reflection of short culture times, no use of mitogens and a greater number of (malignant) lymphocytes in the initial culture system, due to the disaggregation procedure described above.

6.1.2) Numerical changes

Among the many numerical abnormalities, a particularly interesting finding was the much greater prevalence of numerical gains in the low-grade NHL samples. Twice as many of these samples exhibited trisomies of several chromosomes, when compared to those in the high-grade group. Furthermore, trisomies of almost all the chromosomes were observed in low-grade samples, whereas only seven of the chromosomes were involved in trisomies in the high-grade group. This observation does not appear to have been previously documented. It may be an indication of a more general breakdown in the machinery of cell replication in low-grade disease or is perhaps an example of the regional variation found in neoplasia-associated chromosome aberrations (Fifth International Workshop on Chromosomes in Leukaemia-Lymphoma, 1987; Johansson et al, 1991). However, further analysis of the data showed that trisomies were more common in low-grade disease samples from patients who had been previously treated than in similarly treated high-grade samples. The effect of the treatment may therefore have been greater on the chromosomes in low-grade than in high-grade NHL.

The most common numerical chromosome change observed was the presence of an additional chromosome X, present at a higher frequency than in previously reported series, in which the frequency varied between 3 and 22.5% (Offit et al, 1991a; Yunis et al, 1987; Fleischman et al, 1989). The significance of an extra X
chromosome is unclear, although in some NHL cases trisomy X has been reported as the only identifiable anomaly (Mitelman et al, 1991), suggesting that the gain of a copy of this chromosome may be a primary abnormality in NHL. Studies of other malignancies have indicated that additional X chromosomes are present in the active state in neoplastic cells (Dutrillaux et al, 1986; Wang et al, 1990) and the results of the study of X chromosome activation status in NHL cells from this series are discussed below in section 6.2.1.1.

Trisomies involving chromosomes 3 and 12 were observed at frequencies similar to those previously reported in other series (Offit et al, 1991a; Yunis et al, 1987; Fleischman et al, 1989; Fifth International Workshop on Chromosomes in Leukaemia-Lymphoma, 1987). The data presented in this thesis do not support suggestions that +3 is associated with high-grade lymphomas (Schouten et al, 1990a; Fleischman et al, 1989), or that +12 is associated either with lymphocytic (Mitelman et al, 1990) or diffuse large cell lymphoma (Cabanillas et al, 1988; Offit et al, 1991a). Trisomy 12 was twice as common in low-grade when compared to high-grade NHL in this series, but was not particularly associated with the lymphocytic subtype. Notably among the chromosome gains, +12 was more frequent in patients who had not been treated; in spite of the fact that low- and high-grade samples were evenly distributed between the treated and untreated groups. This may reflect clonal selection against trisomy 12, driven by chemotherapy. It is also possible that the relatively higher frequency of trisomy 12 in low-grade NHL could be a manifestation of the different treatment regimes used in the management of these cases; since low-grade NHL tend to be treated over many years while high-grade cases receive more aggressive, shorter term therapy.

Loss of a whole chromosome was not a frequent occurrence in the series. However, when loss of chromosomes 11, 13, or 17 was observed, usually only one of these chromosomes was lost in any particular sample. This is noteworthy, because studies in a variety of malignancies have demonstrated the presence of
tumour suppressor genes on these chromosomes. Chromosome 13 carries the RB-1 gene (Lundberg et al, 1987; Bookstein et al, 1988), chromosome 17 the p53 gene (Baker et al, 1989) and chromosome 11 the WT-1 gene (van Heyningen et al, 1985). Loss of one of these chromosomes occurs in one quarter of the cases in this study and suggests that molecular biology studies of these tumour suppressor genes may be informative. However, loss of chromosome 13 probably involves a tumour suppressor gene other than RB-1, since an extensive analysis of RB-1 in this series failed to identify any abnormalities (Grierson et al, 1995). A possible candidate TSG may exist at the DBM (Disrupted in B-cell Malignancy) locus, recently identified within 13q14 (the site of RB-1) in chronic lymphocytic leukaemias (Brown et al, 1993).

6.1.3) Structural changes
The most common chromosome abnormality detected in the series was the t(14;18)(q32;q21), with frequencies of 59% in follicular low grade and 17% in high-grade NHL. These findings are in agreement with the results of some previously published studies (Offit et al, 1991b; Schouten et al, 1990a), but somewhat lower than those of others (Yunis et al, 1987; Fleischman et al, 1989). It may be that these differences are a reflection of variations in culture techniques or ascertainment procedures between different laboratories. This translocation appears to be the mechanism by which the BCL-2 gene is activated (Tsujimoto et al, 1985) and is likely to be of central importance in the aetiology of follicular low grade NHL, since the BCL-2 gene product acts to prevent apoptotic cell death (Reed, 1994), thus allowing the expansion of subpopulations of cells capable of subsequent progression to malignancy. However, in a series of high-grade NHL with no previous history of low-grade disease BCL-2 rearrangements were uncommon (Lee K et al, 1993), suggesting further genetic changes are required for high grade malignancy.
Chromosome 6 was often observed to have all or part of its q arm deleted. If translocation breakpoints are included then 27% of all cases exhibited deletion or rearrangements of 6q21-25. Similar findings have been previously documented (Offit et al, 1991b; Fleischman et al, 1989; Fifth International Workshop on Chromosomes in Leukaemia-Lymphoma, 1987). There was no evidence to support the suggestions that these abnormalities were primarily associated either with previous treatment (Cabanillas et al, 1988) or immunoblastic lymphoma (Schouten et al, 1990b). In fact the data suggested a more common association of 6q abnormalities with low grade disease.

Recurrent breakpoints on other chromosomes were suggestive of known oncogene involvement. 1p32-36 (L-MYC) (Little et al, 1983), 17p13 (p53) (Baker et al, 1989), 3q27 (BCL-6) (Ye et al, 1993) and 19q13 (BCL-3) (McKeithan et al, 1987) were all noted in several cases. However, the 11q13 locus, the site of BCL-1, was involved in only one case; in spite of being reported at high frequencies in other series (Fifth International Workshop on Chromosomes in Leukaemia-Lymphoma, 1987; Offit et al, 1991a). Again, this may be an example of regional variation. No 10q24 (LYT-10) (Neri et al, 1991) breakpoints were observed but, given a series of this size, the absence of a relatively uncommon rearrangement was not surprising. However, two cases showed rearrangements involving 2p23 (ALK) (Morris et al, 1994), but without a 5q35 translocation partner. The t(2;5)(p23;q35) is found only in Ki-1 lymphoma and would thus be a highly unexpected finding, but the presence of this breakpoint is provocative.
6.2) MOLECULAR CYTOGENETICS

6.2.1) Numerical studies

6.2.1.1) Activation of chromosome X

This part of the study attempted to examine the activation status of X-chromosomes in NHL cells. With other cell types it should have been a straightforward matter to count Barr bodies, but in lymphoid cells these were very difficult to distinguish. The adopted strategy of combining FISH analysis of X-chromosome centromeres in interphase nuclei with Southern blot analysis of the methylation status of the DXS255 locus provided complementary evidence for the presence of additional active X-chromosomes, in spite of the limitations inherent in both these techniques.

A major advantage of the FISH approach was in allowing the NHL cells in the sample to be distinguished from normal cells, by virtue of abnormal numbers of signals. The main disadvantage was the inherent lack of a reliable method of quantification, due to the uncertain position in the cell cycle of individual nuclei and additionally to differences in signal size from cell to cell.

The methylation studies examined the DXS255 locus which, although not expressed, was differentially methylated on active or inactive X-chromosomes (Boyd and Fraser, 1990). The locus was usually informative because it contained a VNTR sequence that was highly polymorphic. In the NHL series, analysis of this locus indicated that in one of the cases there were additional X-chromosomes present in the active state. In other cases this was not so certain, and in fact L022 provided no evidence that this was the case even though the FISH analysis had indicated otherwise. A possible explanation for the contradiction between the two approaches applied to L022 might be due to differential hypermethylation of the Msp I / Hpa II sites in the DXS255 locus in this sample. Hypermethylation of the locus has been reported in a number of cell types (Gale et al, 1992; Hendriks et al,
1991). It has been assumed that there was no allele specific difference in the hypermethylation which might produce differences in the intensities of the hybridisation signals, but if this was not the case then it could account for the data obtained for L022, and also question the reliability of the data from samples L010B and L034, where the proportion of inactive X-chromosomes appeared to be far greater than that determined cytoogenetically. The presence of normal cells in the biopsy samples might also have complicated the situation, especially as the inactivation of the X-chromosome could be a random event in these cells.

Patient L109 was particularly interesting because the malignant cells contained one normal and one abnormal X-chromosome, and both of these chromosomes appeared to be present in the active state. It was also noted that there was clear evidence of LOH of the X-chromosome in this sample. It would appear therefore that the inactive X-chromosome had been lost from this clone, duplication of the active chromosome then occurred, and finally one of the activated chromosomes suffered deletion of part of its p and q arms. This is therefore an interesting example of how the probable evolution of part of the malignant karyotype can be inferred by a combination of FISH and Southern blot analysis.

Although there are a number of reservations concerning the approaches used here, they have provided evidence that additional X-chromosomes might exist in the active state in some NHL cases. Since, under normal circumstances, only single copies of most genes present on the X-chromosome are available for transcription, this raises the interesting possibility that a subversion of the gene dosage effect may contribute to neoplastic development. Although there are few studies of this phenomenon in the literature, additional active X-chromosomes have been reported in colorectal cancers (Muleris et al, 1990) and in two human mammary carcinoma cell lines (Wang et al, 1990). Furthermore, it has also been suggested that one or more oncogenes might be located on the X-chromosome. A recent study analysed structural abnormalities of the X-chromosome in 280 cases of NHL, and has identified Xp22 and Xq28 as likely regions for the location of
such a gene (Goyns et al, 1993). More specifically, a putative oncogene has recently been isolated from a T-cell leukaemia and mapped to Xq28 (Thick et al, 1992). Together these observations suggest a possible role for the X-chromosome in the evolution of some cases of NHL.

6.2.1.2) Interphase cytogenetics

**NHL**

It has often been difficult to obtain metaphase spreads from tumour cells of either sufficient quality or quantity for use in conventional cytogenetics. Alpha satellite probes have now been used successfully to enumerate chromosome copy number in a range of solid tumours (Devilee et al, 1988; Nederlof et al, 1989; Arnoldus et al, 1991) and haematological malignancies (Anastasi et al, 1992). It proved possible to clearly identify numerical chromosomal abnormalities in interphase NHL cells from the series, allowing both chromosome counting and the possibility of determining relative numbers of normal and abnormal cells in a particular lymphoma sample.

**Bone marrow smears**

DNA probes specific for the centromeric regions of a range of human chromosomes were hybridised to a series of archival bone marrow smears taken from children with ALL at diagnosis and in remission. Signals were observed in all cases examined, although a proportion of cells in most samples failed to exhibit detectable hybridisation.

In the remission series, the hybridisation efficiency observed and the accuracy of the technique in identifying normal male and female cells was related to the age of the material, with the oldest performing least well and the freshest the best. In previous work applying FISH to bone marrow smears, the age of the material has ranged from a few weeks (Anastasi et al, 1991) to several years (Lee W et al,
1993), the latter on unstained slides. Interestingly, those authors found no relationship between the storage time of the material and the extent to which hybridisation was successful. However, in the series of diagnostic smears, the unfixed frozen material gave far more consistent results when compared to those slides where coverslip removal and destaining had been required.

Numerical chromosomal abnormalities were detected in all of the ALL samples, with the proportion of cells displaying aneuploidy varying considerably from case to case. This reflected a mixed clonality within the samples, previously demonstrated by chromosome analysis, although there was not an exact correspondence between conventional and interphase cytogenetics. This discordance may be due in part to a low efficiency of hybridisation, particularly on the Romanowsky stained material, but it is also likely that the FISH technique, which is not limited to dividing cells, is reflecting the relative proportions of different clones more accurately, as other workers have found (Anastasi et al, 1992).

These findings indicate that it is possible to obtain accurate information on chromosome abnormalities from routine bone marrow smears after many years in storage, including stained and mounted slides from patients who were diagnosed before chromosome techniques were widely available (Seabright, 1971).

6.2.2) Structural studies

6.2.2.1) Abnormalities of chromosome 14

In this study, der(14) chromosomes were analysed from a series of NHL and demonstrated the efficacy of chromosome painting as a means of identifying the origins of abnormal chromosomes. Although this technique is very powerful it does, however, have its limitations. The hybridisation of chromosome paints involves the use of competitor DNA which has the effect of reducing the
efficiency of hybridisation of the paints compared to that of unique sequence probes. Furthermore
the chromosome paints may not be entirely representative of all regions of a chromosome and thus might produce variable signal intensities between different regions (Rosenberg et al, 1992). Factors such as these may therefore contribute to difficulties in visualising small regions that have been translocated. A good example of this concerns the small region of chromosome 14 (14q32-14qter) that is translocated to the 18q- derivative chromosome as a result of the t(14;18) reciprocal translocation which, as shown here and elsewhere (Bajalica et al, 1993), has consistently been difficult to visualise. Nevertheless chromosome painting has been used to determine the origins of derivative chromosomes observed in both malignant (Brothman and Patel, 1992; Smit et al, 1991; Xu and Wang, 1994) and non-malignant cells (Magnani et al, 1993; Ohta et al, 1993).

In the present study, chromosome painting has allowed us to identify material that had been translocated to form part of the der(14) chromosomes observed in several NHL. This not only allowed for the identification of regions that could not be characterised by Giemsa-banding, but interestingly also revealed abnormalities that had not been suspected. For example in L021, the der(14) was shown not to be due to a simple translocation but rather to be the result of insertion of chromosome 11 material into the q arm of chromosome 14. Furthermore, in L032 an abnormal chromosome that had been identified as a der(14) on the basis of its Giemsa-banding pattern was found not to be composed of chromosome 14 material. These observations are important for a number of reasons, not least of which is that they provide a more accurate picture of chromosome abnormalities present in malignant cells and hence may result in a reassessment of the accepted frequencies of particular chromosome abnormalities.

It is possible that the additional information obtained by the application of FISH techniques could also provide an insight into the evolution of malignant cell karyotypes. In L011 there were two normal chromosomes 3 and a der(14) which
was demonstrated to contain chromosome 3 material. These observations are consistent with a model in which a t(3;14)(q27;q32) translocation had occurred, followed by loss of the resulting 3q- chromosome from the cell and duplication of the remaining normal chromosome 3. The application of other chromosome paints to a range of NHL might therefore allow for common sets of events to be identified that are associated with the evolution and progression of NHL.

A more accurate analysis of the abnormal chromosomes provided by FISH techniques could also lead to the identification of reliable prognostic markers. For example, L011 and L006 both contained der(14) chromosomes typical of the t(3;14)(q27;q32) translocation but without the presence of the reciprocal der(3). Chromosome painting demonstrated, however, that the der(14) chromosomes was the result of the t(3;14) translocation. This translocation is associated with rearrangement of the BCL-6 gene and high grade centroblastic NHL (Ye et al, 1993) and it was therefore of interest that although L011 had been diagnosed with low grade disease, within a few months of biopsy, this had transformed to high grade centroblastic NHL.

The cloning of breakpoint regions involving the immunoglobulin heavy chain gene locus on 14q32 has been a remarkably successful approach for identifying novel genes that are associated with the evolution of NHL (Tsujimoto et al, 1984a, b; Motokura et al, 1991; Ohno et al, 1990; Ye et al, 1993; Neri et al, 1991). The identification of der(14;?)(q32;?) chromosomes that are different to those that have previously been used in these cloning experiments, should therefore allow for the identification of NHL cases which exhibit translocations involving the recombination of other novel oncogenes near to the immunoglobulin locus. The application of chromosome painting would therefore enable a more efficient search to be made for such oncogenes and in this series both L022 and L031 might be examples worth further investigation.
6.2.2.2) Abnormalities of chromosome 6

Aberrations involving the long arm of chromosome 6 are common in NHL (Schouten et al, 1990b) and have also been reported in a variety of other haematologic and solid malignancies (Hatashi et al, 1990; Millikin et al, 1991; Morita et al, 1991; Stenman et al, 1989; Gaidano et al, 1992). In NHL, these 6q abnormalities have been inconsistently correlated with both clinical features and prognosis (Cabanillas et al, 1988; Schouten et al, 1990b); therefore chromosome painting and single gene localisation by FISH were utilised in an attempt to investigate the 6q abnormalities found in this series.

Although painting with sequences specific for chromosome 6 failed to reveal any hidden interstitial rearrangements and only one example of translocated material, additional aberrations cannot be completely ruled out, since it is not always possible to detect small fragments of a chromosome by this method (Bajalica et al, 1993).

The *MYB* gene was mapped to 6q23, refining its previous location of 6q22-23 (Barletta et al, 1987), although it should be noted that other workers have since mapped *MYB* (also by FISH) to 6q24 (Gaidano et al, 1992). The same probe was then applied to NHL metaphases with abnormalities of 6q and in at least one of these cases, unsuspected rearrangements of chromosome 6 were identified in the NHL cells by the presence of aberrant *MYB* hybridisation signals. Recent work has identified various regions of common deletion on 6q in NHL (Offit et al, 1993; Menasce et al, 1994). The former paper attempts to define distinct clinicopathologic subsets based on these regions, as ascertained by conventional cytogenetics. The occurrence of occult rearrangements, such as those detected in the work presented in this thesis, may go some way to elucidating the difficulties of assigning 6q abnormalities to specific clinical or pathological groups.
6.2.2.3) Analysis of double minutes

C-banding of chromosomes is known to reveal heterochromatic regions found in all centromeres and in certain pericentromeric regions (Sumner, 1982). However, the centromere-associated DNA-containing dmin described here were negative for C-banding. This raised the possibility that the centromere-associated DNA in these dmin exists in an abnormal conformational state, or that it may have been altered in some other way.

The dmin are generally regarded as containing a number of amplicons or gene amplification units (Kaufman et al, 1979; Cowell, 1982), that may have arisen by a variety of mechanisms (Carroll et al., 1988; Wahl et al., 1989; Von Hoff et al., 1990; Roelofs et al., 1993). The dmin described here could only be demonstrated to contain centromere-associated DNA sequences because the intensity of the hybridisation signals, coupled with the small size of the dmin, prevented any non-centromeric regions from being observed. It was therefore unclear whether these dmin represented only amplicons of non-coding centromeric alphoid repeats or contained also other sequences. If the latter was the case, then it suggested that a mechanism could exist for the formation of dmin which allowed for the integration of disparate DNA sequences, as has been described in a gastric cancer cell line (Bar-Am et al., 1992).

It has been proposed that the presence of dmin in tumour cells, with their associated amplification of oncogenes (Brodeur et al., 1984; Dolf et al., 1991) or drug resistance genes (Hahn et al., 1992), could be an indicator of poor prognosis (Schwab and Amler, 1990; Nielson et al., 1993). However, if dmin are not always associated with gross gene amplification, then this might suggest that dmin should be reassessed as prognostic markers in malignant disease. A comprehensive study of the frequency of centromere-associated DNA positive dmin and their correlation with prognosis would be of value.
6.3) CONCLUSIONS

By comparison with other NHL series, this study of 40 specimens is small; in the largest series to date, Offit and colleagues reported on 434 specimens in 1991. However, with the notable exceptions of finding both a high incidence of additional copies of the X chromosome and a low incidence of 11q13 abnormalities, the results reported here are very much in agreement with those of Offit et al. This suggests that smaller studies may well be informative if the data is gathered in a consistent manner. Cytogenetics has to date proved a powerful tool for the analysis of genetic change in NHL. As the number of analysed cases in the literature increases, both the identification of new consistent breakpoints, which may be the site of potential oncogenes and the forging of firm associations between specific abnormalities, diagnosis and particularly prognosis become more likely. Probably the most effective approach in the search for chromosomal indicators of prognosis is the analysis of sequential samples from individual cases. At the time of writing, only three studies of this nature have been reported (Sanger et al, 1987; Levine et al, 1990; Whang-Peng et al, 1995). However, most recent series of NHL karyotypes contain examples of cases that have been sequentially sampled, including the data in this thesis and a logical next step would be to collate all the available information, in an attempt to identify those acquired abnormalities associated with transformation from low- to high-grade NHL.

The question of regional variation in the incidence of particular chromosome abnormalities (Johansson et al, 1991) will be less straightforward to resolve, especially since the overwhelming majority of cases are reported from the USA. Apart from adding another variable to the already complex task of deriving firm clinical/pathological associations, this geographic heterogeneity may be important in attempts to define the aetiological effects of different genetic or environmental factors on NHL.
The above questions can only be resolved satisfactorily if chromosome abnormalities can be tabulated correctly. The results of the work under discussion in this thesis make it clear that although conventional cytogenetics can be a powerful tool for the investigation of genetic change in NHL and other malignancies, several areas of potential inaccuracy exist. In the study of metaphase cells it is not always possible to identify every chromosome. Furthermore, unsuspected chromosomal rearrangements, which are not detectable by G-banding, may be present. An additional confounding factor may also arise from the exclusive study of dividing cells, since other clones may exist in the interphase population.

The application of FISH techniques has gone some way towards resolving the difficulty in obtaining an accurate picture of karyotypic change in cancer cells. Chromosome painting is now used almost routinely to elucidate complex or obscure cytogenetic abnormalities. Indeed, in order to incorporate the new information gained from painting into the standard nomenclature, a new system for scoring structural aberrations detected by chromosome painting has recently been proposed (Tucker et al, 1995). The use of interphase cytogenetics has revealed an enormous potential for detecting both numerical and structural chromosome abnormalities in tumour cells. Data concerning the frequency of polysomy 12 and monosomy 18 in NHL, obtained by the application of interphase cytogenetics, have recently been published (Younes et al, 1994a; 1994b) and the report of deletion of the RBL gene in interphase nuclei of chronic lymphocytic leukaemia cells (Stilgenbauer et al, 1993) serves as an example of the sensitivity of this approach in haematological malignancy.

There is considerable future potential for the application of a combined cytogenetic / FISH approach to the study of genetic alterations in NHL. It is envisaged that in addition to applying the techniques described above to a continuing series of NHL biopsies, the use of FISH technology will be further extended. Ratio painting (Dauwserse et al, 1992) will be applied to lymphoma
metaphases to enable a more complete characterisation to be carried out and NHL DNA will be used in CGH (Kallioniemi et al, 1992; du Manoir et al, 1993) experiments, in order to identify occult regions of amplification or deletion. The investigation of genetic change in malignancy at the chromosomal level has now been greatly enhanced and extended by the application of FISH techniques. The capability of examining the complete genome, albeit at low resolution, remains vital to an overall understanding of the genetics of cancer.
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