GENE MAPPING AND THE STUDY OF FRAGILE SITES IN HUMANS USING CHROMOSOMAL *IN SITU* HYBRIDIZATION

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ABSTRACT

In this project several cloned DNA sequences were localized to human chromosomes using the technique of *in situ* hybridization. Special emphasis was made on the relationship of some of these sequences to chromosomal fragile sites.

The first three chapters of this thesis present the historical background and practical considerations to the use of this technique.

Original localizations were made for two unique sequences. The tumour necrosis factor-α gene was localized to 6p12->p22. The gene for the granulocyte colony-stimulating factor (*G-CSF*) was localized to 17q11.2->q21. Further hybridization to chromosomes from patients with the 15;17 translocation specific to acute promyelocytic leukaemia indicated that the coding sequence of *G-CSF* is proximal to the 17q breakpoint, and is not split by the rearrangement.

The α-globin gene cluster was mapped distal to the rare fragile site, *FRA16A*, and to two constitutional chromosome rearrangements involving 16p. Further localization to the region 16p13.2->pter was used to support one of two conflicting published assignments of this gene cluster.

The metallothionein gene cluster (*MT*) was localized proximal to the rare and common fragile sites at the interface of bands 16q21 and 16q22

(*FRA16B* and *FRA16C* respectively). *MT* is therefore probably in band 16q21, not 16q22 as had been previously reported.

The haptoglobin locus was localized distal to *FRA16B*.

In situ hybridization showed that the adenine phosphoribosyltransferase locus is distal to *FRA16B*, and also to the common fragile site in band 16q23, *FRA16D*. This is more distal than the previously recognized shortest region of overlap for this locus.

Two anonymous DNA fragments, identifying the *D16S4* and *D16S5* loci, were mapped distal to *FRA16B*. *FRA16B* defines the proximal limit for the *D16S4* locus.

A probe to the c-*ets*-1 oncogene hybridized distal to the rare fragile site at band 11q23, mapping this fragile site away from the breakpoint of the t(11;22) specific to Ewing sarcoma and related malignancies. This result, together with the *MT* localization result, form the first molecular evidence against a proposed relationship between fragile sites and the specific chromosome rearrangements seen in cancer.

These studies have aided the development of a more detailed human gene map. They have also increased the available knowledge about fragile sites, and provided further means of investigating them.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. All of the work presented in this thesis was carried out by me or under my direct supervision, except where otherwise acknowledged.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Ruth Simmers

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PREFACE

The general methods and details of experiments are presented in the appendices, AI-AIV. Some data are presented in a summarized form in the text, but the bulk of raw data appear in appendix AIII. In most cases a summary of the methods and results is presented in the relevant chapter, or a paper referred to.

Much of the work presented here has been published, or accepted for publication. These papers are appended (Appendix AV). While the chapters presented in this thesis are largely self-contained, there is some reference to these papers, in the interest of avoiding repetition. It is suggested that the relevant papers, as outlined in Appendix AV, are read before each chapter.

CHAPTER 1

INTRODUCTION AND HISTORICAL BACKGROUND

1.1. INTRODUCTION

During the project described in this thesis a number of problems were approached using the method of *in situ* hybridization. The technique involves the hybridization to chromosomes of labelled probes specific to known DNA regions, and detection of the site(s) of hybridization. With this technique, a variety of questions are addressed, thereby increasing our knowledge of the human gene map, and aiding in our understanding of fragile sites.

Much of the work reported here involves the use of fragile sites on human chromosomes, which when used as a target for *in situ* hybridization result in greater resolution for chromosome mapping than that obtained with normal chromosomes, and provide useful information about the positions of certain loci with respect to fragile sites.

This chapter provides an introduction and historical background to the two recurring themes of this thesis: *in situ* hybridization and fragile sites on human chromosomes.

1.2.*IN SITU* HYBRIDIZATION

The first *in situ* hybridization experiments were described by John *et al.* (1969),Pardue, Gall (1970) and Gall and Pardue (1970) and Buongiorno-Nardelli and Amaldi (1970). Certain deoxyribonucleic acid (DNA) sequences were detected and localized in the nuclei of cytological preparations or tissue sections. The nuclei thus probed were at interphase (John *et al.*, 1969; Gall and Pardue, 1970; Buongiorno-Nardelli and Amaldi, 1970) or at metaphase (Pardue and Gall, 1970; Jones, 1970). [Later, the technique was also applied to the localization of cytoplasmic nucleic acids, e.g. Cox *et al.*, 1984.] This discussion will centre on the development of the technique, *in situ* hybridization, where metaphase chromosomes are used. It enables the localization of a DNA sequence of interest to a particular chromosome region.

This new technique relied on the formation of hybrids by the application of a radiolabelled, single-stranded nucleic acid "probe" in solution to the denatured DNA of intact preparations; the excess probe was removed, and the hybrid molecules were detected by autoradiography (e.g. Gall and Pardue, 1970; Pardue and Gall, 1970; Jones, 1970). Methods of obtaining labelled probe included extraction from cells provided with labelled precursors (Jones, 1970; Steffensen, 1977) and labelling nucleic acid templates *in vitro* (e.g. Jones and Robertson, 1970, Saunders *et al.*, 1972A and B). Other labelling methods have been suggested (Hirschhorn and Boyer, 1974). Although most of the early *in situ* hybridization experiments used ribonucleic acid (RNA) probes, DNA probes could also be

used successfully (Jones, 1970). The attainable specific activity of the DNA probes was at that time lower than could be achieved for RNA, so their detection was less efficient (Hsu, 1979).

Many repeated DNA sequences were localized on chromosomes in this way (e.g. Jones and Corneo, 1971; Jones *et al.*, 1973; Gosden *et al.*, 1975A; B). The reiterated nature of the cellular DNA or RNA enabled probes to be prepared by the isolation of sequences characterized by their reassociation constants and performance in density gradients (Jones, 1974; Macaya *et al.*, 1977). Repeated sequences could be successfully localized in this way because of the large amount of target DNA available (Wimber and Steffensen, 1973). [When mapping genes to polytene chromosomes, a low copy number gene is effectively present in many copies on the chromosome, allowing *in situ* hybridization with these relatively low specific activity probes (Wimber and Steffensen, 1973; Pardue and Gall, 1975; Steffensen, 1977).] As there is single stranded RNA in the cellular and mitotic preparations, which would also hybridize to the probe, this was removed beforehand, with ribonuclease (RNase) (Jones, 1974; Pardue and Gall, 1975).

There were limitations to the technique so that for about twelve years only repeated sequences could be reliably localized. The RNA probes, having been obtained by fractionation of the total cellular RNA or DNA population, were by no means pure, and therefore sequences of low copy number would not produce a signal great enough for detection above the level from non-specific sources (Henderson *et al.*, 1978; Buckle *et al.*, 1986; see below). In any case the labelling procedures did not result

in a specific activity great enough to give a signal detectable above the general background level ("noise" from non-specifically bound probe and silver grains exposed or developed by other means), had been used a low copy number sequence probe (Harper and Saunders, 1981).

One advantage of using RNA probes is the ability to remove the single-stranded, unbound probe enzymatically, with RNase, without degrading the RNA/DNA hybrids, preventing the unbound probe from causing background signal (Jones, 1974).

Although the localization of unique sequences by *in situ* hybridization was not yet feasible, it was considered within the bounds of possibility (Wimber and Steffensen, 1973; Steffensen, 1977).

The progress towards mapping sequences of low copy number by *in situ* hybridization consisted largely of attempts at mapping the globin loci.

In 1972, Price *et al.* (1972A) reported that they had mapped the globin genes by *in situ* hybridization. They had extracted ^{3}H-labelled mRNA from rabbit reticulocytes, and believed the globin mRNA to be sufficiently pure to give a result, even though contaminating mRNA was present. Upon statistical analysis of the grain levels over 18 metaphases, two significant regions were obtained, on chromosome 2 and on a B group chromosome (reasoned to be the sites of the α-, and β-, γ- and δ-globin genes respectively).

The presence of the two peaks were, they believed, (Price *et al.*, 1972B) good evidence that the signal from *in situ* hybridization had been great enough to detect above background. Bishop and Jones (1972) however noted that the amount of signal at these regions indicated that it was emitted by a different, repeated sequence, given the very low specific activity of the probe. Price *et al.* (1972A) had acknowledged that the specific activity they gave for the probe was low, but later stated that this figure may have been incorrect (Price *et al.*, 1972B). This low specific activity is the basis of further criticism directed at their localization (Bishop and Jones, 1972; Prensky and Holmquist, 1973; Steffensen, 1977). Bishop and Jones (1972) calculated that the amount of labelled probe with this specific activity, which would be required to produce the amount of signal detected at the two peaks, would be equivalent to 5% of the genome, and that the signal peaks may therefore have been a result of non-specific hybridization.

Price *et al.*'s (1972A) localization of the globin genes also received some support, either by evidence from other methods (Weitkamp *et al.*, 1972; Gandini *et al.*, 1977) or correspondence of observed and expected grain levels (based on hybridization efficiency) (Atwood *et al.*, 1976), or by repeats of *in situ* hybridization experiments, using a higher specific activity cDNA probe prepared from mRNA (Atwood *et al.*, 1975; Price and Hirschhorn, 1975) or mouse chromosomes, for which the location of the α-globin locus was already known (Atwood *et al.*, 1975). Cheung *et al.* (1976), using a non-radioactive detection method (with pure globin mRNA probes), found an excess of label over a B group chromosome. Although there was no obvious reason that Price *et al.*'s (1972A) *in situ*

hybridization should have worked, given the low specific activity of the probe used (Atwood *et al.*, 1975), there was considerable evidence in support of their results.

Deisseroth *et al.* (1977; 1978) used a new method, i.e. the detection of chromosomal DNA in somatic cell hybrids by Southern analysis, to map the α- and β-globin loci (termed *HBA* and *HBB* respectively). In these cases the globin cDNA, although again prepared from reticulocyte mRNA, was purer than that used in previous attempts at localization, and the results of DNA/cDNA hybridization were found to correspond to the presence or absence of chromosome 16 and 11 respectively. Deisseroth *et al.* (1977) summarized the previous reports of *HBA* localization inconsistent with theirs, and possible reasons for their inaccuracy.

The original objections to the localization of the globin genes by Price *et al.* (1972), based on low specific activity of the probe, appeared to have been substantiated by these new and more reliable results, and by many further studies (see later and chapter 5). Yet experiments had repeatedly pointed to the globin loci being on chromosome 2 and a B group chromosome. This might be explained by contaminating sequences present in the reticulocyte mRNA, probably of a repeated nature (Henderson *et al.*, 1978). Calculations of the expected signal level from specific activity and probe size were of some value in ruling out grain peaks at levels inappropriate to the sequence of interest, caused by contaminant labelled RNA. They could, however, not be used to reject a level of signal appropriate to that expected from the probe in

question, but caused by contaminants (Atwood *et al.*, 1976; Henderson *et al.*, 1978).

Tereba *et al.* (1979) were the first to report the successful localization of a unique sequence probe by chromosomal *in situ* hybridization. The probes were prepared from RNA of an endogenous retrovirus, so that the viral genome could be localized to a particular chromosome region. The authors developed a technique to label the relatively pure RNA to a high specific activity: the RNA provided the specificity, and was attached to sea urchin DNA which had been labelled with ^{125}I, via polynucleotide linkers. The sea urchin DNA was in the form of networks, which had been produced by the annealing of single stranded, labelled DNA, by virtue of its many reiterated sequences. Network formation of this DNA meant a large amount of radioactivity attached to the virus RNA, which therefore had a high specific activity. The general application of this technique was not feasible at the time however, as cellular mRNA could not be isolated to as high a purity as the viral RNA (Gerhard al., 1981), for the purpose of constructing a probe.

Among the improvements in molecular biological technique leading to successful low copy number *in situ* hybridization was the application of recombinant DNA technology. The use of labelled, cloned probes provided many advantages: the sequence was free of other contaminating sequences; and the labelled vector provided extra signal, so that efficiency could be greatly increased without increasing the level of background (Malcolm *et al.*, 1977). Reannealing of complementary DNA in probe solutions

after denaturation could readily be offset by the use of higher probe concentrations, as the clones were easy to prepare (Malcolm *et al.*, 1977). The use of cloned probes eventually allowed the ready chromosomal localization of many unique and low copy number sequences.

Papers reporting successful localization of low copy number native chromosomal sequences (c.f. the localization of the DNA of an endogenous retrovirus by Tereba *et al.*, 1979), by *in situ* hybridization using cloned probes, began appearing in 1981. Malcolm *et al.* (1981) reported successful mapping of the β-globin locus by *in situ* hybridization. These authors then reported mapping the α-globin locus using the same method (Barton *et al.*, 1982). In 1981, Gerhard *et al.* (1981) also mapped the α-globin locus, using *in situ* hybridization. Significant labelling was achieved by a method more similar to the now classic method of Harper and Saunders (1981).

Harper and Saunders (1981) employed a cloned probe. A large probe insert (14.9kb), and the presence of dextran sulphate in the hybridization solution, made feasible the detection of specific signal from the sequence, present in one or two copies in the haploid genome. The rate of DNA renaturation in solution can be accelerated by the presence of dextran sulphate, a viscous substance which excludes, and therefore concentrates, the DNA (Wetmur, 1975) This feature had been used to greatly enhance hybridization of DNA probes to DNA on filters (e.g. Southern blots, Southern, 1975) (Wahl *et al.*, 1979). As well as accelerating the rate of hybridization, dextran sulphate allows the complementary single stranded DNA sequences to reanneal in the form of

networks, when the fragments are partially complementary as in the case of nick translated DNA (Wahl *et al.*, 1979).

Nick translation is an *in vitro* reaction which can label DNA to a high specific activity (Rigby *et al.*, 1977). Nick translated probes were first used for *in situ* hybridization by Glover *et al.* (1975) and Macgregor and Mizuno (1976). By including dextran sulphate in the hybridization mixture, the hybridizing molecule becomes effectively larger and much more radioactive. It is this property which was used to advantage in the methods of Harper and Saunders (1981) and Gerhard *et al.* (1981).

The probes used by Malcolm *et al.* (1981) and Barton *et al.* (1982) were cRNA copies of clones which contained genomic DNA homologous to about 5kb of human DNA in the globin gene clusters. Mapping of the low copy number genes was possible because of the large regions covered by these probes, as well as their purity. The authors acknowledged that a combination of DNA probes and dextran sulphate might give better signal, but also ultimately more background, as unhybridized RNA probe could be removed with RNase. They also raised a number of other theoretical objections to the use of DNA probes. The papers of Harper and Saunders (1981) and Gerhard *et al.* (1981) did, however, report much greater specificity of signal, and since this time most reported *in situ* hybridization protocols have made use of DNA probes and dextran sulphate, largely based on Harper and Saunders' (1981) method (e.g. Zabel *et al.*, 1983; Law *et al.*, 1986; Sparkes *et al.*, 1987).

Gerhard *et al.*'s (1981) paper, although using a neater technique, producing less background, than that of Malcolm *et al.* (1981) and Barton *et al.* (1982), localized the α-globin gene cluster largely because it was a means of testing the method, as this locus was known to be on chromosome 16. Therefore the authors did not examine the regional localization on chromosome 16 (Gerhard *et al.*, 1981). Malcolm *et al.* (1981) and Barton *et al.* (1982) did regionally localize the β- and α-globin loci, by visually dividing the chromosomes into equal portions, and noting the distribution of silver grains. Their localization of the α-globin cluster to the distal half of 16p in this manner (Barton *et al.*, 1982) has since been repeatedly supported (see chapter 5). However, their localization of the β-globin cluster to the proximal quarter of 11p (Malcolm *et al.*, 1981) was in agreement with the previous report of Gusella *et al.* (1979), but has since been shown to be probably incorrect, the locus being more distal on 11p (e.g. de Martinville and Francke, 1983; Morton *et al.*, 1984; Zabel *et al.*, 1985; c.f. Chaganti *et al.*, 1985; Lin *et al.*, 1985).

The method used by Harper and Saunders (1981) also produced more informative results than others used at the same time. Cell synchronization, resulting in longer chromosomes, increased the mapping resolution. The use of techniques for banding the chromosomes after *in situ* hybridization subdivided the chromosomes into more identifiable regions.

1.3.RECENT ADVANCES IN *IN SITU* HYBRIDIZATION

Among the advances in *in situ* hybridization technique since this project commenced, is the use of alternative probe detection methods. The combination of nick translation and dextran sulphate produce a readily detected signal when radioactive precursors are used. Alternative methods of *in vitro* labelling of probes can result in probes of higher specific activity to those produced by nick translation. For example, synthesis of an RNA probe using a probe with an SP6 promoter (Melton *et al.*, 1984) can result in single-stranded RNA probes of high specific activity (Little, 1984). However, it must be remembered that, unlike probing DNA on filters, where single copy fragments can be concentrated on a filter, chromosomes contain only one piece of a single copy sequence on any chromatid, so any probe has to be detectable above background under these restrictions. The SP6 labelling system has not been applied to chromosomal *in situ* hybridization, although it would certainly be applicable to the localization of reiterated sequences by *in situ* hybridization (c.f. Cox *et al.*, 1984). On the other hand, Sparkes *et al* (1986) and Lin *et al.* (1985) have reported the use of probes labelled to high specific activities, by the random primer method and oligolabelling respectively, with which dextran sulphate and the principle of network formation were applicable to *in situ* hybridization. This report of Lin *et al.* (1985) shows extremely high specificity of labelling.

Non-radioactive methods of labelling and detecting DNA are desirable from a safety point of view, and the detection of hybridized probe can

be potentially quicker, as the slides need not be exposed for extended periods of time. The saving of time can be considerable in the case of low copy sequences, and Landegent *et al.* (1985) have described such a method. More recently, success with biotin labelled probes has been reported for low copy number sequences (McGee *et al.*, 1986; Nicholls *et al.*, 1987). Because there is scatter of ß-particles associated with tritium detection by autoradiography, these methods can also provide greater resolution in *in situ* hybridization. However, the procedures are at present troublesome and not very reliable (Hultén, 1987).

In situ hybridization has other applications apart from the mapping of loci on chromosomes. For example some probes have been applied to the chromosomes of patients, to assess the nature of chromosomal abnormalities: a probe can give an indication of the identity of otherwise ambiguous chromosome material (e.g. Mattei *et al.*, 1985A; Vorsanova *et al.*, 1986; Pellissier *et al.*, 1987). *In situ* hybridization techniques are at present too time consuming to be directly applied in routine clinical situations (Hultén, 1987).

1.4.*IN SITU* HYBRIDIZATION AS A TOOL FOR GENE MAPPING

Although *in situ* hybridization is extremely useful for gene mapping, especially in the absence of appropriate somatic cell hybrids, there has been some criticism of the validity of *in situ* hybridization results.

There is some evidence that chromosomal DNA sequences are not all equally accessible. A "position effect" has been described with

translocations: sequences located near the site of chromosome translocations have produced different levels of signal on the normal and translocated chromosomes, a difference attributed to differences in accessibility as a result of the three-dimensional chromosome structure (Emanuel *et al.*, 1985; Davis *et al.*, 1986). Such differences in hybridization efficiency appear to have resulted in Harper and Saunders (1981) making an incorrect localization. Goode *et al.* (1986) and Carritt *et al.* (1986), using Southern analysis, found that the locus, *D1S1*, detected by Harper and Saunders (1981) with the λCh4A-H3 probe, is probably a sequence, in two or four copies, related to that from which the probe derives on chromosome 3. The probe clearly hybridizes *in situ* to the chromosome 1 locus. No excess of silver grains on chromosome 3 was reported by Harper and Saunders (1981), and only a minor (but significant) peak on chromosome 3 was reported by Donlon and Magenis (1984), after *in situ* hybridization. The level of silver grain signal can not necessarily be equated with the length and degree of homology of hybridizing sequences. Goode *et al.* (1986) suggested that this is a result of DNA sequence and chromosome structure affecting *in situ* hybridization efficiency to varying degrees.

Another problem with using *in situ* hybridization alone is that sequences with incomplete homology to the probe might exist within the genome, and either (a) result in a secondary (or major) peak, which can only be explained using other methods, such as Southern analysis or use of somatic cell hybrids, or (b) not present any significant labelling, leaving their presence unhinted at. Ideally other methods should be used besides *in situ* hybridization, to confirm that a significant site

of labelling indicates the actual locus (Goode *et al.*, 1986; Devilee *et al.*, 1986).

Some inconsistencies and errors exist in the literature, apart from that mentioned above, as a result of *in situ* hybridization data. Conflicting localizations have resulted in some cases, when different clones have been used as probes for the same locus (e.g. Caccia *et al.*, 1984, c.f. Le Beau *et al.*, 1985A; Le Beau *et al.*, 1985B, c.f. Isobe *et al.*, 1986). In the case of the p53 gene, when a probe from the murine gene was used, a major grain peak occurred over 17q, and in some cases a minor peak was observed over 17p (Le Beau *et al.*, 1985B). This site of minor hybridization was found to be the actual site of the locus when a human p53 gene probe was used, both by *in situ* hybridization (Isobe *et al.*, 1986; McBride *et al.*, 1986) and Southern analysis (Benchimol *et al.*, 1985; McBride *et al.*, 1986).

In some cases, localizations made using *in situ* hybridization are in conflict with other reports, by only a few bands. For example, there are conflicting localizations for the β-globin locus (see section 1.2). Zabel *et al.* (1985) suggested that complex chromosomal rearrangements, which remain undetected by cytogenetic analysis, can give misleading results, and noted that the use of chromosomes from only one individual can give a regional localization slightly different to that given by chromosomes from other individuals. Münke *et al.* (1984) have suggested that protrusion of loops of DNA from the chromosome may give a grain peak away from the actual chromosomal site of a locus. They further suggested that the relative lengths of the bands vary with the state of

compaction of the chromosomes, so that if banding is poor or not used, a misleading impression of the location of a silver grain may be given. In relation to both of these points, the longer the chromosomes, the better the resolution obtained, a principle which applies even without such inherent imprecision.

The use of chromosome markers which increase resolution (chapters 5 and 6) helps overcome the possible lack of correspondence of grain peaks with the chromosomal locus. In my opinion, such lack of correspondence can be due to the spread of grains, and to imprecise localization of silver grains due to observer bias because of expected results or the surrounding chromosomal landmarks (see chapter 5). Imprecise breakpoint localization of translocations used for *in situ* hybridization or any other mapping technique may also lead to incorrect mapping results. In chapter 4, two original localizations are described. Prometaphase chromosomes were used to optimise resolution, and other data available indicate that the grain peaks obtained with the probes are from hybridization to the genes of interest, rather than to related sequences.

In conclusion, *in situ* hybridization can be a powerful technique, but the results may be misleading. It cannot be used to determine copy number, but may hint at it (e.g. Board and Webb, 1987). Ideally separate evidence, available either before or after *in situ* hybridization is carried out, will support (or cast doubt on) the assumption that a specific signal indicates the actual site of the gene.

1.5.FRAGILE SITES

A "fragile site" on a chromosome (a term coined by Magenis *et al.*, 1970) is a non-staining gap of variable width, at a precise point, usually involving both chromatids (Sutherland, 1979A), which is inherited in a Mendelian co-dominant fashion (Sutherland, 1983). Although every cell of a fragile site carrier contains the DNA message for the fragile site, it is only seen in a percentage of cells (Giraud *et al.*, 1976), usually fewer than 50% (Sutherland, 1983). Classification of a marker as a fragile site also depends upon the observation of acentric fragments, deleted chromosomes, triradial figures and similar structures demonstrating fragility at mitosis, under appropriate culture conditions (Sutherland, 1979A).

Scanning electron microscopy has shown there to be a definite chromatin fibre across the fragile X (Harrison *et al.*, 1983). From this and other evidence, it appears that fragile site expression is an anomaly of packaging (Harrison *et al.*, 1983; Nussbaum and Ledbetter, 1986).

The fragile site nomenclature originally proposed (Sutherland, 1979A) uses the triplet "fra" before the location of the fragile site as described by ISCN rules (ISCN, 1981; 1985), e.g. fra(16)(p12) on chromosome 16 at band 12 on the short arm. This system was supplemented for the designation of gene symbols for fragile sites (Berger *et al.*, 1985). Because of the ambiguities caused by the existence of some bands with more than one different fragile site, the new nomenclature uses the triplet *FRA*, followed by the chromosome and a letter assigned to the

particular fragile site. For example, fra(16)(p12) is now termed *FRA16A* (Berger *et al.*, 1985).

Dekaban (1965) provided the first report of a fragile site in the literature. This was observed as a gap (a "weak region")in one or both chromatids on the long arm of a C group chromosome, probably a chromosome 9, in 22 to 27 percent of cells. It was considered to be an acquired abnormality, which had been induced by clinical irradiation in the precursor of the clone of blood cells in which it was seen. It was not detected in fibroblast cells cultured from the same patient. The possibility that it was inherited was considered, but could not be tested.

Further isolated cases of fragile sites were reported (e.g. Day *et al.*, 1967; Lejeune *et al.*, 1968). That of Lejeune *et al.* (1968) included a clear photograph and description of a triradial figure of the fragile site observed near the centromere on the long arm of chromosome 2 (2q): the material distal to the fragile site was present in two copies. The authors concluded that it was a result of selective endoreduplication of this segment. The morphology is however a result of breakage at the fragile site followed by non-disjunction (Ferguson-Smith, 1973; 1977; Noël *et al.*, 1977). Lejeune *et al.* (1968) also demonstrated that this fragile site was heritable.

Lubs (1969) reported a family with mental retardation in the male members segregating with a heritable fragile site near the terminal end of the long arm of the X chromosome, which they termed the "marker X".

This was not the first time the presence of a fragile site had been associated with mental retardation (e.g. Lejeune *et al.*, 1968), and it was not until 1976 that association between the fragile X and mental retardation was confirmed (Giraud *et al.*, 1976; Harvey *et al.*, 1977). The fragile X has been precisely localized to band Xq27.3 (Brookwell and Turner, 1983; Krawczun *et al.*, 1985), and is termed fra(X)(q27) (Sutherland, 1983) or *FRAXA* (Berger *et al.*, 1985).

Sutherland (1983, 1985A) attributed the lag from the original association between the fragile X and mental retardation, and confirmation, to a change in common laboratory procedure at around this time: more refined tissue culture media were adopted. Until the report of Sutherland (1977) showing that the newer media suppressed the expression of certain fragile sites, there was no clue as to what caused them to be expressed; and many fragile site carriers will have remained undetected by cytogenetic analysis at this time. Sutherland (1979A) found a number of agents to inhibit fragile site expression, including folic acid and thymidine.

There is now a variety of tissue culture conditions which can be utilized to detect these fragile sites, in particular the fragile X, in various tissue types (Sutherland, 1979A; Glover *et al.*, 1981; Tommerup *et al.*, 1981; Jacobs *et al.*, 1982; Lejeune *et al.*, 1982; Jacky and Sutherland, 1983; Sutherland *et al.*, 1985A; Sutherland and Baker, 1986). The fragile sites thus affected are now known as the folate sensitive fragile sites (Sutherland, 1983). These belong to the category of

fragile sites known as rare (or "heritable") fragile sites. (Sutherland and Hecht, 1985), which are present in only some individuals.

The conditions affecting expression of the fragile sites differ, such conditions forming the basis of classification. A common mechanism of expression is implied for fragile sites induced under the same conditions (Jacky and Sutherland, 1983). Other classes of rare fragile sites include the distamycin A inducible (three known, including one newly described at 8q24.1) and the bromodeoxyuridine requiring (one known) fragile sites (Sutherland *et al.*, 1984; Sutherland and Hecht, 1985; Takahashi *et al.*, 1987.).

Phenotypically normal homozygotes have been observed for some of the rare fragile sites (Schmid *et al.*, 1980; Sutherland, 1981; Izakovič, 1984), but as yet no homozygote for an autosomal folate sensitive fragile site has been found, so it is not known whether such homozygosity would be deleterious (Sutherland, 1979B; Sutherland and Hecht, 1985).

The other branch of the major dichotomy is the set of common (or "constitutive") fragile sites (Glover *et al.*, 1984), which probably occur in the homozygous state in all individuals (Sutherland and Hecht, 1985). Again there are different groups of these fragile sites, induced by different agents (Glover *et al.*, 1984; Yunis and Soreng, 1984; Sutherland *et al.*, 1985B; Pelliccia and Rocchi, 1986). Glover *et al.* (1984) recognized these non-random "hot spots" (Sutherland, 1983) as true fragile sites. Some have been mistakenly treated as rare fragile

sites in the literature (e.g. Wegner, 1983; Rudduck and Franzén, 1983; Shabtai *et al.*, 1983).

It is only relatively recently that a high prevalence of fragile (X)-linked mental retardation (one of the terms for this syndrome, coined by Kaiser-McCaw *et al.*, 1980) was recognized (Turner *et al.*, 1980; Gerald, 1980), and today it is recognized as the commonest inherited form of mental retardation (Sutherland, 1985A). Synonyms for this disorder include the Martin-Bell syndrome, marker (X)-linked mental retardation (Sutherland and Hecht, 1985) and the fragile X syndrome (Fryns, 1984; Turner *et al.*, 1986). Thus, fragile sites, previously only a chromosomal curiosity, became important to medical science. Although the fragile X is the only one of these clearly associated with a clinical problem, there is much that can be learnt about the fragile X from the study of the other fragile sites, especially those falling into the same category, namely the folate sensitive fragile sites (Sutherland, 1985A).

There have also been suggestions that carriers of the autosomal fragile sites are predisposed to malignant diseases, (see chapter 6), another reason for closer investigation of the nature of fragile sites.

1.6.*IN SITU* HYBRIDIZATION IN GENE MAPPING AND FRAGILE SITE RESEARCH

Based on X-linked inheritance, the X chromosome was for a long time (1911-1968) the only chromosome to which genes were assigned (Wilson, 1911; Renwick,1971). The use of hypotonic treatment for chromosome

harvest to produce good quality metaphase spreads (Hsu, 1952), and the subsequent discovery of the correct chromosome number, 46, in man (Tjio and Levan, 1956), paved the way for systematic cytogenetic classification and study (Denver Conference, 1960). The first human autosomal gene assignment was then made in 1968 (Donahue *et al.*, 1968; Ying and Ives, 1968).

The advent of chromosome banding (Caspersson *et al.*, 1970), enabling the identification of individual human chromosomes, was a further step which has been important to the history of human gene mapping, a process which may be of immense practical and intellectual value (Bodmer, 1986). Techniques which have been important in the development of the human gene map (see HGM8, 1985) include somatic cell hybridization and Southern analysis (Deisseroth *et al.*, 1977; 1978; Anderson *et al.*, 1976), linkage analysis (Renwick, 1971; White *et al.*, 1985), recombinant DNA technology (Southern, 1982), the use of chromosome aberrations (Ferguson-Smith and Aitken, 1982), and *in situ* hybridization (see section 1.2). In addition, recombinant DNA technology has led to a rapid accumulation of DNA probes, enabling many new known and anonymous DNA sequences to be mapped (Bodmer, 1986).

In situ hybridization compares favourably with other established methods of making original gene localizations, in both speed and accuracy. There is a waiting period before slides hybridized with radioactive probes can be developed, yet once established, the procedure is straightforward, and does not require, for example, the establishment of somatic cell hybrids, large family studies, or polymorphic markers. In

chapter 4 two original localizations of genes encoding immunologically important proteins will be described. In both cases, possible functional implications for the genes are revealed by the chromosomal regions to which they map. Knowledge of the chromosomal location of a gene is a relevant aspect of its molecular biology, and may reveal or suggest important and previously unknown information about the gene and its neighbours. Linkage to uncloned genes of interest might also be indicated, leading to improved carrier detection and prenatal diagnosis, or an approach to cloning the gene.

When used with particular chromosome markers which physically separate the DNA on either side of a specific point, the resolution of *in situ* hybridization can be increased, and the technique becomes a more powerful tool.

In combination with a constitutional or malignant chromosome translocation, the regional location of a DNA sequence can be more narrowly defined: usually to either the region proximal (on the side closest to the centromere) (e.g. Dayton *et al.*, 1984) or distal (on the side furthest from the centromere) (e.g. Nicholls *et al.*, 1987) to the translocation breakpoint. Such rearranged chromosomes can also be used for these purposes by methods other than *in situ* hybridization (Ferguson-Smith and Aitken, 1982). *In situ* hybridization might alternatively reveal the fact that the chromosomal translocation used occurs *within* ("splits") the region homologous to the probe (e.g. Diaz *et al.*, 1986). *In situ* hybridization to chromosome translocations will be described in chapters 4 and 5, where the these rearrangements are

used to both enable narrower regional localization and find out whether the breakpoint occurs within the region homologous to the probe.

The precision of any mapping technique which uses chromosome translocations depends on the accuracy in defining the chromosome breakpoints. When fragile sites are used for *in situ* hybridization, this is less of a problem, as their location is usually clear.

Fragile sites have been used in linkage analysis, to make chromosomal assignments and exclusions, and to regionally map loci by virtue of the fragile site being a chromosome marker at a known location (Magenis *et al.*, 1970; Mulley *et al.*, 1983A and B; Mulley, 1985). Such linkage studies rely on the availability of polymorphic markers and suitable families, and the assumption that the gene for the fragile site occurs at the fragile site, and does not interfere with recombination frequencies (Mulley, 1985). The fragile site DNA does appear to occur at the site of cytogenetic expression (Sutherland, 1983; Sutherland and Mulley, 1984; Nussbaum and Ledbetter, 1986; Oberlé *et al.*, 1986); this is particularly evidenced by the agreement between mapping locations obtained using cytogenetic expression and those using inheritance (Mulley, 1985). Evidence suggesting interference with recombination frequencies is controversial (Mulley, 1985; Simmers *et al.*, 1987C; Davies, 1986; Brown *et al.*, 1987), but appears to be due to occasional incorrect classification of fragile site status (G.R. Sutherland, J.C. Mulley, personal communication). Linkage studies may be used to infer the position of DNA sequences with respect to fragile sites (Mulley, 1985), but *in situ* hybridization to chromosomes expressing fragile sites

is the more efficient and more reliable method, requiring only a suitable gene probe and chromosomes expressing the fragile site of interest. It is therefore an invaluable technique in fragile site research.

It is of intrinsic interest to define narrower regional gene localizations, and the use of fragile sites, as described above, can be of great value in achieving this aim. Of greater practical interest is the resulting ability to identify those markers which flank a fragile site. These are potentially very useful in genotyping individuals for fragile sites, which are not always penetrant cytogenetically (Mulley, 1985). This is particularly important in the case of the fragile X, and since this project commenced, many localizations have been made with respect to fra(X)(q27) using *in situ* hybridization (see chapter 5).

The fragile site falls somewhere between any pair of flanking markers, by definition, narrowing down the chromosomal region of interest and giving direction to a systematic search for the DNA at this fragile site. It is this application which prompted the development of the *in situ* hybridization technique reported here, and experiments of this nature will be described in chapter 5.

In certain cases, a fragile site might be thought to occur within a defined gene or DNA sequence. This would be the case for a putative clone of the fragile site, or a sequence corresponding to a known site of chromosome rearrangement which is thought to coincide with a fragile site. *In situ* hybridization experiments can help determine whether the

sequence is in fact at the fragile site, or maps away from it. Chapter 6 will describe such experiments testing for correspondence between the locations of certain breakpoints in cancer chromosome rearrangements and fragile sites in the same chromosome band.

1.7.SUMMARY AND CONCLUSIONS

In this chapter the development of *in situ* hybridization has been followed, and its use as a tool in gene mapping examined. *In situ* hybridization is a powerful technique, and it has only recently become possible to use it to localize sequences of low copy number. Errors in localization have occasionally been made using it, but with other information the validity of a result may be supported.

Fragile sites were also introduced. They can increase the resolution of *in situ* hybridization, aiding in more narrow regional gene localizations. They are also genes in their own right, and may be readily ordered with respect to other loci along a chromosome using *in situ* hybridization.

CHAPTER 2

DATA COLLECTION AND ANALYSIS

2.1. INTRODUCTION

The first chromosomal *in situ* hybridization experiments localizing reiterated sequences, present in many copies in the genome, resulted in obvious clusters of silver grains over the sites of specifically bound probe. Statistical analysis was not necessary to show where the probe had hybridized. When *in situ* hybridization is carried out with a probe hybridizing to only one or a few sequences, however, the signal is not evident when viewing one or a small number of cells. In any one cell, there may be more signal from background sources or nonspecifically bound probe than from the probe hybridized to the sequence of interest, and there will not necessarily be signal over every copy of the sequence in every cell. It is therefore necessary to examine many cells in detail and record the locations of all silver grains. The location of specific signal is then determined by examining these data for sites of statistically significant accumulation of signal above background. This approach has been taken since the first attempt at low copy number chromosomal localization by *in situ* hybridization of Price *et al.* (1972).

Before describing the development and assessment of the *in situ* hybridization technique and its application in the following chapters, I shall describe the methods used in collecting and assessing the data.

2.2.DATA COLLECTION OVER NORMAL CHROMOSOMES

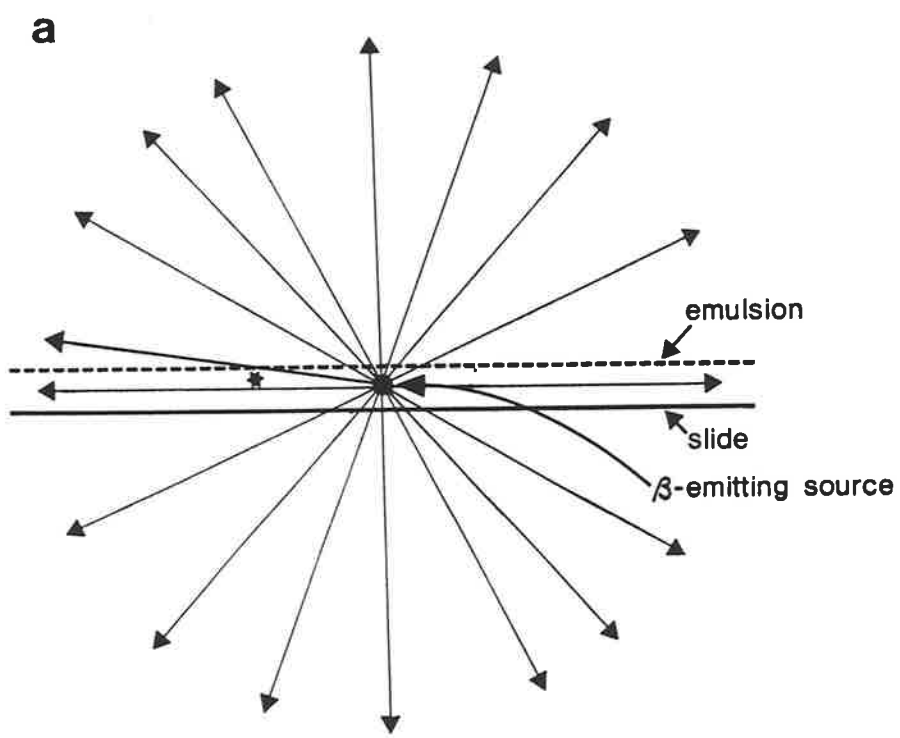
After *in situ* hybridization, the developed and stained slide has a number of black silver grains in a focal plane above the level of the chromosomes. The first decision to be made was the criteria under which silver grains were to be scored.

Ideally the DNA probe will have bound to its homologous chromosomal sequence and produced specific silver grains, but there will be "background" silver grains present (as noted above). Those emitted from the site of hybridization will mostly be present over the chromatin around this sequence. There may also be some, resulting from β-particle emission at an angle close to the slide (see figure 2.1), which do not overlap with, or "touch" the chromosome. Scoring grains that do not touch the chromosome but might be the result of signal emitted from it can be subject to bias or variations in judgement. Therefore in all experiments only those silver grains judged to "touch " (i.e. to overlap at some point with) the stained chromatin were scored.

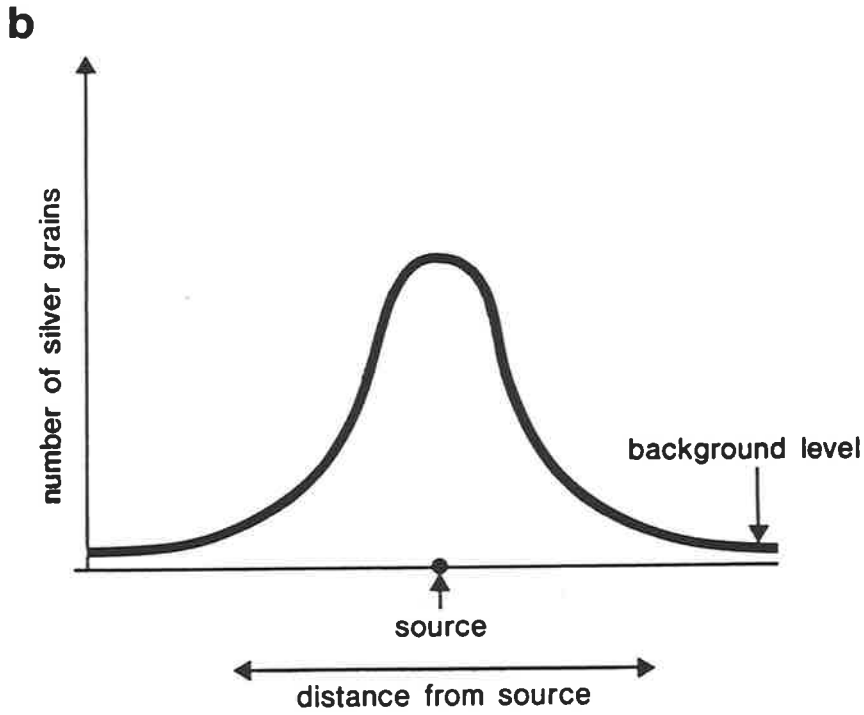
Another approach to this problem would be to score those silver grains at a measured distance from the chromatid, calculated from the path length of the emitted radioactive particles to include all possible grains from bound probe (Eccles *et al.*, 1984), but this procedure would be cumbersome.

Figure 2.1.

(a) Diagram of a side view of a microscope slide covered with emulsion showing the directions of emission, in two dimensions, of β-particles from ^{3}H. The direction of emission will in a small proportion of cases be at an angle close to the slide, which may cause the resulting silver grain to be at a greater than normal distance from the source (*). (b) A graph showing the resulting relative densities of silver grains in the emulsion at increasing distances from the source in a straight line. A constant background level of silver grains is included. (Modified from Rogers, 1973.)



NOT DRAWN TO SCALE



Silver grains are not seen in the same focal plane as the chromosomes, and in some cases the decision of whether the silver grain touched the chromosome could only be made by focussing between the two objects.

In the initial *in situ* hybridization experiments to be described, and those utilizing fragile sites to increase resolution, chromosome banding techniques were not used. Chromosome groups were recognizable on the basis of morphology. The chromosomes of interest (Y and 16) each had unique morphology and could be recognized without banding. In later experiments banded chromosomes were analysed. In both cases the resulting data consisted of the number of silver grains scored over each of a set of defined chromosome regions. Such a region will be termed a "grain scoring region" (GSR), and may consist of a group of chromosome arms, for example, all short arms of C group chromosomes, which are not readily distinguishable. This concept is distinct from that of individual pairs of chromosomes or chromosome arms, which might or might not constitute a GSR. A GSR might consist of many relatively short arms (e.g. all Cp arms), so its total length is greater than a GSR consisting of a single, much longer chromosome (e.g. chromosome 1) (see table 2.1)

The total length of each region differs, and the resulting difference in the expected numbers of silver grains over each region must be taken into account. The data may be presented as a histogram. For unbanded chromosomes, where the GSRs are large and disproportionate, the number of grains scored over each GSR can be divided by its total length, so that any large excess above the expected grain level per unit length will be obvious (e.g. fig. 1 in Simmers *et al.*, 1986A).

Table 2.1.

Relative lengths of GSRs (grain scoring regions) on unbanded chromosomes, calculated from ISCN, 1981.

GSR	relative length
1	9.11
2p	3.35
2q	5.26
3	6.97
Bp	3.46
Bq	9.24
Cp (female)	14.14
(male)	13.18
Cq (female)	26.36
(male)	24.74
Dp	1.91
Dq	9.00
16p	1.33
16q	1.81
17/18p	1.69
17/18q	4.06
F	4.71
Gp	1.00
Gq	2.50
Yp	0.24
Yq	0.85
Total (female)	105.90
(male)	104.41

Table 2.1 shows the groups of chromosomes and chromosome arms that are morphologically distinguishable in unbanded metaphases and define the GSRs used in this project on unbanded chromosomes. The relative lengths have been calculated from the relative lengths and centromere indices provided in ISCN (1981).

When grains are scored according to band, the GSRs are again unequal, but there are hundreds of these and the data are clearest if represented against idiograms of chromosomes showing actual relative band size (e.g. fig. 4.1 and fig. 1 in Simmers et al., 1987D). The differences in band size will not matter for the purpose of statistical analysis, and the histogram mainly serves to clearly indicate possible regions of significance.

A silver grain may overlap two GSRs. The approach was taken in such a case that the grain was equally likely to be a result of signal from either GSR, and scored as a half grain on each. The exception to this rule occurred when banded chromosomes were used for chromosome identification. When a grain covered adjacent bands unevenly, it was assigned to that which was covered the most by the silver grain. The scoring of half grains is a compromise when using the discrete Poisson distribution (see below), but still serves the desired purpose.

Slides of metaphases with fragile sites were sometimes scored to assess the level of specific labelling on the chromosome carrying the fragile site. In such cases metaphases for scoring were chosen in which this

fragile site was not expressed, as expression would increase the distance from the centromere to the telomere, thereby increasing the effective length of the scoring region. This necessitated ensuring that the number of metaphases with unexpressed fragile sites on the slide was great enough, which was particularly important if the frequency of expression was high.

When multiple grains occurred at the same location, the actual number of grains was scored or estimated. Some authors have scored "hybridization events" (Jhanwar *et al.*, 1983; Yang-Feng *et al.*, 1986), i.e. given a cluster of silver grains the same value as a single grain. By scoring the number of grains in a cluster, however, the figure obtained for each site is more representative of the amount of probe hybridized there. Such a relationship would cease once the emulsion is close to saturation with silver grains over the site.

2.3.THE EFFECT OF CHROMOSOME LENGTH ON GRAIN NUMBER

When scoring silver grains over chromosomes, the sampling areas in each metaphase are largely determined by the chromosomal morphology. The recognizable regions are of different size within a cell, and the same chromosome varies in size and shape between cells. I will examine here some resulting peculiarities in the data.

Any GSR which includes a telomere presents an unusual sampling area, illustrated in figure 2.2. A silver grain occurring to the proximal side of any interstitial point

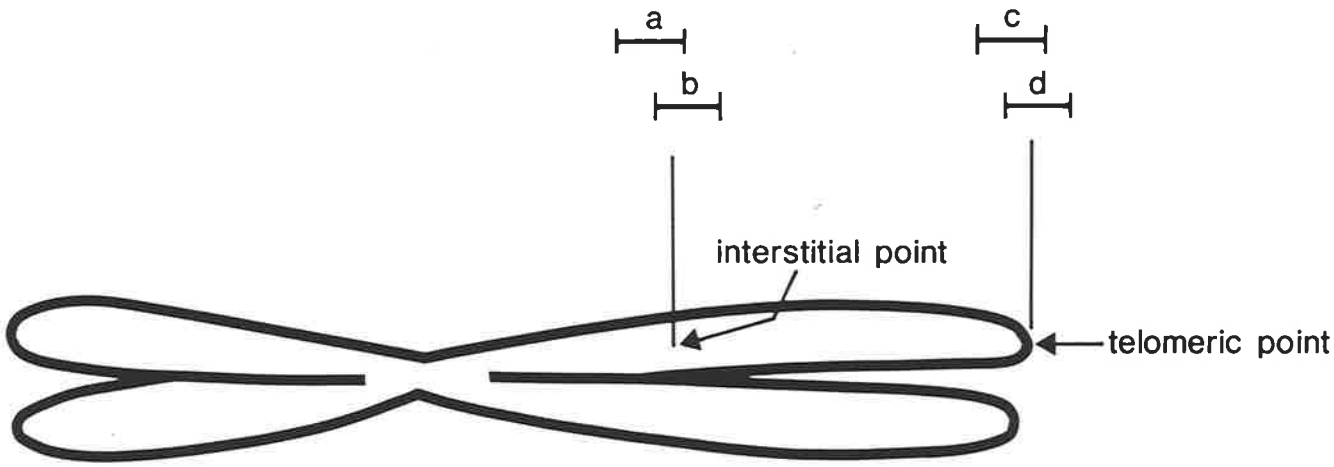


Figure 2.2.

Diagram showing theoretical interstitial and telomeric points. See text for discussion.

would be classified to that side, and a grain on the distal side would correspondingly be classified to the region distal to the point. A silver grain which covers this interstitial point (fig. 2.2(a) and (b)) would be assigned to the side over which it covers the largest area (proximal and distal to the point respectively, in this example). A silver grain which "touches" a telomeric point (fig. 2.2(c) and (d)) would always be assigned to the proximal side of this point. This means that the effective sampling area of each chromosome arm is related to its relative length plus a constant which depends on grain size. This constant is a greater proportion of the effective sampling area the smaller a chromosome arm is. Consequently, in a representation of grains according to chromosome or chromosome arm length (for example, as detailed in table 2.1), the smaller chromosomes and arms will tend to show grain levels above the mean, and larger chromosomes and arms levels below the mean. This general trend can be seen in papers showing this type of histogram (e.g. Malcolm *et al.*, 1981; Barton *et al.*, 1982; de la Chapelle *et al.*, 1983; fig. 2 in Simmers *et al.*, 1986A; figure 2.3). Silver grains scored against banded chromosomes would also show this trend at the telomeric bands, unless the scoring method corrected for it.

In histograms showing data for the gross chromosomal regions there is also a trend for the magnitude of difference between the grains per relative length and the mean to be greater for the smaller GSRs. This is because the value of a single grain on the ordinate (grains/relative length) is inversely proportional to the relative length of the GSR, and smaller sampling areas can be expected to give greater proportional

deviations from the mean by chance. This has important implications for some statistical approaches, and will be discussed later.

It was initially thought desirable to correct for the effect of scoring silver grains at telomeres. This can be done in at least three ways. One approach is to derive the effective constant to add to the relative length by scoring silver grains over a large number of metaphases. This would be time consuming in the first instance, and the true value of the constant would vary both within and between slides because of (a) the variation in chromosome lengths between metaphases and in the resulting magnitude of the constant expressed in terms of relative length, and (b) differences in silver grain sizes due to developing conditions. This is therefore not a suitable method. Alternatively the telomere could be made to have the same value as an interstitial region of the chromosome. This could be done in one of two ways: (i) by not scoring those silver grains over the telomere that have less than half their area covering part of the chromosome, or (ii) by giving all the silver grains occurring over the telomere a half grain value. The expected number of silver grains from method (ii) would equal that expected from method (i), given a random grain distribution. However, as more background would be expected over the chromosomes than off them, due to non-specific DNA-DNA binding, method (ii) would probably be less accurate.

In practice, the signal from a probe bound to a chromosome near the telomere would be downplayed if any of these methods were applied. Because of this, and the complications mentioned above, it was decided to give all silver grains occurring over chromosomes a unit value. This

is also consistent with most other reported methods of scoring. The phenomenon should be noted however, especially when dividing chromosomes into smaller regions of equal length, (e.g. Yokoi *et al*, 1986). They divided the long arm of chromosome 21 into three equal regions, and localized the gene in question to the telomeric third. If the coding DNA in such a situation is actually at the interface of a telomeric region and the middle region, and the regions are short enough for some silver grains from the specifically bound probe to occur over the telomere, there would be a misleading excess of grains over the telomeric third. When scoring grains over two halves of a defined region however (e.g. see chapters 5 and 6), each half has the equivalent of a telomere at one end (i.e. a fragile site or a telomere, in the examples in chapters 5 and 6), and therefore the two halves are effectively the same length.

2.4.STATISTICAL ANALYSIS OF DATA FROM NORMAL CHROMOSOMES

Few papers reporting *in situ* hybridization have clearly described a statistical method of determining significance of a grain peak. Although a large excess of silver grains might be evident over a region after scoring a number of cells, this will not always be the case, and minor peaks need to be discounted or otherwise explained. For meaningful analysis of *in situ* hybridization data, an appropriate statistical approach was considered necessary, especially in the initial stages of developing the technique.

The "background" for *in situ* hybridization consists of silver grains, produced by non-specifically bound probe, and other factors (e.g. background irradiation, pressure and overdevelopment), resulting in a random distribution of silver grains over the chromosomes. The aim of the statistical analysis was to determine whether a peak of silver grains over a GSR was significantly greater than the background level.

A silver grain is produced by the photographic development of a "latent image". The photographic emulsion applied over the slide consists of a suspension of silver bromide crystals in gelatin (Rogers, 1973). The transfer of energy to a crystal will cause some of its halide ions to be converted into silver atoms, constituting the latent image; and during development of the emulsion this latent image catalyses the conversion of the silver halide crystal into metallic silver (Rogers 1973). Assuming that the amount of non-specifically bound probe is low enough at any position for the chance of it producing two silver grains to be negligible, the occurrence of each silver grain is a rare random event. The distribution of background silver grains is expected to conform to a Poisson distribution.

I will examine the application of the Poisson distribution to the data illustrated by figure 1 in Simmers *et al*. (1986A). Table 2.2(a) shows the grain data for this histogram.

The sampling area for the silver grains is all the chromosomes from 60 metaphases. This was divided into smaller sampling areas, including for example, the 120

Table 2.2(a).

Grain data obtained for chromosomes (46,XX) probed with 0.2μg/ml pULB1148. Below are the calculations for comparing to a Poisson distribution.

GSR	relative length (L)	number of grains (g)
1	9.11	47
2p	3.35	25
2q	5.26	30
3	6.97	39½
Bp	3.46	29
Bq	9.24	65½
Cp	14.14	80
Cq	26.36	145
Dp	1.91	17
Dq	9.00	73
16p	1.33	12½
16q	1.81	67
17/18p	1.69	11½
17/18q	4.06	29½
F	4.71	31
Gp	1.00	6½
Gq	2.50	22

$\Sigma L = 105.90$ $\Sigma g = 731$

Excluding 16q data:

$\Sigma L = 104.09$ $\Sigma g = 664$

$\frac{\Sigma g * 1.81}{\Sigma L} = 11.55$ *=expected grain count on 16q*

Table 2.2(b).

Grain data obtained for chromosomes (46,XX) probed with 0.2ug/ml pULB1148, tabulated for chi-square analysis. Expected values are the mean number of grains per relative length (6.38) multiplied by the relative length of the GSR.

GSR	relative length (L)	observed number of grains (O)	expected (E) $=(\Sigma O/\Sigma L)L$	$\frac{(O-E)^2}{E}$
1	9.11	47	58.11	2.12
2p	3.35	25	21.37	0.62
2q	5.26	30	33.55	0.38
3	6.97	39½	44.46	0.38
Bp	3.46	29	22.07	2.18
Bq	9.24	65½	58.94	0.73
Cp	14.14	80	90.20	1.15
Cq	26.36	145	168.15	3.19
Dp	1.91	17	12.18	1.91
Dq	9.00	73	57.41	4.23
16p	1.33	12½	8.48	1.91
16q	1.81	67	11.55	266.21
17/18p	1.69	11½	10.78	0.48
17/18q	4.06	29½	25.90	0.50
F	4.71	31	30.05	0.03
Gp	1.00	6½	6.38	0.00
Gq	2.50	22	15.95	2.29
	$\Sigma L=105.90$	$\Sigma O=731$		$X^2=288.48$

$$\chi^2_{0.05[15]}=25.00$$

$$P<<10^{-3}$$

excluding 16q data:

$$X^2=22.27$$

$$\chi^2_{0.05[14]}=23.68$$

n.s.

copies of 16p and the 360 copies of Dq (table 2.1). For the purposes of this project this partitioning was adequate as only chromosome 16 was to be examined for specificity of labelling. It should be noted however that over a long GSR, such as that containing all group C short arms, the specific signal may not be great enough to form a significant peak against the expected value.

The null hypothesis is that the grain peak over 16q (r) is consistent with the random distribution of grains over other chromosome regions (expected value = λ), i.e., $r=\lambda$. The alternative hypothesis is $r>\lambda$. As the randomness of the grain level over 16q is in question, this value (r=67) is compared to the mean provided by the grain number from the much larger sampling area of the other chromosome regions over 60 metaphases. The expected number of grains over 16q, with a relative length of 1.81, is determined by:

$$\lambda = \frac{\Sigma g * 1.81}{\Sigma L}$$

where g is the number of grains over a GSR and L is the relative length of the GSR, and all data are used excluding those from 16q (see table 2.2(a)). The λ value obtained is 11.55. In a table showing Cumulative Poisson Probabilities (White *et al.*, 1974), under a λ value of 12.0, the r value giving a probability of 0.05 is 16. The probability of 16 grains at random occurring over 16q is just under 0.05. The probability of 67 grains occurring over 16q at random is much less than 10^{-5} and the null hypothesis is rejected.

It is important to use the unaltered grain number in comparison with the Poisson distribution, as pairs of λ and r altered by the same factor do not carry the same probabilities. Such an alteration occurs if grain numbers are divided by the relative lengths; a step included for example by Bonner *et al.*, 1984; Morton *et al.*, 1984A and B and Bartram *et al.*, 1985). The inappropriateness of this method is illustrated by the probabilities for the following pairs of λ and r, where $r=2\lambda$: $\lambda=8.0$, r=16, P=0.00823; $\lambda=4.0$, r=8, P=0.05113; $\lambda=2.0$, r=4, P=0.14288. The probability changes (through significance to non-significance at the 0.05 level) with the magnitude of the grain number, if the observed and expected values are scaled up or down.

A small GSR can accommodate a low number of silver grains before showing a high peak relative to other regions. If this is not taken into account, such a peak could be found significant with an inappropriate test. This is illustrated in figure 2.3. For the peak of 14 silver grains over 16p, P=0.03. The peak of 3 grains over Yp, as is evident on the histogram, has a larger grains/relative length value than the 14 grain peak over 16p. However, using the cumulative Poisson probabilities to compare the three grains against the expected number (1.46) for a relative length of 0.24, the peak is not significant (P=0.19).

This method for evaluating the data using the Poisson distribution appears to be close to that used for example by Bernheim *et al.* (1984), and by Rabin and colleagues (e.g.

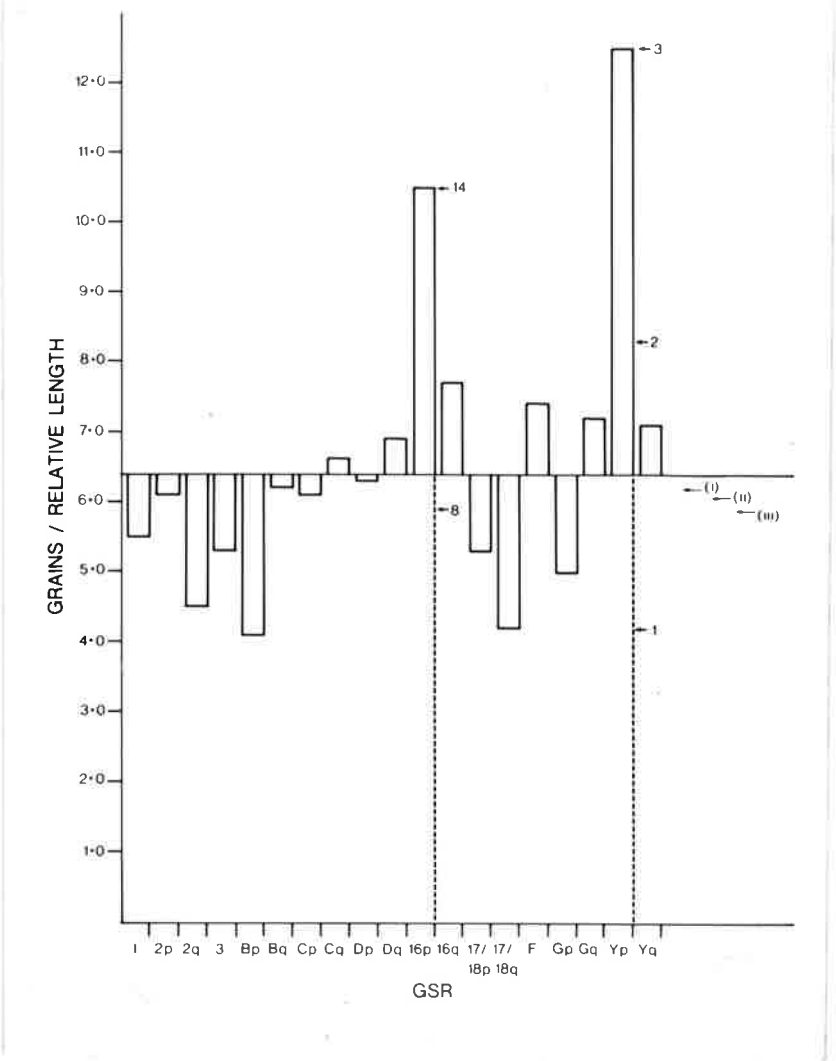


Figure 2.3.

A histogram showing silver grains per relative length (g/L) scored over chromosomes (46,XY) in 55 metaphases, probed with 0.2µg/ml pHPζ. The base line is the mean for all chromosome regions. The short arrows indicate the number of individual silver grains represented at that height on 16p and Yp. The long arrows indicate (i) the mean value of g/L excluding 16p data; (ii) the mean value of g/L excluding Yp data; and (iii) the mean value of g/L excluding both 16p and Yp data.

Rabin *et al.*, 1985A-C; Lieberman *et al.*, 1985; Pravtcheva *et al.*, 1986). Rabin and colleagues have divided the chromosomes, which were banded and identifiable, into equal GSRs, with a length corresponding to the diameter of a silver grain, rather than scoring over the unequal bands. The grain level for a GSR could then be compared to a Poisson distribution. The essential differences between their system and the system applied here are (i) the inclusion of a GSR adjoining each telomere, and (ii) the use of equal GSRs. The use of flanking GSRs, as in (i), would lower the effective lengths of the telomeric GSRs, so their grain levels are not artificially raised. Again, the effective sampling size of these terminal GSRs would be larger than the interstitial ones, but a high grain level would not be disproportionately attributed to the adjacent chromosomal GSR. The advantage of using equal GSRs is that the mean (expected) value for each is equal. Therefore the grain number for each sampling area need not be adjusted. A disadvantage occurs in that the chromosomes must be partitioned by external criteria, disregarding the chromosome bands or morphology.

The GSRs, or sampling areas, reported in this thesis, were chosen on the basis of chromosome morphology: they were a whole chromosome or chromosome arm when unbanded chromosomes were used, and individual bands when banded chromosomes were used. These regions are more easily defined by visual criteria than regions of equal length defined by external criteria.

Testing for significance of the grain level over a particular chromosome region has two applications: (i) the probe has not been previously localized and a peak of grains represents the candidate region for the original localization; and (ii) the sequence is known to map to a particular region and a significant peak over this region will indicate that the *in situ* hybridization procedure was successful.

By definition a grain level significant at the 0.05 level will occur 5% of the time. When the location of the gene is known, as in (ii) above, this level of significance is sufficient. If the location is unknown however, as in (i) above, all GSRs are under surveillance. If the 17 (female) or 19 (male) GSRs given in table 2.1 are used, 0.85 (17*0.05) or 0.95 (19*0.05) of these regions are expected to show a signal level significant at the 0.05 level given an entirely random grain distribution. Clearly this is unacceptable, and the criterion for significance must be more stringent. This is even more important when scoring on chromosome bands. For example, in a 400 band karyotype, if a 10^{-4} level of significance is applied to the grain count over any band, 0.04 bands ($400*10^{-4}$) are expected to have a significant grain level by chance alone, a principle outlined by Steffensen and Wimber (1971). The 10^{-4} level of significance will be applied in original localizations.

Another method of testing peaks from *in situ* hybridization data for significance has been to apply chi-square tests to the set of observed and expected grain numbers, both with and without the region(s) to be tested (e.g. Halley *et al.*, 1984; Bartram *et al.*, 1984). This is illustrated in table 2.2(b). Clearly the grain distribution is still

near significance without including the 16q data. This is probably due to the contribution of the telomeres to deviation of the grain numbers from expected. Some authors have incorrectly used single $\frac{(O-E)^2}{E}$ values as the sample X^2 statistics (e.g. de la Chapelle *et al.*, 1983; Geffrotin *et al.*, 1984; Robakis *et al.*, 1986).

Student's t-test has been applied by some authors in the analysis of *in situ* hybridization data (e.g. Humphries *et al.*, 1984; Devine *et al.*, 1985). This is inappropriate as the grain numbers must be adjusted for the relative lengths to calculate an expected value for a GSR, and the calculated standard deviation is not representative of that of the GSR to be tested. The larger the relative length of the GSR, the smaller is the contributing deviation of the observed from the expected grain number, as the sampling area is larger. Hence, the calculated probability of a grain level depends in part on the relative length of its GSR, rather than on chance alone (refer to fig. 2.3).

2.5.LOCALIZATION WITH RESPECT TO FRAGILE SITES

A major part of this thesis consists of the localization of genes or DNA sequences with respect to fragile sites. This is possible with *in situ* hybridization because of the increased resolution between the DNA on either side of the fragile site, provided by expression of the fragile site.

Data collection consisted of searching for metaphases where the fragile site was expressed, and scoring all silver grains on either side of the fragile site. The region for inclusion of the silver grains in these data was chosen to be approximately equal on both sides, so that the grain numbers were directly comparable. These regions are illustrated in figure 2.4. In all cases grains were scored over the entire acentric fragment distal to the fragile site, as this region was not much further than the distance of scatter of the silver grains from the known region of hybridization. The region for scoring proximal to the fragile site was chosen as the rest of the chromosome arm (from fragile site to centromere) in the cases of fra(16)(p12) and both fra(16)(q22)s (chapter 5), as the fragile site divided the chromosome arm into approximately equal sections. In the cases of fra(11)(q23) and the gap at 11q23.1 induced in lymphoblastoid culture (chapter 6), and fra(16)(q23) (chapter 5), the scoring region proximal to the fragile site or gap was chosen to equal the acentric fragment. Silver grains that touched any part of these regions were scored, so that grains over the telomere were included, as were those over the centromere (where this was the end point of the scoring region), or those over the interstitial end point of a proximal GSR. In this way, if silver grains were distributed entirely at random on the chromosome of interest, the expected numbers of grains scored both distal and proximal to the fragile site would be equal.

Most silver grains could clearly be classified as either "proximal" or "distal" (touching the proximal or distal scoring region respectively), however, those occurring over

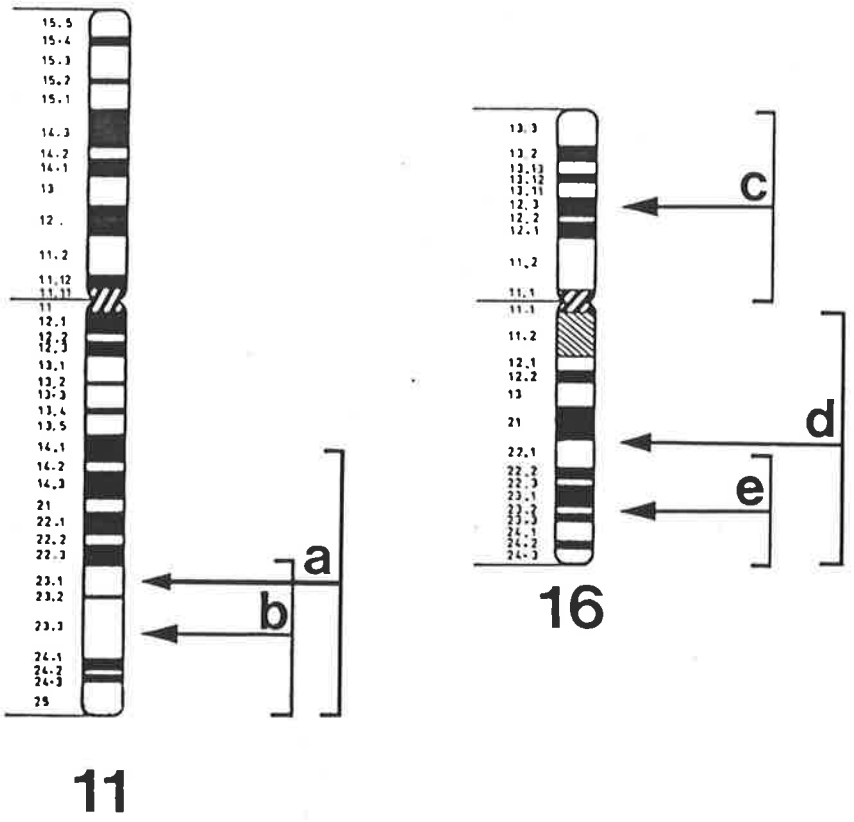


Figure 2.4.

Idiograms of chromosomes 11 and 16. The locations of the fragile sites and gap used for localization by *in situ* hybridization are shown: (a) BrdU-inducible gap at 11q23.1 in lymphoblastoid culture; (b) fra(11)(q23) at 11q23.3; (c) fra(16)(p12); (d) fra(16)(q22) *(FRA16B* and *FRA16C)*; (e) fra(16)(q23). Flanking these points the regions for grain scoring with respect to the fragile site are indicated.

the fragile site, and touching both proximal and distal regions, or touching neither, were classified as "central" grains, and served no informational purpose in determining whether the level of silver grains was greater on one side or the other.

In situ hybridization for localization of DNA seqences with respect to the fragile X has been reported in detail by Szabo *et al.* (1984), Mattei *et al.* (1985B) and Purrello *et al.* (1985). Szabo *et al.* (1984) and Purrello *et al.* (1985) determined that either the band proximal or that distal to fra(X)(q27) showed significant label, by calculating the probability that the observed level of label had occurred over that region by chance, based on the background grain level over all chromosomes. There is a weakness in this statistical approach. The probe is expected to hybridize in the vicinity of the fragile site. It is likely that silver grains will occur on the side of the fragile site opposite the site of hybridization (see chapter 5). A significant level of label adjacent to the fragile site is therefore much more likely to be a result of grain scatter across the fragile site than of the random grain distribution observed over the other chromosomes, and a more stringent test should be applied.

The method used for testing the significance of an excess of silver grains over one of the regions flanking a fragile site as compared to the opposite flanking region was based on comparing the two values against each other. A chi-square test was performed with the null hypothesis that the two values were equal (or in the rare case that tri-radial figures were seen, that the two values were proportional to the

number of sampling regions on each side), i.e that resolution was not increased by the fragile site for the grain level observed.

In some cases the data were further employed to gauge whether a locus found to be distal to a fragile site was closer to the fragile site or to the telomere. The method of scoring silver grains over such an acentric fragment distal to a fragile site has been outlined in Simmers *et al.* (1987C). If the resolution provided by the length of the distal acentric fragment was large enough and the locus far enough from the mid-region of this fragment, this was predicted to be able to provide further information on the location of the DNA sequence of interest, using a chi-square test as described in the previous paragraph.

2.6.SUMMARY AND CONCLUSIONS

In this chapter methods have been outlined for the collection and analysis of silver grain data for *in situ* hybridization. Rules for the inclusion of grains in the data were established, such that each silver grain "touching" a chromosome region could be assigned to a "grain scoring region" (GSR). The testing of a grain peak for significance involved the comparison of the observed count with that expected under a Poisson distribution, with a level of significance appropriate for the number of GSRs under examination. When comparing the grain levels over two GSRs a chi-square test was used.

CHAPTER 3

ADAPTATION OF THE *IN SITU* HYBRIDIZATION TECHNIQUE

3.1. INTRODUCTION

Before *in situ* hybridization could be used for chromosome localization studies, it was necessary to adapt a suitable technique from selected, previously described protocols. Harper and Saunders (1981) carefully applied and assessed the use of dextran sulphate for *in situ* hybridization of low copy number sequence probes. A protocol was developed for use in this project based on the combined *in situ* hybridization procedures of Harper and Saunders (1981), Trent *et al.* (1982), Donlon *et al.* (1983) and Zabel *et al.* (1983), which had all been used for single or low copy number sequence localization.

To assess the suitability of this protocol, probes to repeated sequences were first used. This allowed both the assessment of the methods on a system which required less sensitivity than that for the detection of low copy number and unique sequences, and a relatively short exposure period. The protocol was then tested using a probe to a low copy number sequence, before it was used for the mapping of DNA sequences.

This chapter will present the steps taken in adapting these protocols, particularly for use with chromosomes expressing fragile sites. The general methods used for *in situ* hybridization are given in appendix AI,

and there will also be some discussion on these in this chapter, as well as on some of the observations made after use of these methods, which were not related to the purpose of the experiment (which is reported in a later chapter). The procedures and results discussed, if not presented below, can be found in Appendices AIII and AIV.

3.2.MATERIALS AND METHODS

3.2.1.Probes used

The two anonymous probes from chromosome 16 were prepared by others in this laboratory. The probes for the granulocyte colony-stimulating factor and tumour necrosis factor genes were used in collaboration. In all other cases the probes were obtained from other researchers who had previously described them (see Acknowledgements.)

Repeated sequence probe, **pHY2.1.** This probe consists of a 2.1kb *Hae*III human genomic insert in pBR328, which hybridizes to a 2.1kb *Hae*III restriction fragment from human males, representing a 2.47kb tandem repeat present in 2 000 copies on the Y chromosome (Cooke *et al.*, 1982). A related sequence with homology to the probe is also present in about 100 copies on the autosomes, as tandem repeats (Cooke *et al.*, 1982; Burns *et al.*, 1985).

Repeated sequence probe, **pX1r-101-12.** The *Hin*dIII insert from pX1r-101 (Trendelenberg *et al.*, 1978) has been cloned into the plasmid pBR322 (D.R. Smyth, personal communication). This insert contains 10.55kb

representing almost all of the rDNA repeat from *Xenopus laevis*, and 2.35kb of the *Xenopus*-specific spacer (Trendelenberg *et al.*, 1978). The rDNA repeat encodes the 45S rRNA precursor, the human homologue of which is present in about 300 copies (Schmickel, 1973) at the satellite stalks of the five pairs of acrocentric chromosomes (Henderson *et al.*, 1972; Evans *et al.*, 1974).

Low copy number probe, pHPζ consists of the plasmid pBR322 with a 400bp insert consisting of part of intron 1 and all of exon 2 of the Ψζ-globin gene on the short arm of chromosome 16 (T. Maniatis, personal communication).

3.2.2. *In situ* hybridization

The *in situ* hybridization protocol used was that detailed in appendix AI.5. It is based on the method of Harper and Saunders, and the similar methods of Trent *et al.* (1982), Donlon *et al.* (1983) and Zabel *et al.* (1983).

To first ascertain to what extent the *in situ* hybridization procedure would interfere with fragile site morphology and identification, slides expressing the fragile (X) were processed without probe. Solid stained metaphases expressing the fragile (X) were pre-located, destained and processed using the *in situ* hybridization protocol (appendix AI.5), with carrier DNA but no probe in the hybridization mix. These metaphases were solid stained, relocated and examined afterwards.

The repeated sequence probes, pHY2.1 and pX1r-101-12, were hybridized *in situ* to male metaphase chromosomes as described in appendix AIII.1.

To test the procedure using a probe specific for a sequence of low copy number, the ζ-globin probe was hybridized to normal chromosomes (appendix AIII.2), which were then solid stained and identified by group.

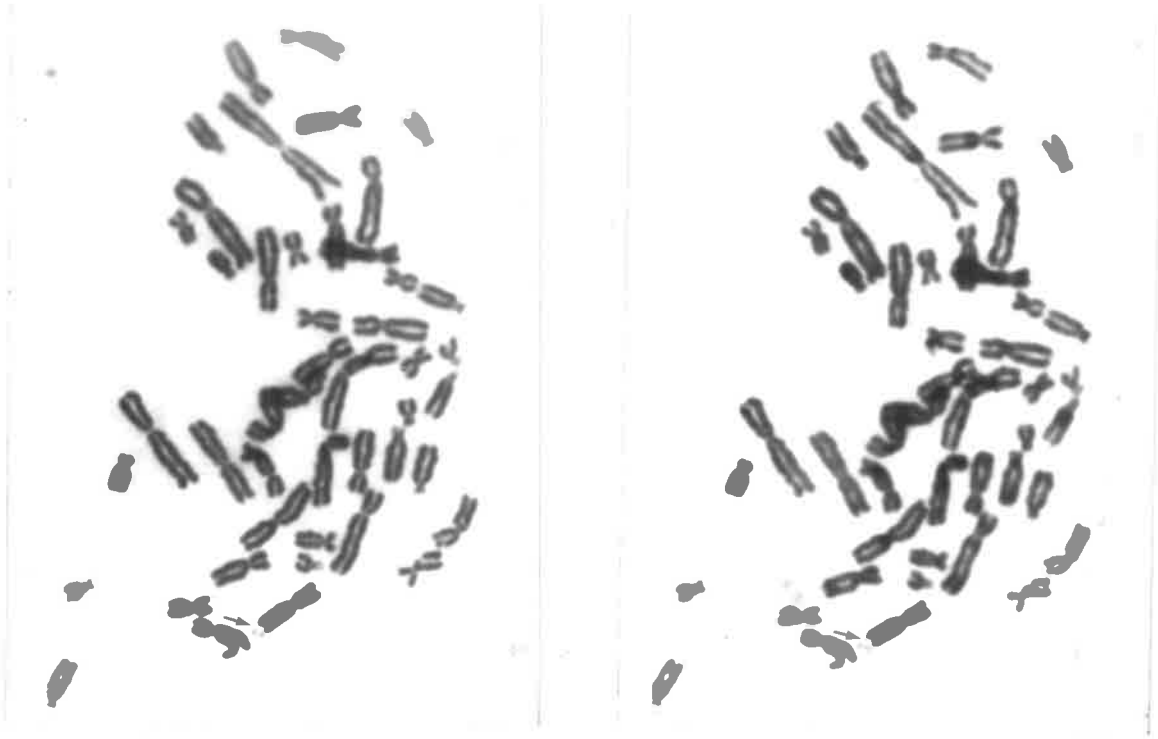
3.2.3.Determination of appropriate emulsion development conditions

Slides with chromosomes from individual F18B (46,XY, with a long Yq; see appendix AII), probed with 0.2μg/ml pHY2.1, were exposed for 24hr, and developed for three to seven minutes, at a constant temperature (19^{o}C), with no agitation, in order to establish a suitable period of development for these set conditions of temperature and agitation. Two slides were developed for each set time, except that only one was developed for seven minutes.

3.3.RESULTS

3.3.1.Morphology of fragile sites after *in situ* hybridization

111 metaphases expressing fra(X)(q27) were located before being treated with the *in situ* hybridization procedure without a probe. In 100 (90%) of these, the fragile site was apparently unchanged morphologically (fig. 3.1(a)) after

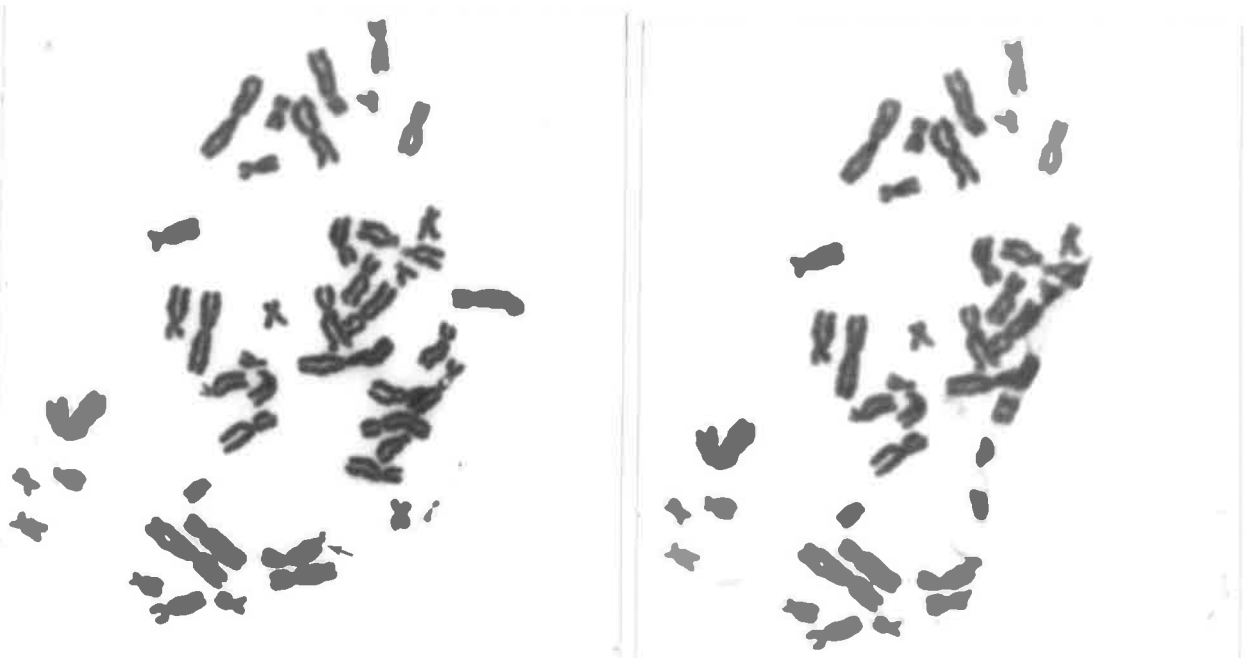


a

Figure 3.1.

Examples of metaphases before (left) and after (right) *in situ* hybridization treatment without a probe, with the fra(X) arrowed. (a) A metaphase, apparently unchanged after treatment. (b) The fragile X chromosome is damaged after treatment, but a portion of this chromosome including the fragile site is unchanged. (c) Part of this metaphase has lifted off the slide, damaging the fragile site.

b



c

treatment. Thirty-two of the 111 cells were noticeably altered, part of the metaphase usually having come off the slide (fig. 3.1(b)). The whole metaphase had been lost in five cases.

3.3.2.Repeat sequence probes

Slides probed with pHY2.1 had a number of silver grains over Yq in many metaphases after 5-8 days of exposure (fig. 3.2(a); see appendix AIII.1), and dense labelling was evident after a much longer exposure period (100 days), making the Y chromosomes difficult to see under the grains, which were difficult to score accurately. The signal and background levels are presented in appendix AIII.1, for slides probed at 0.35 and 0.7μg/ml. In this experiment, one aim of using the repeat sequence probes was to be able to assess the success of the technique quickly, without scoring many cells. Therefore signal and background levels were scored only for these two slides (appendix AIII.1). These levels were highly significant.

Slides probed with pX1-r101-12 did not exhibit obvious label over the short arms of the acrocentric chromosomes after 5-18 days. Most metaphases developed after 100 days' exposure did exhibit label over the region of some of the secondary constrictions. (Grains were not scored, because of the sparsity of the metaphases.)

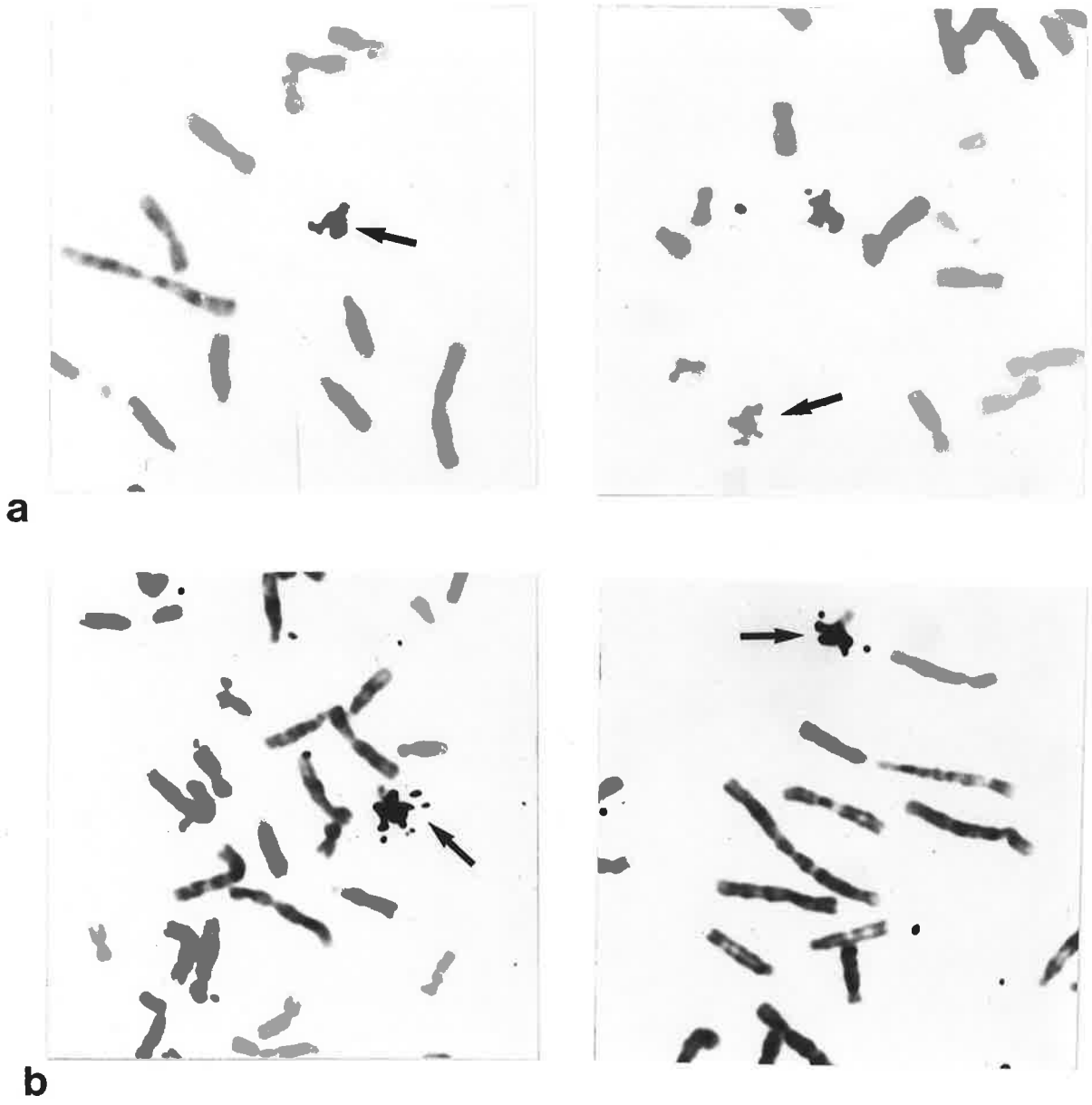


Figure 3.2.

Metaphases probed with pHY2.1, showing label over Yq (arrowed), from (a) the first attempt at *in situ* hybridization and (b) later examples of *in situ* hybridization, after the technique was well established.

3.3.3.Low copy number sequence *in situ* hybridization

The first slide probed with pHPζ, developed after 26 days' exposure, had 14 grains over 16p in 55 metaphases. Although the level of grains in excess of background is not great (see fig. 2.3), this is a significant result, with a probability of random occurrence equal to 0.04 (discussed in chapter 2; see appendix AIII.2). Both longer exposure of the slide and a greater concentration of probe gave better signal and signal:background results (see appendix AIII.2).

3.3.4.Conditions of development

Slides H9.1 and H9.2 (appendix AIII.8, haptoglobin probe), both probed and dipped under the same conditions, were developed for 3 min and 5 min respectively. There was an increase in background (slide H9.1, 12.52 grains per cell; slide H9.2, 15.27 grains per cell), but no increase in signal (slide H9.1, 1.22 grains per cell; slide H9.2, 0.93 grains per cell), with the longer development time. Later, in order to reduce any variability due to temperature and degree of agitation, a series of slides probed with pHY2.1 was developed for different periods. The results are presented in figure 3.3. Twenty-five cells were scored from each slide. From these results a development period of 4min was chosen, for use at 19^{o}C with no agitation.

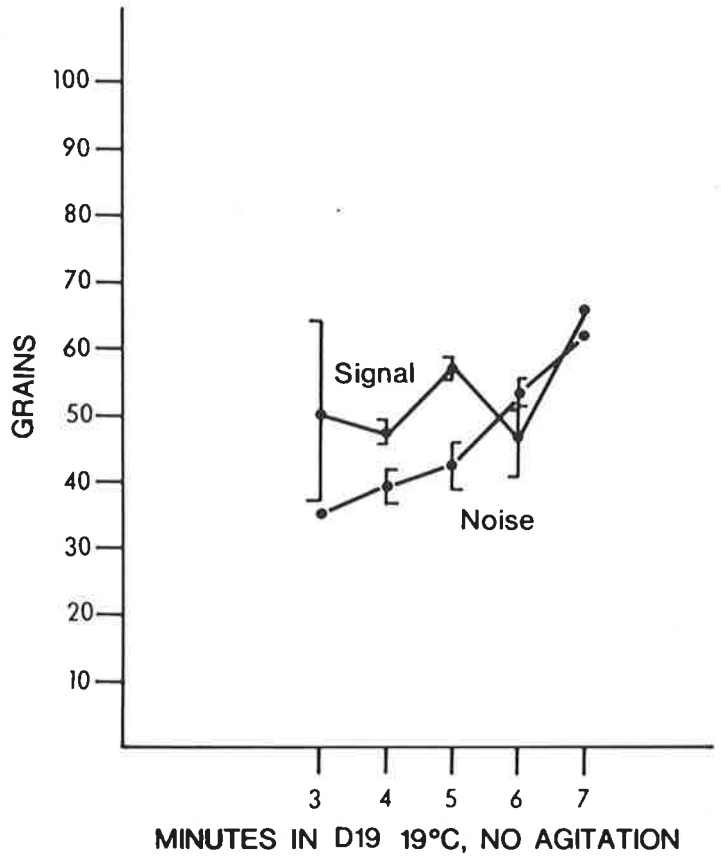


Figure 3.3.

The signal and background levels for chromosomes (46,XY) probed with pHY2.1 and developed for increasing lengths of time.

3.3.5.Patterns of labelling observed on other slides

Appendix AIV gives the "signal" and "background" levels scored for some probes hybridized at a range of concentrations (methods given in chapters 4, 5 and 6, and appendix AIII), as well as the average values of signal and background per cell (for comparison between slides and probes) and the signal:background ratios.

Table 3.1 summarizes signal and background levels for the chromosomes probed with the *APRT* probe at 0.4μg/ml. These levels had not decreased greatly when the probe was used ten months after nick translation, compared to its use immediately after nick translation.

The probe *MT2Ar* hybridized very well to 16q, the site of the *MT* gene cluster (see appendix AIII.10). There were also clusters of silver grains, apparently not specifically bound to any of the sites of loci with homology to the probe. The number of these clusters increased with increasing probe concentration. This phenomenon was common to all chromosome preparations hybridized with this probe, but did not affect chromosomes probed with pHY2.1 at the same time.

Often, with increasing probe concentration, dense concentrations of silver grains were observed, over what appeared to be regions of bacterial contamination. An example is provided by slides from H9 (appendix AIII.8): culture F7F gave this pattern of background, but culture F22A did not. Over slides with this pattern of labelling, a

Table 3.1.

(From appendix AIII.9.) The levels of signal and background after *in situ* hybridization with 0.4ug/ml of the *APRT* probe used (a) immediately after and (b) ten months after the same nick translation.

Slide	exposure (days)	no. of cells	signal/ cell no.	background/ cell no.
(a) H14.12	36	45	0.56	5.18
(b) H26.35-H26.38	32	240	0.45	4.42

lower level of background was seen over F7F metaphases than over those of F22A.

3.3.6.Banding

The appearance of chromosomes after *in situ* hybridization, which had been G-banded with trypsin and pre-photographed before *in situ* hybridization, varied. These chromosomes were often pale (e.g. fig. 5.11). Stronger staining solutions or increased pH of the staining buffer helped in obtaining darker chromosomes in such cases. In many cases chromosomes which had been banded and pre-photographed still showed some G-banding after *in situ* hybridization and staining (e.g. figs 5.11 and 5.12).

In some chromosome preparations obtained after *in situ* hybridization, C-banding and sometimes G-banding could be seen, in chromosomes which had not been banded before *in situ* hybridization, and for which solid staining was intended (see fig. 3.2(b)). Conditions of the *in situ* hybridization technique described here, which were found to improve the extent of C-banding after *in situ* hybridization, include longer denaturation, and denaturation at a higher temperature or higher or lower pH.

3.4.DISCUSSION

3.4.1.Assessment of the *in situ* hybridization protocol

The morphology of the fragile (X) before and after treatment with *in situ* hybridization conditions did not generally differ, apart from mechanical loss of part or all of some metaphases. This result is important to the experiments described in chapters 5 and 6, where probes were localized using fragile sites. In particular, the chromosome outline did not swell, obscuring the fragile site or decreasing resolution. Loss of chromosomes was not a great problem. Careful handling of the slides, particularly when applying and removing coverslips, helps to avoid loss of the metaphases from the slides. When locating metaphases prior to *in situ* hybridization, for examination afterwards, the number pre-located should be in excess of those required, to take into account those that may be lost.

The Y-specific probe, pHY2.1, was well suited to testing the *in situ* hybridization procedure, or for testing variations in conditions. The signal from the first experiment with this probe was evident after only five days. In later experiments, after the technique was well established (using the same protocol), exposure for only one day resulted in a high level of signal over the Y chromosome heterochromatin, such that accurate scoring of grain numbers was impossible (fig. 3.2(b)). The use of conditions allowing single copy gene localization enables rapid detection of the highly reiterated Y-specific sequence using a radioactive detection method. The presence of

repeats with homology to the probe on other chromosomes (Cooke *et al.*, 1982; Burns *et al.*, 1985) is acknowledged, but as the copy number is comparatively low, the signal is not evident after the short exposure periods used for the Y chromosome signal. pHY2.1 has been used by other authors to develop alternative detection methods (Burns *et al.*, 1985; Ferguson *et al.*, 1986), where the high level of hybridization of the probe was used to advantage.

Schmidtke and Schmid (1980) have suggested that the repeats homologous to the probe pHY2.1 are located on the distal part of the Y chromosome heterochromatic material. However, in the present study the silver grains were usually observed over most of this heterochromatin.

The ribosomal probe, pX1-r101-12, produced obvious label over the short arms of the acrocentric chromosomes, but after a much longer exposure time than that required for visualization of specific labelling with pHY2.1. pX1-r101-12 is therefore not practical for rapid, non-quantitative testing of the *in situ* hybridization technique.

The reason for the much higher level of labelling with the Y-specific probe appears to lie in copy number, there being on average 2 000 copies of the sequence per chromatid, clustered as a tandem repeat on a human Y chromosome (Cooke *et al.*,1982). There would be on average however, only 30 (i.e. 300/10) tandem repeats of the ribosomal sequence per chromatid, at each rDNA site in a human diploid metaphase, giving a lower level of hybridization, even when the 5-fold longer size of the part of the insert with human homology in the ribosomal probe is taken into account.

Species variation between *X. laevis* and man would also result in slightly incomplete homology of the ribosomal probe with its human counterpart. The ribosomal probe was probably capable of giving better signal than that observed, given that (a) this was the case with pHY2.1, once the technique had been well established, and (b) Henderson *et al.* (1972) and Evans *et al.* (1974) obtained more efficient hybridization with a less efficient system, probing for the same sequences.

The use of good quality chromosome preparations for *in situ* hybridization appears to be important to the success of hybridization.

In retrospect, the ζ-globin probe was not a potentially reliable low copy number probe with which to test the *in situ* hybridization procedure. The insert is relatively short (400bp), and has complete homology with part of intron 1 (not including the 14bp repeat present in intron 1) and all of exon two of both the ζ- and Ψζ-globin loci, as described by Proudfoot *et al.* (1982), totalling 800bp of double-stranded human sequence homologous to the probe, on each chromatid. Nevertheless, the probe did give a level of signal discernible above the substantial (about ten grains per cell) background. The high background level found on these slides is probably attributable to a high level of exposed silver crystals in the emulsion before exposure, or to development of unexposed silver crystals. (No slide was included for measuring the initial background level in this experiment.) To illustrate this, slide H7.1, exposed for 111 days, did not have a higher level of background than slide H7.3, which was exposed for only 26 days.

3.4.2.Probes

3.4.2.1.Detection system

A radioactive method of probe detection was used, as this was the most reliable for *in situ* hybridization (see chapter 1). There are two radioisotopes in general use for *in situ* hybridization, ^{3}H (tritium) (e.g. Harper and Saunders, 1981) and ^{125}I (e.g. Gerhard *et al.*, 1981). Both have a low maximum energy of emission (18.5keV for ^{3}H; 35keV for ^{125}I) (Rogers, 1973), and therefore single copy sequences being probed require a lengthy exposure period. Their usefulness lies in the small path length that results from this low energy, enabling good resolution. An ^{125}I-labelled probe enables a shorter exposure period than one labelled with ^{3}H (e.g. Eccles *et al.*, 1984; McBride *et al.*, 1986), but resolution is compromised. Li *et al.* (1986) have compared ^{3}H with ^{35}S and ^{32}P for *in situ* hybridization using light and electron microscopy. An ultrathin layer of emulsion allowed sufficient resolution with the two high energy isotopes (^{35}S and ^{32}P), but still that of ^{3}H was better. In addition, the signal obtained from the high energy isotopes was poor, probably because of the low probability of the β-particles, with a high energy at emission, losing energy to the emulsion, as explained by Rogers (1973, 1979).

Tritium was chosen for this project for its superior resolution, and also because of its comparative safety. The half distance of tritium in NTB-2 emulsion (the distance from the source within which half the density of silver grains in that direction occur) is 0.38μm, and the

range of a tritium-emitted β-particle seldom exceeds 1μm in emulsion (Rogers, 1973). This compares favourably with the size of a chromosome (chromatid width being in the order of 1-2μm), so that silver grains will be clustered over a relatively narrow chromosome region. The long half life of tritium (12.3 years) enables probes to be used for months after nick translation. For example, the *APRT* probe (appendix AIII.9) was used successfully ten months after nick translation (slides H26.10-40), with the resulting level of labelling not greatly reduced, compared with slides probed immediately after nick translation (slides H14.1-25) (table 3.1.).

3.4.2.2.Effect of probe size

Those probes which gave the best signal:background ratios (see appendix AIII) include pα3'HVR.64, the 5.4*ETS1* probe, and the two probes with a λ vector, ACH207 and ACH224. They have large inserts (pα3'HVR.64 insert 4.0kb, S.T. Reeders, personal communication; 5.4*ETS1* insert 5.4kb, D. Stehelin, personal communication; ACH207 insert 4.7kb and ACH224 insert 5.0kb, V. Hyland, personal communication), and therefore a large homologous chromosomal region with which to hybridize. The repeated nature of the sequence homologous to the pα3'HVR.64 probe (see chapter 5) probably also contributed to the high level of signal obtained with this probe. The large vector (with a length of 41.7kb) of the λ probes, rather than contributing to probe concentration alone, probably also contributed to network formation. Zabel *et al.* (1983) concluded that vector size is important to the level of signal, because of the contribution to network formation. The α- and ζ-globin probes, JW101

(insert about 800bp, Wilson *et al.*, 1978) and pHPζ (insert 400bp, T. Maniatis, personal communication), with small inserts, did not produce a high level of labelling.

3.4.2.3.Probe concentration

The probe concentration giving the highest signal with low background (Gerhard *et al.*, 1981) was usually chosen for test slides (appendix AIII), based on the previous signal:background results (appendix AIV) for that probe or other probes of similar insert size and extent of homology of the target chromosomes. If the potential hybridization efficiency was uncertain, a concentration of 0.4µg/ml was chosen, which was considered likely to give good signal, without high background, based on experience with other probes (see appendix AIV).

Calculations of *in situ* hybridization efficiency have appeared for experiments using RNA probes (e.g. Coté *et al.*, 1980; Malcolm *et al.*, 1981) and DNA probes without dextran sulphate (Atwood *et al.*, 1976). However, such calculations are not possible when dextran sulphate is used with a DNA probe, as 1:1 hybridization of probe to chromosomal DNA is not expected (Barton *et al.*, 1982). Raap *et al.* (1986) reported a 40% loss of chromosomal DNA during heat denaturation, which, in addition to renaturation of complementary sequences, explains the incomplete efficiency of *in situ* hybridization.

3.4.3.Conditions of hybridization

3.4.3.1.Chromosomal denaturation

The *in situ* hybridization method described by Harper and Saunders (1981) follows that of Chandler and Yunis (1978) to a large extent. An important feature of the technique is denaturation at 70°C. The inclusion of formamide in the denaturation solution allows the temperature for heat denaturation to be reduced from 100°C. The principle allowing this reduction of temperature is stringency: increasing the formamide concentration will decrease the stringency of the solution, so that nucleic acid strands anneal less efficiently (Jones, 1974), and can be denatured at a lower temperature. Lowering the salt concentration and increasing the temperature are also ways of increasing stringency (Jones, 1974). Chromosomal denaturation by acid treatment results in better morphology, but poorer hybridization efficiency, than denaturation by NaOH or heat treatment (Jones, 1974; Pardue and Gall, 1975; Steffensen, 1977). The reduction of denaturation and hybridization temperatures allowed in the presence of deionized formamide improves the morphology obtained after heat denaturation, and washing (Chandler and Yunis, 1978). Dehydration in cold (-20°C) ethanol solutions prevents the chromosomal DNA from reannealing (Steffensen, 1977).

Denaturation by low pH appears to cause extraction of the basic histone proteins from the chromosomes; whereas denaturation by high pH extracts non-histone proteins, leading to loss of morphology and staining ability

(Pardue and Gall, 1975; Steffensen, 1977). Such extraction of histone proteins, as may also be attained by acid fixation of the chromosomes (this is applicable to the method of chromosome harvest and storing used in this project), removes some of the chromosomal proteins to which the nucleic acid probe might bind non-specifically (Pardue and Gall, 1975; Steffensen, 1977). The non-histone proteins appear to be necessary for good quality chromosome morphology, so the inclusion of a DNA carrier in the hybridization solution (i.e. sheared salmon sperm DNA in the protocol detailed in appendix AI.4) counters the possible non-specific binding which might otherwise occur when these proteins are left intact (Pardue and Gall, 1975).

3.4.3.2.Stringency of hybridization and washing

The consistent use of hybridization and washing solutions at pH7.0 should prevent any dissociation of nucleic acid hybrid which might otherwise occur at a higher or lower pH.

Variation of washing stringency from the standard conditions (H14.15-21: see appendix AIII.9) did not result in any detectable decrease in background level.

Slides from H9 and H14 (haptoglobin gene and *APRT* gene localization experiments, see appendix AIII.8 and AIII.9) were hybridized at 42^{o}C. An initial wash with a temperature a few degrees higher than that of hybridization should be, but was not, used. These steps also vary from the standard protocols followed, probably allowing less effective

hybridization of the probe. Hybridization at 42°C was therefore abandoned in later experiments.

There is an optimal temperature (other conditions affecting stringency remaining unaltered) at which maximal hybridization between two sequences occurs (Moar *et al.*, 1975). Controlled high stringency is important when there are known sequences which will otherwise cross-hybridize with the probe, and signal from these is unwanted (Cremer *et al.*,1986). Such distinction was not needed in the experiments reported in this thesis. The original *in situ* hybridization protocol, which created no such problems in localization, and is in common use, was adhered to, and variations of dubious advantage were not introduced.

3.4.4.Autoradiography

3.4.4.1.Emulsion

As tritium has a low energy of disintegration, a sensitive emulsion is not necessary. Therefore Kodak NTB-2 emulsion was chosen, in preference to Ilford L4, as it has a lower sensitivity rating (a rating of 3 on the Ilford scale), to avoid some background labelling from external sources, as recommended by Rogers (1973) for tritium. Resolution is lower with NTB-2 than with L4 emulsion, because of the larger size of the silver crystal in NTB-2, but this difference in resolution is negligible at the light microscope level (Rogers, 1979).

A 2:1 dilution of emulsion with water gives an emulsion thickness of about 1-2μm, which gives increased resolution compared to the use of Kodak emulsions undiluted (Rogers, 1973). For quantitative autoradiography with tritium, the emulsion should be thick enough to include all potential silver grains from the tritium source (Rogers, 1973). However, this is more important for cases where grain scoring regions are separated from each other, e.g. histological specimens. In chromosomal autoradiography, each metaphase, consisting of several GSRs, is itself covered by a small area of emulsion, effectively of uniform thickness, and the variations between metaphases in the thickness of the overlying emulsion affect each class of GSR equally.

3.4.4.2.Duration of exposure

There is an increasing trend towards development after a relatively short exposure period (e.g. Caubet *et al.*, 1985; Le Beau *et al.*, 1986), often resulting in low background levels, and shortening the wait for results. These benefits must be considered against the possible disadvantages. Similar magnitudes of signal and background grains might be obtained from a short exposure period as from a longer one by scoring proportionately more cells (and a greater expenditure of time and effort are therefore necessary, the disadvantage of which must be compared against the advantage of an earlier result). Regardless of the length of exposure, the data will be more powerful the greater the combined signal (S) and noise (B) counts, where S and B are related by a constant, as a difference between the two which is a certain proportion of S (if a difference exists) will be more significant (less likely to

have occurred by chance). It can be seen from figure 3.4, however, that the signal:background ratio should increase with the length of exposure, provided the rate of exposure of the emulsion as a result of specific signal is still constant, and is at least twice the rate of exposure due to background. This implies that a longer exposure will give a greater difference between signal and background than will scoring extra cells after a shorter exposure. The rate of exposure starts decreasing when 10% of the silver halide crystals in the region of emulsion over a source have been exposed (Rogers, 1979), so development is desirable before this state is reached. Development before this stage also gives optimal resolution (Rogers, 1979).

In the experiments reported in this thesis, relatively long exposure periods were usually opted for. This was particularly important in cases where slides were pre-photographed, or for localization using fragile sites, as only a limited number of cells were to be useful, and a maximal amount of labelling was required. When carrying out original localization experiments, these constrictions were not as limiting, but long exposure periods were chosen for the reasons mentioned in the previous paragraph.

3.4.4.3.Conditions of development

The level of background in the emulsion depends in part on the conditions of development (Rogers, 1973). The degree of agitation, and the time and temperature of development are important. Under ideal conditions of each, all of the

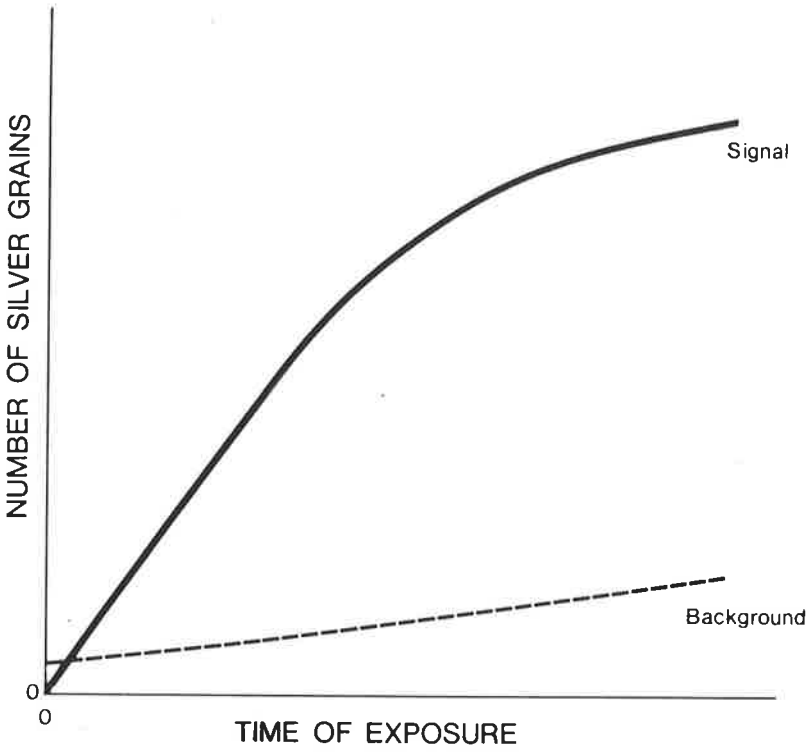


Figure 3.4.

The relative rates of increase of signal and background levels shown as a graph, time of exposure vs. number of silver grains. Background increases from a level initially higher than signal, but at a slower rate. The signal grains saturate the emulsion (illustrated by levelling off of the signal curve) before the background grains do, and the rate of increase of background is then greater than the rate of increase of signal.

latent images will have been developed, and development stopped before any of the unexposed silver halide crystals have been developed. Initially a development time of 5min was chosen, at room temperature, with constant agitation. Slides developed together would have received the same treatment, but differences in agitation between batches, and in temperature (although controlled) between days would decrease reproducibility.

In the earliest *in situ* hybridization experiments the emulsion had been developed for 5min (see appendices AIII.1, AIII.2). The decrease in background corresponding to the development of slide H9.2 for 3min (see results), without decrease in signal, was the basis for subsequent development of slides for 3 min. In the results presented in figure 3.3 for slides probed with pHY2.1, the level of background clearly increased with time of development. The level of signal did not show such a clear relationship, but overdevelopment possibly contributed to the high level at seven minutes (such a small region of emulsion over Yq would not be affected greatly by random development of unexposed silver halide crystals). A development time of 4 min was chosen from these results, so that development was finished before the rapid increase in development of unexposed silver halide crystals, but was not short enough to result in underdevelopment, or too short to be accurately reproducible.

3.4.5.The effect of fragile site inducing agents on *in situ* hybridization

The DNA-binding agents which induce fra(16)(q22)(*FRA16B*), including distamycin A and Hoechst 33258 (Sutherland *et al.*, 1984; Schmid *et al.*, 1986), were not generally used, as it was not known how the binding of these agents to the DNA would affect hybridization. Chromosomes from two individuals, in which *FRA16B* had been induced with Hoechst 33258, were used in experiments H35 and H40 (see appendix AIII.10, *MT2A*u and *MT1B*u probes, slides H35.14-15 and H40.12-15). On most of these slides the same labelling trend was seen as for the slides with *FRA16B* induced with BrdU (H35 and H40). The data are small however, and do not indicate whether the levels of labelling are different. A more detailed study of this effect was not carried out.

Bromodeoxyuridine (BrdU), on the other hand, is incorporated into DNA in place of deoxythymidine residues. BrdU was often included in cell culture, either for cell synchronization or for induction of *FRA16B*. Zabel *et al.* (1983) found that its incorporation into early replicating bands does not change the relative efficiencies of hybridization to dark and light bands.

3.4.6.Chromosome identification after *in situ* hybridization

Most chromosome banding techniques will not work, or work with reduced efficiency after *in situ* hybridization (Malcolm *et al.*, 1986). Synchronization of the cultures, which would result in prometaphase

chromosomes, or enable banding after *in situ* hybridization by the method of Zabel *et al.* (1983), was considered neither necessary, compatible with, nor easily adaptable to the requirements for induction of some fragile sites. For these reasons, as well as the difficulty in establishing a banding technique for use after *in situ* hybridization, chromosomes were not always banded after *in situ* hybridization. In the first experiments testing the viability of the *in situ* hybridization technique, probes to chromosome regions identifiable without banding (Y, 16 and the short arms of the acrocentric chromosomes) were used. When fragile sites were used for localization, in the majority of cases these were on chromosome 16, for which banding was not needed for identification. However, when the fragile site on 11q was used for localization, its expression was the only criterion available for recognition of chromosome 11.

Banded chromosomes were required however, when chromosome recognition was more crucial: when a probe of unknown location was used, or when a probe was hybridized to a chromosome which could not be identified without banding. Where identification of chromosomes other than chromosomes 16 or Y was necessary, chromosomes were either G-banded and photographed before *in situ* hybridization, or G-banded after *in situ* hybridization with the method of Zabel *et al.* (1983). The use of cell synchronization to obtain prometaphase chromosomes suitable for banding (Zabel *et al.* (1983)) aided in the degree of resolution obtained.

Researchers initially identified chromosomes after *in situ* hybridization either by recognition of morphologically similar groups (e.g. Price *et*

al., 1972; Steffensen *et al.*, 1974), or by banding and photographing selected metaphases before hybridization, to be relocated after hybridization and autoradiography (e.g. Jones *et al.*, 1974; Gosden *et al.*, 1975B).

Banding with pre-photography before *in situ* hybridization is still a suitable method of chromosome identification, and was sometimes used in this project. The location and photography of sufficient metaphases, including a surplus in case some are lost, is an extensive and time consuming process. However, the inconvenience is offset after hybridization by the rapid relocation of the metaphases to be scored. Another advantage is that the choice of cells is not biassed by the silver grain pattern. The appearance of these pre-photographed chromosomes after *in situ* hybridization varied, and they were often pale (e.g. fig. 5.11; c.f. Devilee *et al*, 1986). Reduction in the denaturation time of these chromosomes may improve their stainability after *in situ* hybridization (L. Webber, personal communication).

Chandler and Yunis (1978) described the first technique for banding chromosomes after *in situ* hybridization. Bands were obtained by staining with Wright's stain without pre-treatment. It had been previously uncertain whether banding was possible after *in situ* hybridization (Hirschhorn and Boyer, 1974). Chandler and Yunis' (1978) method was also used by Harper and Saunders (1981). A number of other banding techniques for use after *in situ* hybridization have been described, including those of Bernheim and Berger (1983) (where chromosomal denaturation is achieved as part of a standard R-banding

technique), Kiel-Metzger and Erickson (1984) and Cannizzaro and Emanuel (1984).

Some of the published post-hybridization banding procedures damage the emulsion to some extent, requiring that metaphases be photographed before banding, recording the positions of silver grains (e.g. Lee *et al.*, 1985; Popescu *et al.*, 1985). This photography and relocation process appears to involve the same inconveniences as banding and photographing before *in situ* hybridization. However, in the first case the end result is a photograph of silver grains and a slide of banded chromosomes, whereas in the second, a photograph of banded chromosomes and a slide of chromosomes with silver grains are obtained. The latter seems preferable, as the scoring of silver grains by microscopy is much more reliable than scoring from a photograph: the scatter of silver grains is three-dimensional, the grains usually being out of focus on a photograph, and their location with respect to the underlying chromosomes not accurately represented.

The G-banding method (Zabel *et al.*, 1983) was used after *in situ* hybridization when original localizations were made (chapter 4). The early replicating bands, into which the BrdU had been incorporated, are negatively stained, and the late replicating bands are positively stained.

C-banding was developed directly from *in situ* hybridization (Arrighi and Hsu, 1971), and was sometimes seen in chromosomes after *in situ* hybridization. Such banding was not predictable however, and usually

not sharp enough to use for chromosome identification. By altering the conditions of chromosome denaturation, the extent of C-banding could be improved (see section 3.3.5), but the general morphology of the chromosomes suffered under these conditions. Such limited identification of chromosomes may prove useful in some cases, when more detailed banding is elusive, but was not used for chromosome identification in the experiments described in this thesis.

3.4.7.Patterns of background labelling

Increasing the concentration of probe in the hybridization solution generally increases the level of non-specific hybridization (Harper and Marselle, 1986). In some cases there was obvious clustering of silver grains, the level of which increased with probe concentration.

The heavy clustering of silver grains after even low concentrations of the probe *MT2Ar* appears to have been due to some property of the probe, being common to all chromosome preparations hybridized with this probe, but not affecting chromosomes probed with pHY2.1 at the same time. It was the only probe with which background labelling at low probe concentrations made signal/background scoring almost impossible (see appendix AIII.10).

An interesting contrast in background labelling levels was seen between slides which could be distinguished by being from separate tissue culture batches (see Results, section 3.3.4). Apparently patches of bacterial contamination provided a target for non-specific hybridization

of the probe, lessening the amount of non-specifically bound probe over other areas. Such contamination would have occurred before *in situ* hybridization: it could have occurred during cell culture or RNase treatment, but the similarities in the extent of bacterial contamination and its labelling observed between cells from the same culture supports the former.

3.5.SUMMARY AND CONCLUSIONS

This chapter described the adaptation of a protocol for *in situ* hybridization. It was first tested using probes to repeated sequences, so that a rapid result was obtained. This was successful, as was low copy number *in situ* hybridization using the same procedure. It was also found that treatment of the chromosomes in this way did not affect the morphology of fragile sites.

The chosen *in situ* hybridization procedure was successful, and did not need modification. Variations in probe concentration and conditions of exposure and development had an effect on the resultant signal and background levels. The use of a series of probe concentrations allowed the assessment of an optimal one for general use. The length of exposure needed could be assessed during each experiment; the arguments for short vs. long exposure periods were presented.

Various methods were used for chromosome identification, and the choice depended on the extent of identification that was needed, and what techniques could be reliably applied. The use of chromosome 16 for the

bulk of the work presented in this thesis removed the need for banding in those experiments, and this was important when fragile site expression and identification were of primary importance.

CHAPTER 4

LOCALIZATION OF THE *TNF* AND G-*CSF* GENES

4.1. INTRODUCTION

During this project, two probes to genes of immunological interest were used to make original gene localizations. This chapter describes these experiments, and the implications of the chromosomal locations of the genes for the proteins, tumour necrosis factor (TNFα) and granulocyte colony-stimulating factor (G-CSF).

4.2. MATERIALS AND METHODS

TNFA probe. This probe consists of the 800bp *Eco*RI fragment from the clone λ42-4 (Pennica *et al.*, 1984) in the plasmid SP64. This 800bp cDNA sequence includes the entire coding region for mature TNFα.

G-CSF probe. This cDNA clone is the sequence for *G-CSF* described by Souza *et al.* (1986) (1.6-1.7kb in length), inserted into the plasmid pXMT2 using *Eco*RI adaptors, at the *Xho*I restriction site (G. Wong, personal communication).

In situ hybridization. The *TNFA* and *G-CSF* probes were hybridized *in situ* to normal chromosomes from male individuals, as described in appendix AIII.3 and AIII.4. The locations of silver grains were identified after development by comparison with photographs of the

banded metaphases taken before hybridization (for *G-CSF* localization), or by banding the chromosomes after development as described (appendix AI.7) (for both *TNFA* and *G-CSF* localization).

On the basis of the localization result obtained for *G-CSF*, the *G-CSF* probe was hybridized to chromosomes from patients with acute promyelocytic leukaemia (APL), with the t(15;17) (karyotypes given in appendix AII), as described in appendix AIII.5 and Simmers *et al.* (1987B). The chromosomes were banded with alkaline phosphatase buffer (in another laboratory, see Acknowledgements), pre-photographed, probed, and relocated after development and solid staining for comparison with the photographs.

4.3.THE TUMOUR NECROSIS FACTOR GENE

4.3.1.Results

The test slides were developed after 26 days' exposure, and G-banded successfully. Forty-nine metaphases were scored from slides H29.11 and H29.12, both probed at 0.4μg/ml. The results are shown in figure 4.1.

Of the 158 silver grains scored over the 49 metaphases from slides H29.11 and H29.12, 25 (16%) were localized over the region 6p12-p22, with a peak of 16 grains over band 6p21. This result is highly significant (relative length of 6p12->p22 = 1.5; λ=1.9; $P<<10^{-5}$). There were no significant peaks elsewhere. This result localizes *TNFA* to within the region 6p12-p22. The most likely location of *TNFA* is within

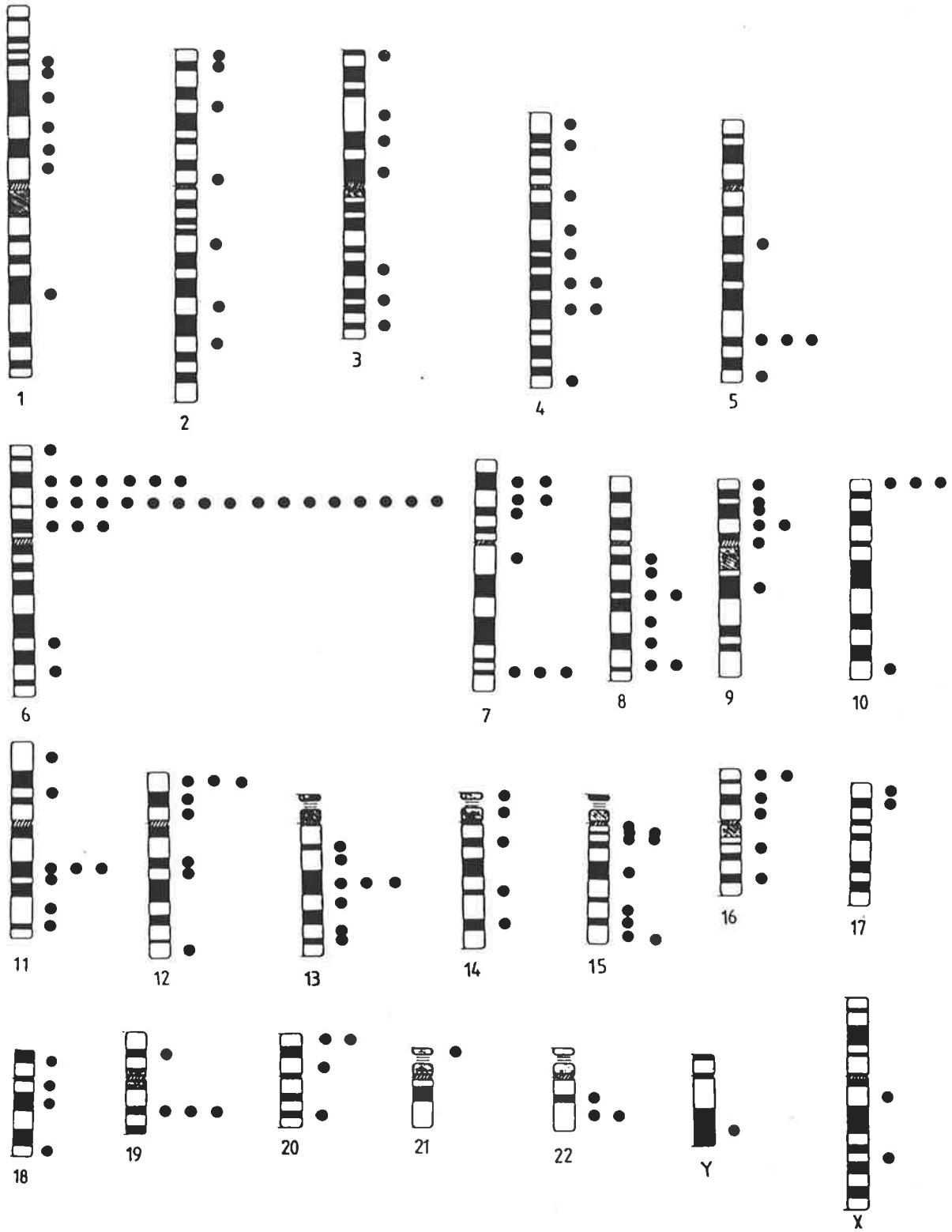


Figure 4.1.

An idiogram of the human karyotype, showing the silver grains scored over all the chromosomes probed for *TNFA*, in 49 metaphases.

band 6p21. Figure 4.2. shows examples of metaphases with silver grains over this region.

4.3.2.Discussion

TNF is a cytotoxic protein, produced by macrophages,, to which a great number of functions have been attributed, being involved in a complex set of polypeptide mediator circuits (Old, 1987). Separate lines of research have unwittingly involved this same protein: TNF has proved to be identical to cachectin (Beutler *et al.*, 1985), and probably identical to one of the differentiation inducing factors produced by leukocytes (Takeda *et al.*, 1986). In addition, natural cytotoxic activity appears to be due to the release of TNF (Ortaldo *et al.*, 1986; Patek *et al.*, 1987).

The attributes of TNF which were originally studied include its ability to suppress lipoprotein lipase, thereby mediating endotoxic shock, and the ability to induce haemorrhagic necrosis of tumours (Beutler and Cerami, 1987; Carswell *et al.*, 1975). Clinical trials are in progress to assess TNF as a treatment for cancer, especially in conjunction with interferon (Beutler and Cerami, 1987; Old, 1987; Ruddle, 1987).

Lymphotoxin (TNFβ) has 28% sequence homology with TNFα, although by rearranging the alignment of the two sequences, 35% homology is found (Nedwin *et al.*, 1985A), with 46% sequence homology between the coding regions of the two genes (Pennica *et al.*, 1984). The two proteins have been renamed TNFα (previously TNF) and TNFβ (previously

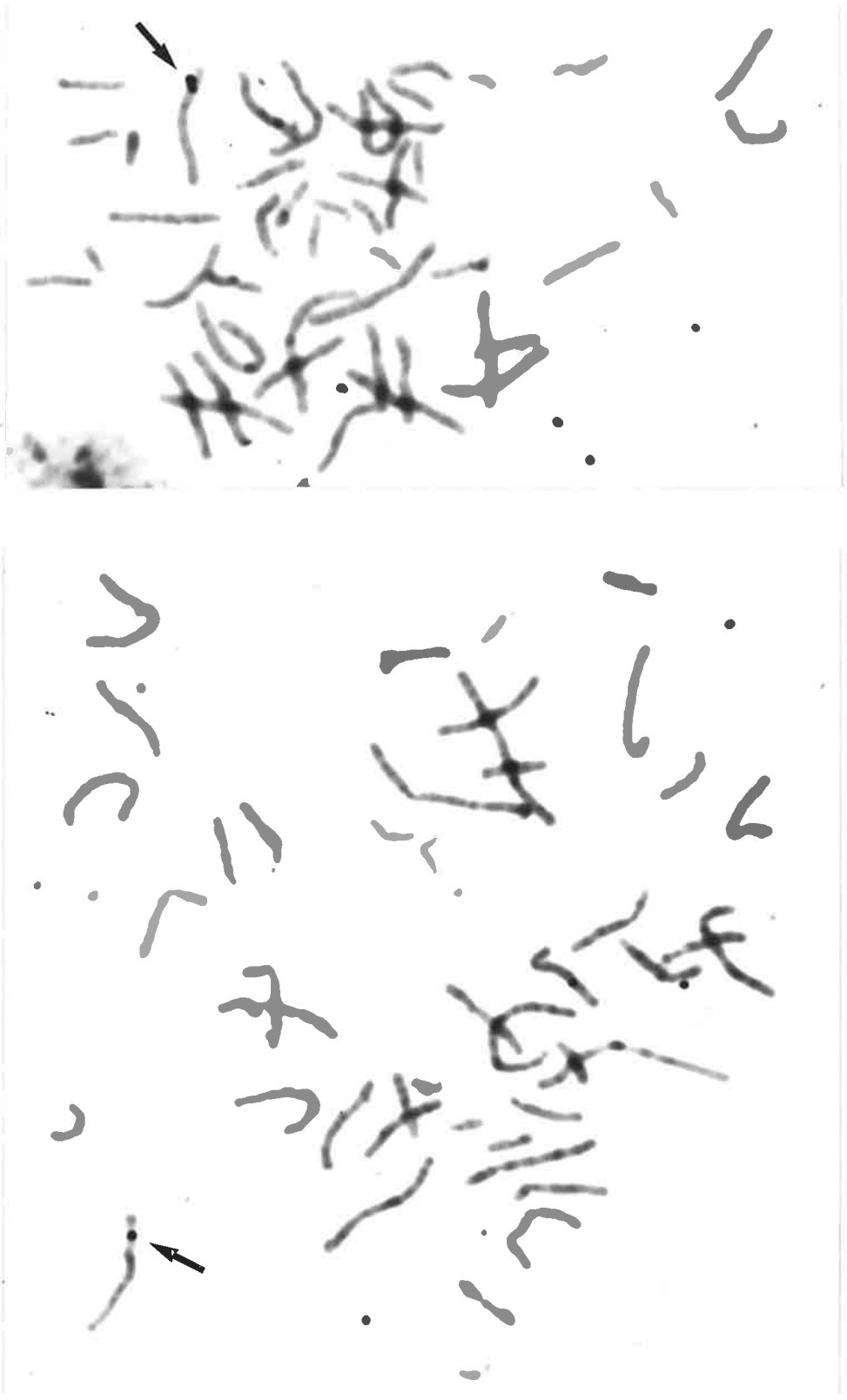


Figure 4.2.

Metaphases after hybridization with the *TNFA* probe, showing silver grains over the region 6p21-p22 (arrow).

lymphotoxin) (Shalaby *et al.*, 1985). The genes for these two proteins will therefore be termed *TNFA* and *TNFB*, respectively. The two genes for these proteins are very close, in tandem arrangement (Nedospasov *et al.*, 1986B). The murine *TNFA* and *TNFB* genes, which closely resemble their human counterparts in structure and tandem arrangement, are separated by only 1.1kb (Nedospasov *et al.*, 1986A). The human genes are similarly close (Nedospasov, 1986B; Goeddel *et al.*, 1986). A maximum homology of 56% between the nucleotide sequences occurs in the last exon of both (which encodes more than 80% of both proteins) (Nedwin *et al.*, 1985A). The two genes probably arose from a single tandem gene duplication event (Nedwin *et al.*, 1985A; Nedospasov *et al.*, 1986A; 1986B).

TNFB had previously been assigned to chromosome 6 (Nedwin *et al.*, 1985B), and both *TNFA* and *TNFB* had been localized to the region 6p23->q12, by Southern analysis of somatic cell hybrids (Nedwin *et al.*, 1985A). Although there is some homology between the two sequences (see above), Southern analysis indicates that there is a single *TNFA* gene, neither probe cross-hybridizing with the other gene (Pennica *et al.*, 1986; Nedwin *et al.*, 1986A). Thus, the *in situ* hybridization results presented above for the *TNFA* probe probably indicate the true location of *TNFA*. Even in the event of limited hybridization to *TNFB*, the two loci are so close that the overall grain distribution would not have been affected.

The result localizing *TNFA* to 6p21->p22 was reported (Simmers *et al.*, 1986B), noting the proximity of this locus to the major

histocompatibility complex. This finding has since been confirmed (Spies *et al.*, 1986).

The shortest region of overlap (SRO) for the *HLA* region was defined as 6p21.3 at Human Gene Mapping Workshop VII (Robson and Lamm, 1984). TNFα has been found to increase the level of expression of the HLA class I and II antigens in some normal and tumour cells (Collins *et al.*, 1986; Pfizenmaier *et al.*, 1987; Pujol-Borrell *et al.*, 1987). The localization of *TNFA* to the same band as the *HLA* genes was therefore of interest, suggesting perhaps that it is within or near the major histocompatibility complex (MHC). Nedwin *et al.* (1985A) suggested that a location for *TNFA* and *TNFB* adjacent to the *HLA* genes might be useful for coordinate regulation of their respective products. Spring *et al.* (1985) have localized genes of the MHC more precisely to regions within 6p21.3, using a series of B-cell line mutants. They were found to span over half of this sub-band, the genes for class I, HLA-DQ and HLA-DP antigens being defined by a series of deletions.

Spies *et al.* (1986) localized both *TNFA* and *TNFB* to within the bounds of the MHC. Using a different series of deletion mutants for various regions of the MHC, of known *HLA* status, both genes were mapped to one of three regions: on one or the other side of the class III region or in an uncloned region of the class I genes. Müller *et al.* (1987) found that the murine *TNF* genes are also within the MHC of the mouse. Using a panel of mouse strains recombinant within the MHC, finer localization detail was possible: *TNFA* and *TNFB* were localized about 70kb upstream of the *H2-D* gene (i.e., at the proximal end of the *D/Qa* region gene

cluster), close to a number of other genes in the MHC which are also structurally and functionally unrelated to classes I and II genes (Müller *et al.*, 1987). The presumed position for the human *TNF* genes close to *HLA-B*, deduced from the mouse data (Müller *et al.*, 1987), is concordant with the results obtained by Spies *et al.* (1986) for the human *TNF* genes (Müller *et al.*, 1987).

Although there is a functional relationship between TNF α and lymphotoxin, and the HLA antigens (see above), there is no evidence for any functional significance of the proximity of their genes (Nedospasov *et al.*, 1986A; Müller *et al.*, 1987). The enhancing effect of TNFα and lymphotoxin on the expression of the HLA antigens in any case appears to be mediated via a protein intermediate (Collins *et al.*, 1986), apparently β_2-interferon (Scheurich *et al.*, 1986; Kohase *et al.*, 1986; May *et al.*, 1986).

4.4.THE GRANULOCYTE COLONY-STIMULATING FACTOR GENE

4.4.1.Results

Slide H43.1, which was developed after 26 days, showed no significant labelling over any region. [Seven (6%) of the 121 silver grains were over 17q on this slide (35 metaphases) (λ=2.25; P=0.01), a peak which is not significant at the 10^{-4} level.] The remaining slides were therefore exposed for a longer period.

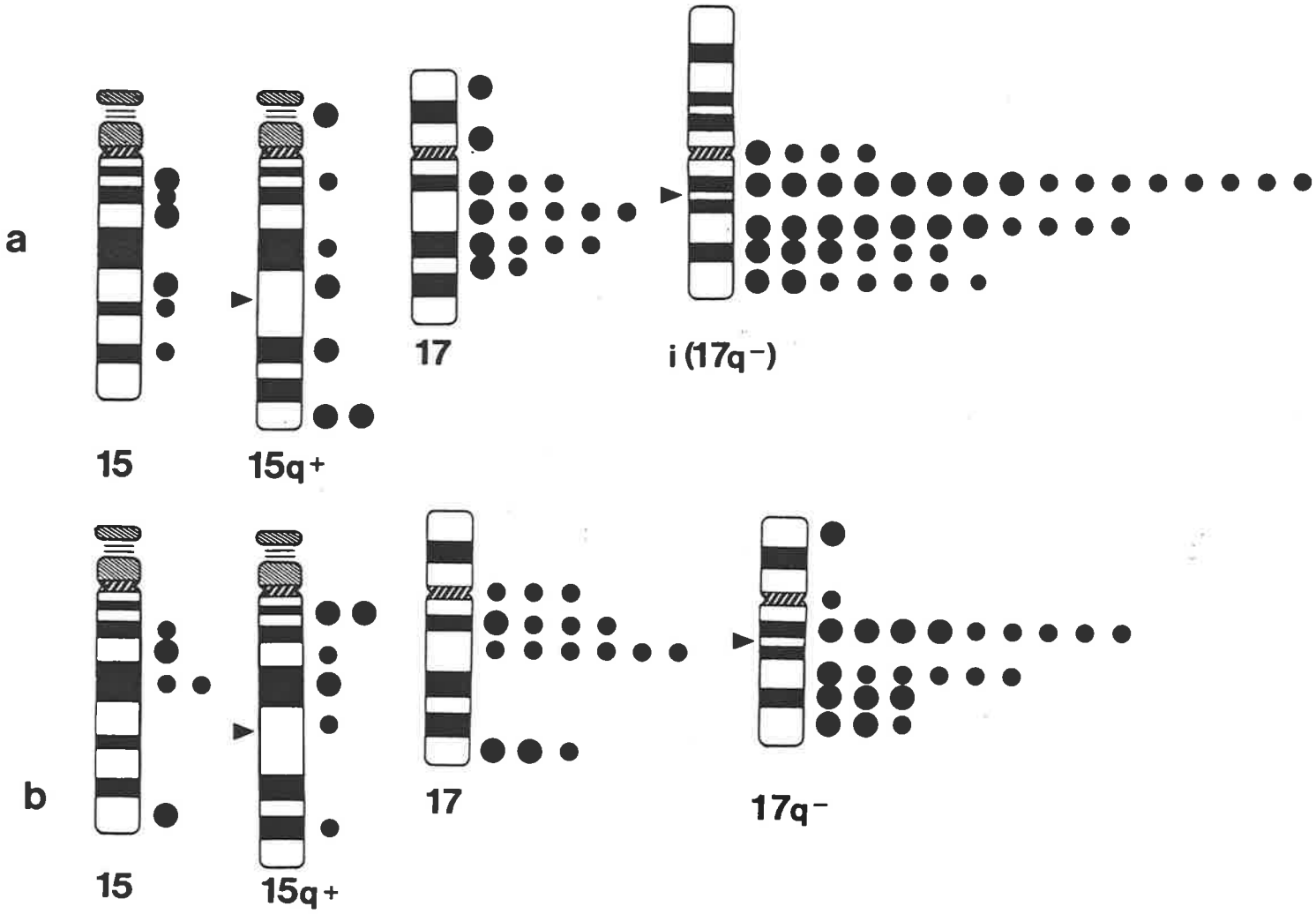
Fifty-eight metaphases and prometaphases were scored for silver grains over slide H43.2, probed with 0.4μg/ml of the *G-CSF* probe and exposed for 39 days. These metaphases had been G-banded with trypsin and photographed before *in situ* hybridization, and the silver grains were scored over the relocated metaphases. Twenty-nine (13%) of the 226 silver grains were over the long arm of chromosome 17 (fig. 1 in Simmers et al., 1987D). This represents a probability much less than 10^{-5} of random occurence (λ=3.89; $P<<10^{-5}$). A minor peak (9 grains) was seen over 4p (relative length 1.80). This peak was, however, not significant under the rules defined in chapter 2 (i.e. P 10^{-4}): λ=3.36; P=0.008

A further 98 metaphases were examined on slide H43.12. Thirteen (5%) of 251 silver grains were observed over 17q (and two over 17p). (This is less efficient labelling of these chromosomes, which were G-banded after *in situ* hybridization by the method of Zabel *et al.*, 1983). The locations of these silver grains on 17q and those from slide H43.2 are represented against 17q in figure 1, Simmers *et al.*, 1987D. There is a peak of 18½ silver grains over band 17q12, with a range over 17q11.2-17q21 encompassing the likely site of the locus.

The results of the experiment hybridizing the *G-CSF* probe to chromosomes from APL are given in figure 4.3. and in Simmers *et al.* (1987B). The slides were all hybridized *in situ* together, under the same conditions, but dipped in emulsion, developed and scored separately in this laboratory and another (see Acknowledgements). Both sets of data were combined for the purpose of analysis.

Figure 4.3.

The distribution of silver grains (large dots represent NTB-2 grains; small dots represent L4 grains) scored over the normal and derived chromosomes 15 and 17 of (a) patient SD (49 metaphases scored) and (b) patient ML (59 metaphases scored), probed for *G-CSF*. Note that in (a) two copies of the der(17) occur in the i(17q$^-$), and that about 20% of metaphases also had an extra copy of this chromosome. As it is not possible to differentiate mophologically between the two arms of the isochromosome, the label is represented on only one arm. The arrows indicate the regions of translocation.


a
15
15q+
17
i (17q−)
b
15
15q+
17
17q−

The relative lengths used for statistical analysis (calculated using ISCN, 1981) are: for individual ML, 17q = 1.01, 17cen->17q21::15q22->15qter = 0.9, 15q15->qter = 1.2, 15q15->15q22::17q21->17qter = 1.3; for individual SD, 17q = 1.01, 17cen->17q21::15q22->15qter = 0.9*2.2 = 2.0, 15q15->qter = 1.2, 15q15->15q22::17q21->17qter = 1.3, total relative length = 107.23. The levels of signal on the normal chromosome 17s obtained in this laboratory were not high, but those on the der(17)s were (fig. 4.3). Together, the data show that the *G-CSF* gene is proximal to the breakpoint on 17q (see Simmers *et al.*, 1987B; figs 4.4 and 4.5).

4.4.2.Discussion

Granulocyte colony-stimulating factor (G-CSF) was first detected in the serum of mice treated with endotoxin (Lotem *et al.*, 1980; Burgess and Metcalf, 1980). Purification of this factor (Nicola *et al.*, 1983) allowed the determination of its functional characteristics: G-CSF is predominantly a stimulator of granulocyte colony formation, and will also stimulate the initial proliferation of other haemopoietic progenitor cell types (Nicola *et al.*, 1983; Metcalf and Nicola, 1983). In contrast to other colony stimulating factors, it induces differentiation in granulocytes (Burgess and Metcalf, 1980; Metcalf and Nicola, 1982).

The second of five introns in the *G-CSF* gene includes two alternative splice donor sites, so that two different mRNAs can be generated from a common precursor RNA (Nagata *et al.*, 1986B). Southern analysis has

indicated that there is only one *G-CSF* gene in the human genome (J. Smith, personal communication; Nagata *et al.*, 1986A). Thus the site of chromosomal hybridization of the probe (on 17q) is likely to be the *G-CSF* gene.

The result localizing *G-CSF* to 17q11.2->17q22 is of particular interest in light of a specific translocation breakpoint seen in the chromosomes of APL.

A rearrangement involving chromosome 17 or 18 was observed in the chromosomes from a patient with APL (Engel *et al.*, 1967), and this was later recognized as a translocation between 15q and 17q (Okada *et al.*, 1977, Kaneko *et al.*, 1977). This rearrangement is highly specific for APL (II IWCL, 1980, IV IWCL, 1984A), and probably occurs in every case (Larson *et al.*, 1984). This leukaemia is characterized by a high proportion of abnormal promyelocytes in the bone marrow (Bennett al., 1976).

In addition to the effects of G-CSF on normal cells, this protein can induce the normal differentiation of myeloid leukaemia cells or cell lines, with loss of tumorigenicity (Lotem *et al.*, 1980; Metcalf, 1980; Metcalf and Nicola, 1981; Nicola *et al.*, 1983). In particular, recombinant human G-CSF was found to cause differentiation of APL cells into mature cells (Souza *et al.*, 1986). It therefore seemed likely that the t(15;17) occurs at or near the *G-CSF* gene, so that a decrease in the normal level of G-CSF causes reduced differentiation and increased proliferation of myeloid cells, resulting in leukaemia, characterized by

immature cells. Analogous to this possibility, GM-CSF, which has a major role in cell proliferation rather than differentiation, has been found to be expressed constitutively in some leukaemias, causing proliferation by autocrine stimulation (Lang *et al.*, 1985; Young *et al.*, 1987). Also, rearrangement of *G-CSF* was observed in a squamous carcinoma cell line, (not of haemopoietic origin), but there was constitutive expression, rather than decreased expression, of G-CSF by this cell line (Nagata *et al.*, 1986).

To determine whether the t(15;17) occurred through the coding region of the *G-CSF* gene, the probe was hybridized *in situ* to metaphases from two patients with APL, one with the t(15;17), and one with the variant iso(17q-) (Kondo and Sasaki, 1979; IV IWCL, 1984A).

The results indicated that the *G-CSF* gene remains on the derived chromosome 17. There was no significant hybridization to the derived chromosomes 15. A translocation with a breakpoint near the end of the region homologous to the probe might not leave enough DNA for hybridization to one of the resulting pieces; nor would a breakpoint outside the region of probe homology be detected by this method. However, further evidence that the *G-CSF* gene is not involved comes from Southern analysis of DNA from APL cells with the t(15;17). No rearrangement of the gene or the surrounding DNA has beeen found (M.F. Shannon, personal communication, see Simmers *et al.*, 1987B).

There has been considerable controversy over the assignment of the breakpoints for the t(15;17) specific to APL. This controversy has been

attributed to the similarity of the bands in this region on the two chromosomes involved and the generally poor morphology of bone marrow chromosomes (Rowley, 1984; Testa, 1984). While some assignments of the 17q breakpoint included 17q22 (Golomb *et al.*, 1979; II IWCL, 1980) and 17q12 (Kondo and Sasaki, 1979; Ohyashiki *et al.*, 1985A), many authors have favoured a localization at the proximal end of band 17q21 (e.g. Okada *et al.*, 1977; IV IWCL, 1984A; Larson *et al.*, 1984), most having noted the possibility of another interpretation. Misawa *et al* (1986) were able to analyse the breakpoints of a variant APL translocation, where analysis was easier because of the dissimilarity of the bands in the two regions involved. They assigned the breakpoints of the t(15;17) to 15q22 and 17q21.1.

Rowley *et al.* (1977) suggested the use of the t(15;17) in APL for regional mapping of genes on chromosome 17. More recently, this has come to realization. Sheer *et al.* (1983) utilized this translocation to further define the regional localizations of a number of genes by Southern analysis of somatic cell hybrids.

Mitelman *et al.* (1986) analysed the t(15;17) of APL, as well as an apparently identical constitutional t(15;17). Both were interpreted to be t(15;17)(q22.3;q11.2), with breakpoints more proximal than previous interpretations. The photographs of chromosomes 15 and 17 given in figure 1 (Mitelman *et al.*, 1986), being of high resolution, are convincing, but it is not indicated whether the chromosomes are from the constitutional translocation or the APL translocation. The constitutional t(15;17) and that of APL are not identical, as the c-*erb*A

locus maps between the two breakpoints on 17q (Dayton *et al.*, 1984; Sheer *et al.*, 1985); the localization of both breakpoints was used as evidence that this oncogene maps to 17q11.2 (MItelman *et al.*, 1986).

A number of genes localized near the 17q breakpoint of APL have been considered likely to be involved in this rearrangement, and have been localized with respect to the breakpoint by *in situ* hybridization in the process of assessing whether they were involved in the translocation. Thus, c-*erb*A was localized proximal to the breakpoint on 17q (Dayton *et al.*, 1984; Le Beau *et al.*, 1985B), as was c-*erb*B-2 (Kaneko *et al.*, 1987). The nerve growth factor receptor gene (*NGFR)* is also on 17q, from 17q12->q22 (Huebner *et al.*, 1986). Neither *NGFR* nor the other oncogenes mentioned above were found to be rearranged in cells with the APL translocation (Huebner *et al.*, 1986; Le Beau *et al.*, 1985B; Sheer *et al*, 1985; Fukushige *et al.*, 1986). There are more oncogenes that have been mapped to the region of the 17q breakpoint in APL (Gosden *et al.*, 1986). As noted (Le Beau *et al.*, 1985B; Sheer *et al.*, 1985; Kaneko *et al.*, 1987), the translocation might still occur through a region close to any of these genes, or close to the *G-CSF* gene, causing a change in the level of expression of the gene. Ideally, Northern analysis would be used to give an indication of whether this is so (Kaneko *et al.*, 1987).

Another leukaemia translocation involving 17q, t(17;21)(q21;q22), was used for further localization of *NGFR*, distal to the breakpoint on 17q (Huebner *et al.*, 1986). The authors have argued that this translocation breakpoint on 17q is identical at the level of the DNA from the

cytogenetically indistinguishable t(15;17) of APL, and that *NGFR* is therefore also distal to the 17q breakpoint in APL. Yet it has not been shown that the two breakpoints are identical. *NGFR* is therefore not necessarily distal to the breakpoint in APL, although there is no evidence to suggest that the breakpoints are not the same. Dayton *et al.* (1984) found the c-*erb*A oncogene to be proximal to both of these breakpoints on 17q.

The results of *in situ* hybridization to normal chromosomes, of the probes which are proximal to the APL breakpoint on 17q, resulted in grain peaks over or distal to 17q12 (present results; Dayton *et al.*, 1984; Fukushige *et al.*, 1986). Unless the morphology of the chromosome bands has influenced a more distal assignment of the silver grains than occurred, Mitelman *et al.*'s (1986) assignment of the APL 17q breakpoint and c-*erb*A to 17q11.2 is likely to be incorrect.

The clustering of a number of genes controlling cellular development near the 17q breakpoint in APL has prompted a number of studies investigating their involvement in this cancer. Although no gene has yet been shown to be involved, including another oncogene, c-*fes*, which is distal to the breakpoint on 15q (Sheer *et al.*, 1983) there is presumably some sequence of importance to either cellular growth or differentiation, or to a function typical of the normal cell type, on 17q, the expression or structure of which is affected by the rearrangement in APL (Rowley, 1984). Rearrangement, amplification, and elevated expression of the c-*erb*B-2 oncogene (see above) have been

found, not in cells from APL, but in a solid tumour cell line (Fukushige *et al.*, 1986).

The critical recombinant, defined as that conserved in a number of more complex rearrangements (variants of the simple t(15;17)), is apparently the der(15), suggesting that the DNA sequences important to the APL phenotype are actually distal to the breakpoint on chromosome 17 and proximal to that on chromosome 15 (Le Beau *et al.*, 1985B; Misawa *et al.*, 1986). If this is so, none of those genes mentioned above, which have been suggested to contribute to the development of APL, would be involved in the critical rearrangement, because they are all proximal to the 17q breakpoint. Several variant translocations have also been observed in APL, which appear to involve the same chromosome 17 breakpoint, without the involvement of chromosome 15 (De Braekeleer, 1986A). This supports the possibility that the region proximal to the breakpoint on 17q contains a sequence important to the resultant APL phenotype. However, it is possible that 15q was in fact involved in these variant rearrangements, but that this was difficult to detect (de Braekeleer, 1986A).

While the results of hybridization to the translocated chromosomes did not define the localization of *G-CSF* more precisely, they did reveal that the locus is proximal to the breakpoint in the t(15;17). Together with Southern analysis results, the gene was shown probably not to be involved in this translocation. The possibility of translocation through a regulatory sequence adjacent to the coding region, however, has not been excluded.

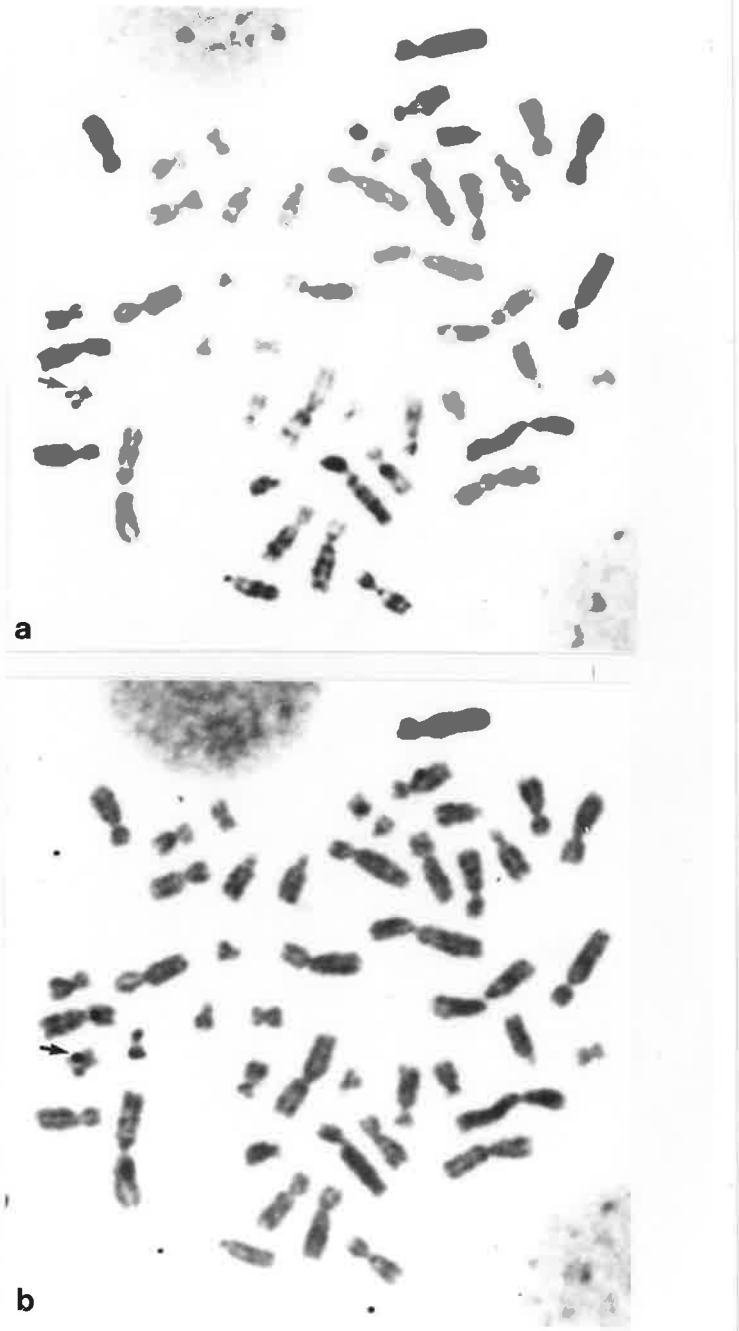


Figure 4.4.

A metaphase from patient ML both before (a) and after (b) *in situ* hybridization. Labelled site of the *G-CSF* gene is indicated (large arrow on der(17)).

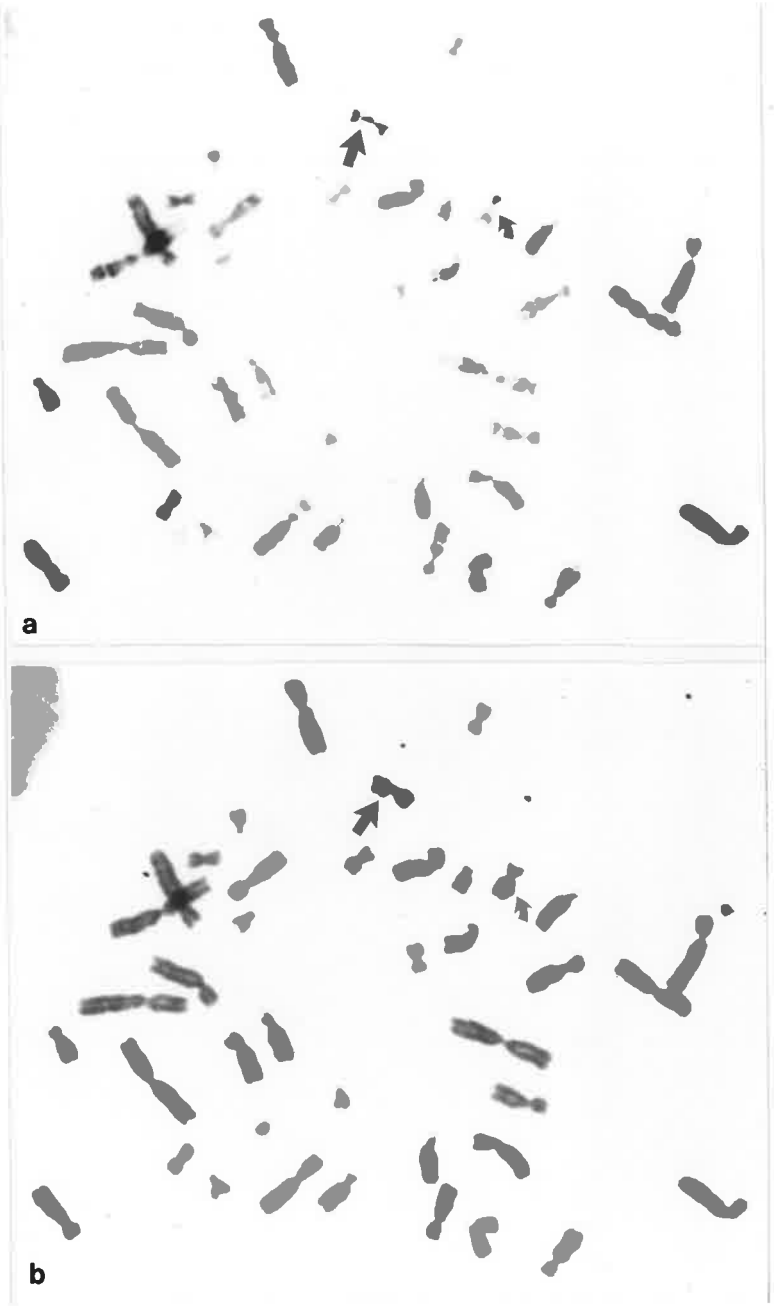


Figure 4.5.

A metaphase from patient SD both before (a) and after (b) *in situ* hybridization. Labelled sites of the *G-CSF* gene are indicated (large arrow on der(17) and small arrow on the normal 17).

4.5.SUMMARY AND CONCLUSIONS

In situ hybridization was used to make two original gene localizations. The *TNFA* gene was mapped to 6p21->p22, the region of the major histocompatibility complex. The *G-CSF* gene was mapped to 17q11.2->q21, and was found to be proximal to the breakpoint in the t(15;17) of acute promyelocytic leukaemia.

CHAPTER 5

MAPPING OF CHROMOSOME 16

5.1. INTRODUCTION

Chromosome 16 has four known fragile sites, two common and two rare, all expressed under different culture conditions, as defined by HGM8 (see fig. 5.1) (Berger *et al.*, 1985). The research programme of which this project is a part has a primary interest in fragile sites, and is therefore particularly interested in chromosome 16. This chapter will describe experiments regionally localizing DNA sequences on this chromosome, with particular reference to these fragile sites. A greater understanding of the map of chromosome 16 is thereby obtained, helping to give direction to methodologies for eventual understanding of fragile site DNA.

Another significant advantage in using chromosome 16 for *in situ* hybridization is that it is easily identifiable without chromosome banding.

A number of DNA probes were used to further localize sequences already known to be on chromosome 16, by *in situ* hybridization. The chromosome was divided into smaller, precisely defined regions, by the use of chromosome markers, both fragile sites and translocation breakpoints. These experiments will be reported in this chapter.

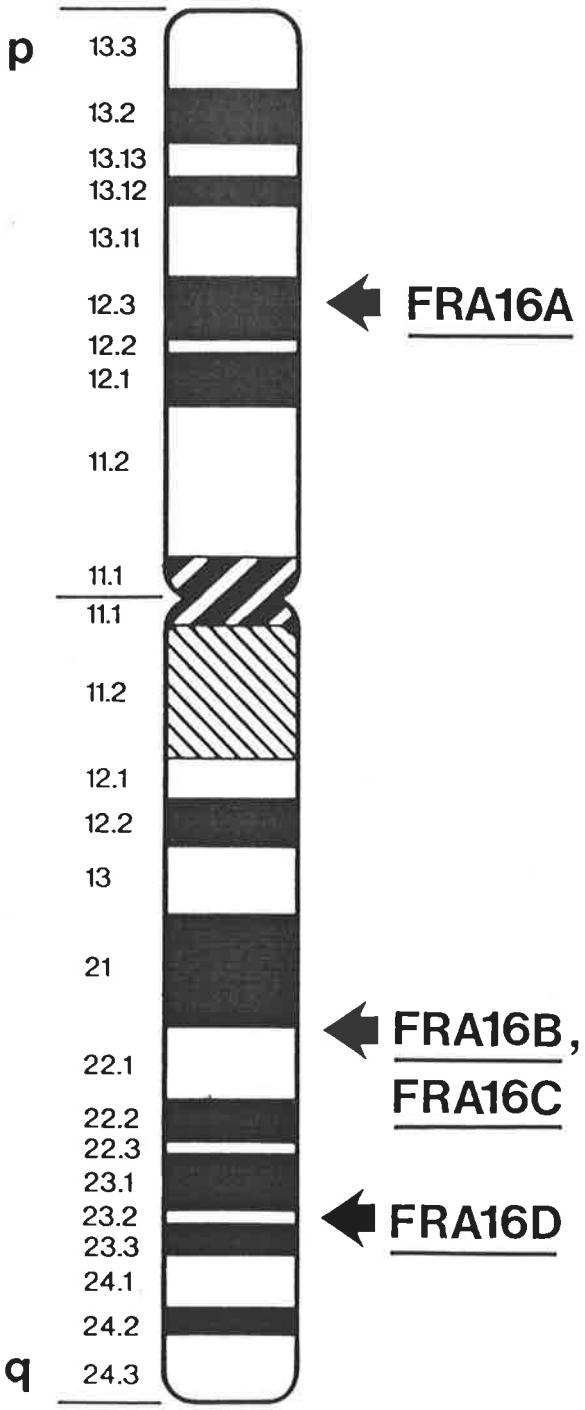


Figure 5.1.

Chromosome 16 showing the four fragile sites. The fra(16)(p12) locus, *FRA16A*, two fra(16)(q22) loci, *FRA16B* (rare) and *FRA16C* (common), and the fra(16)(q23) locus, *FRA16D* are indicated.

5.1.1.fra(16)(p12): *FRA16A*

This fragile site has been reported three times (Sutherland and Hecht, 1985): originally by Day *et al.* (1967) as "fragmentation" of the short arm of chromosome 16 of one individual, showing varying degrees of aberration. It was described as a heritable fragile site present in a large pedigree by Sutherland (1979B). Sutherland and Hecht (1985) also described this fragile site in two siblings. fra(16)(p12) is a rare folate sensitive fragile site (Sutherland, 1979A), at 16p12.3 (Sutherland and Hecht, 1985).

The HGM8 (1985) nomenclature for fragile site loci defines fra(16)(p12) as *FRA16A* (Berger *et al.*, 1985).

5.1.2.fra(16)(q22): *FRA16B*

There are two fragile sites at 16q22, therefore they will be distinguished by using HGM8 (1985) nomenclature: *FRA16B* and *FRA16C* (Berger at al., 1985).

FRA16B is one of two rare, distamycin A inducible fragile sites (Sutherland and Hecht, 1985). It was first described by Magenis *et al.* (1970). Hecht *et al.* (1971) found it to be on average 76% of the way along 16q, so they designated it 16qh(.76). Chromosome banding allowed more precise localization, to the junction of bands 16q21 and 16q22, using both G-banding (Magenis and Chamberlin, 1979) and R-banding (Croci, 1983). This location is termed 16q22.1 under ISCN (1981) rules.

Differences in terminology of this fragile site (fra(16)(q21) or fra(16)(q22)) have probably resulted from the use of different banding techniques (Croci, 1983).

Expression of *FRA16B* can be induced or enhanced by the addition of distamycin A (Schmid *et al.*, 1980) or other AT-specific DNA ligands (Sutherland *et al.*, 1984; Schmid *et al.*, 1986), or BrdU (Croci, 1983) to the culture medium.

5.1.3.fra(16)(q22): *FRA16C*

This common fragile site was first reported among a total of 51 common fragile sites observed after induction with fluorodeoxyuridine (FdU), with enhancement by the inclusion of caffeine (Yunis and Soreng, 1984). This set of fragile sites is an expansion of that seen by Glover *et al.* (1984) after treatment with aphidicolin (see below).

FRA16C is also localized to the junction of bands 16q21 and 16q22, defined as 16q22.1 (Yunis and Soreng, 1984). Yunis and Soreng (1984) suggested that the rare fragile site *FRA16B* is a mutation of *FRA16C*.

5.1.4.fra(16)(q23): *FRA16D*

This is one of the common fragile sites, originally seen at low frequencies under conditions of thymidylate stress (Glover *et al.*, 1984; Sutherland and Hecht, 1985). Glover *et al.* (1984) found that aphidicolin, which is an inhibitor of DNA polymerase α (Lönn *et al.*,

1983), is a reliable inducer of these fragile sites. fra(16)(q23) is the second most frequently expressed of this set of fragile sites, after fra(3)(p14) (see Glover *et al.*, 1984). It is localized at 16q23.2 (Yunis and Soreng, 1984; Berger *et al.*, 1985).

The HGM8 nomenclature for the fra(16)(q23) locus is *FRA16D* (Berger *et al.*, 1985).

5.2.MATERIALS AND METHODS

5.2.1.Probes

ζ-globin gene (HBZ)probe. See chapter 3, section 3.2.1.

α-globin gene (HBA) probe. The probe JW101 was used. This contains an *HBA* cDNA insert of about 800bp, including a full α-globin coding region (Wilson *et al.*, 1978).

pα3'HVR.64 probe. This probe is from the hypervariable region 3' to the α-globin gene complex (Higgs *et al.*, 1981). It is a 4.0kb *Hinf*I fragment from pSEAI (Nicholls *et al.*, 1985), inserted into the plasmid pSP64 by blunt end ligation (S.T. Reeders, personal communication).

Haptoglobin gene probe (HP). This probe, pULB1148, consists of a 1.4kb cDNA insert, including the coding regions for the α2FS and β chains of haptoglobin, a signal peptide and a non-coding tail, in pBR322 (vander Straten, 1983).

Adenine phosphoribosyltransferase gene (APRT) probe. The *APRT*-specific probe is a subclone of the 2.2kb *Bam*HI repeat-free fragment from the genomic clone λHuap15, containing most of the *APRT* gene (Stambrook *et al.*, 1984; P.J. Stambrook, personal communication) in the plasmid M13, which had been made double-stranded.

Metallothionein gene (MT) probes. (See fig. 5.2). Three probes were used. The first, in a plasmid vector, contains about 150bp of cDNA homologous to almost the entire coding region of *MT2A*, as a *Bam*HI-*Hin*dIII fragment (Karin and Richards, 1982B). It cross-reacts with all the members of the *MT* gene family, because of their high sequence homology. (R.I. Richards, personal communication). This probe will be referred to as *MT2A*r ("r" for repeated).

The expression vector pHSI consists of about 770 bp of DNA from the 5′ flanking region of *MT2A* in pUC13 (A.K. West, personal communication; Karin and Richards, 1982A.) The probe specific for *MT1B* consists of an 800 bp *Hin*dIII-*Bam*HI insert from the 5′ flanking region of the *MT1B* gene in pUC13. (A.K. West, personal communication; Heguy *et al.*, 1986.) These clones probe specifically for *MT2A* and *MT1B* respectively (A.K. West, personal communication) and will be referred to as *MT2A*u and *MT1B*u respectively ("u" for unique).

ACH207 (D16S4) and ACH224 (D16S5) probes. These probes were isolated from a flow sorted library (Hyland et al., 1987B). The λCharon21A vector (41.7kb) contains a repeat-free

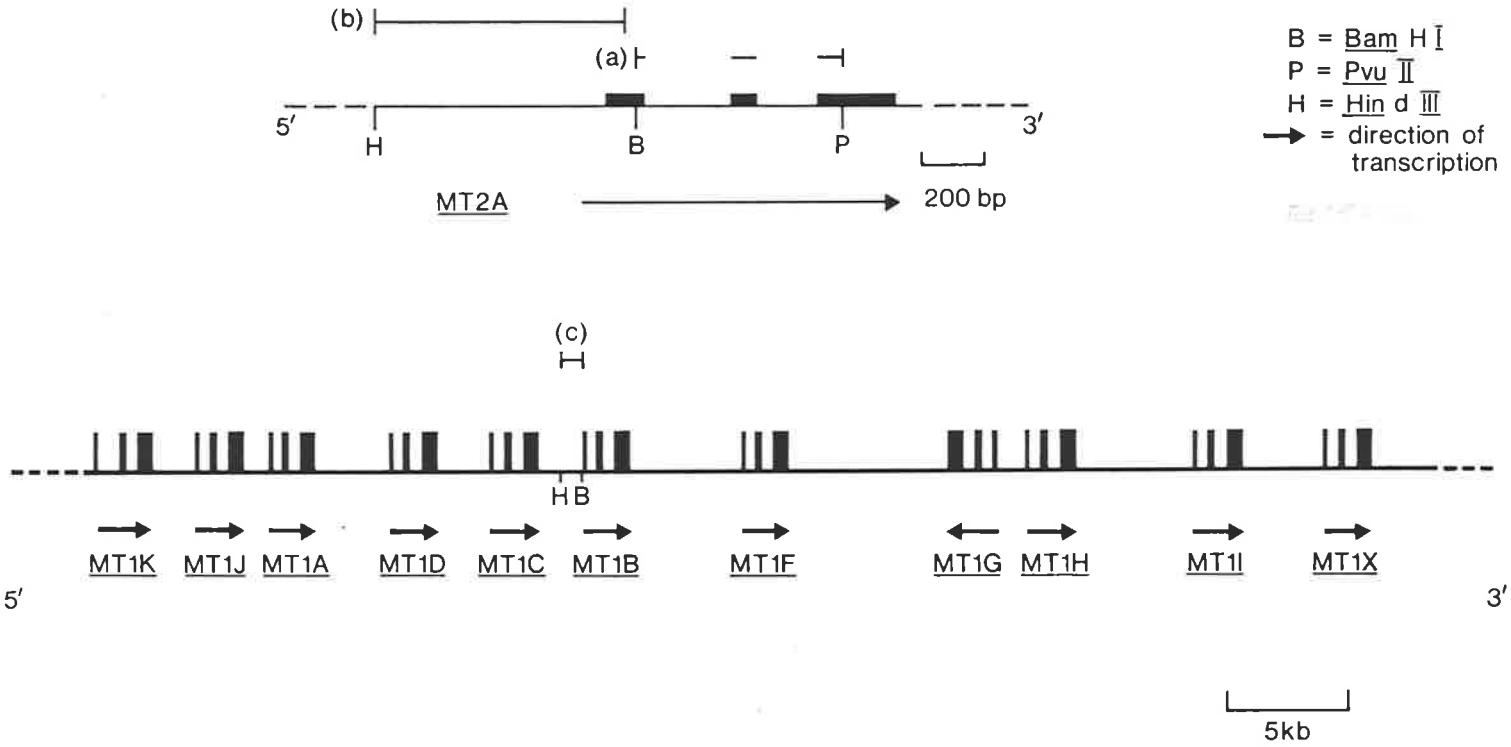


Figure 5.2.

A diagram showing the organization of the metallothionein gene cluster, and the regions from which the three probes described here were taken: (a) MT2Ar; (b) MT2Au; (c) MT1Bu. (R.I. Richards and A.K. West, personal communication).

insert, at the *Hin*dIII cloning site, in both cases. The insert of ACH207 is 4.7kb, and that of ACH224 is 5.0kb (Hyland *et al.*, 1987B).

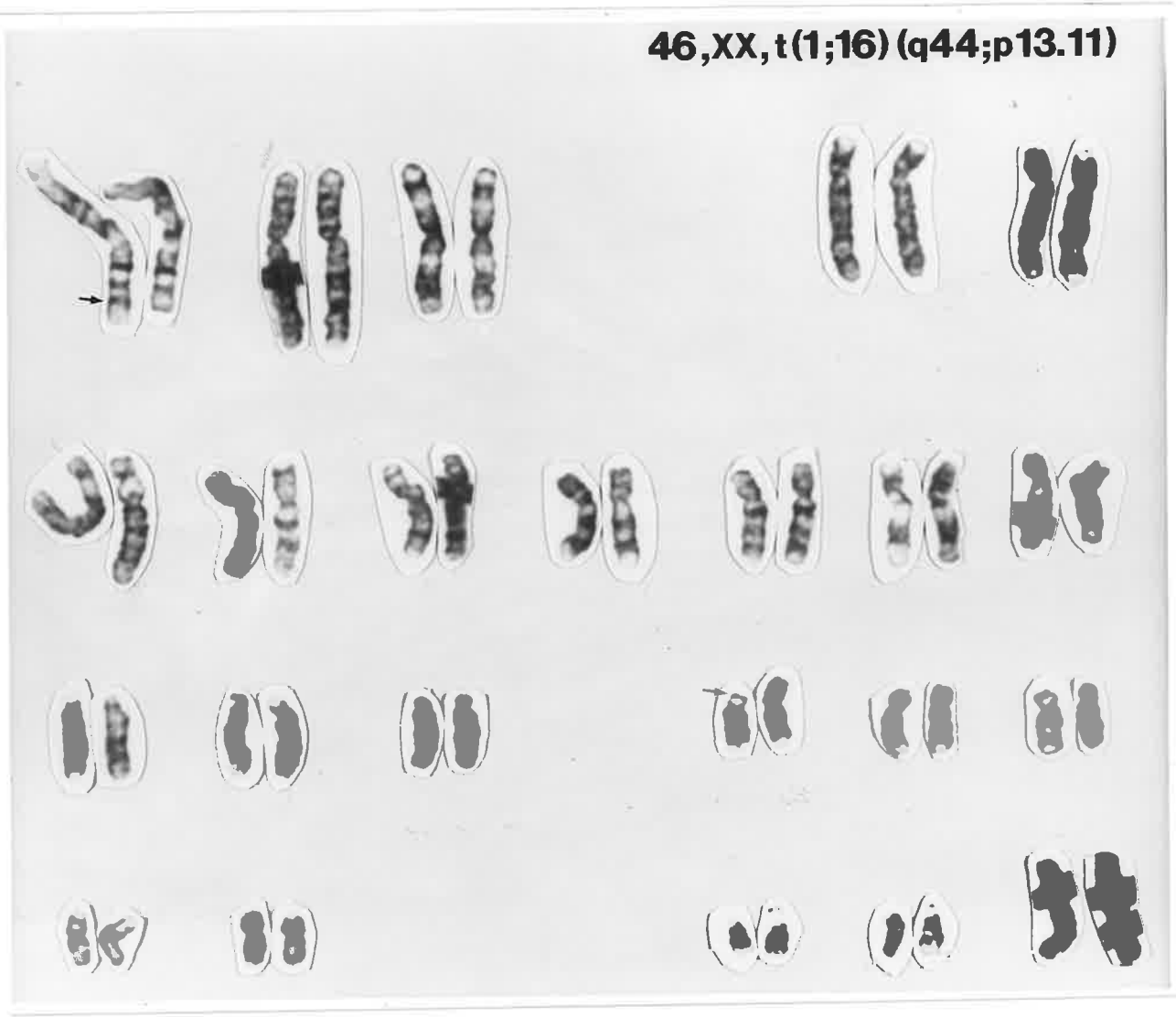
5.2.2.*In situ* hybridization

The above probes were hybridized *in situ*, using the methods described in appendix AI, to chromosomes expressing fragile sites, and to cell lines with constitutional chromosome translocations, in order to obtain narrower regional localizations for the loci represented by the probes, and to determine where they mapped relative to certain nearby fragile sites on chromosome 16. The experiments carried out towards this aim are summarized below. Each probe was also hybridized to normal chromosomes. These slides were dipped at the same time as the test slides, and developed sequentially to determine the appropriate period of exposure (see chapter 3). The metaphases, including those expressing fragile sites, were produced by cell culture and harvest as described in appendix AI.1-3, using the chromosomes described in appendix A.II.

HBA and HBZ. The *HBA* and *HBZ* probes were hybridized to chromosomes expressing the fragile site, fra(16)(p12) (see appendix AIII.6). These probes, and the probe from the α3'HVR were also hybridized to chromosomes from the lymphoblastoid cell lines GM2324 and GM6227 (from the Human Genetic Mutant Cell Repository (Camden, New Jersey)), which both have translocations involving 16p (see figure 5.3(a), (b) and appendix AII for karyotypes), as described (appendix AIII.7). Trypsin G-banded metaphases from the two cell

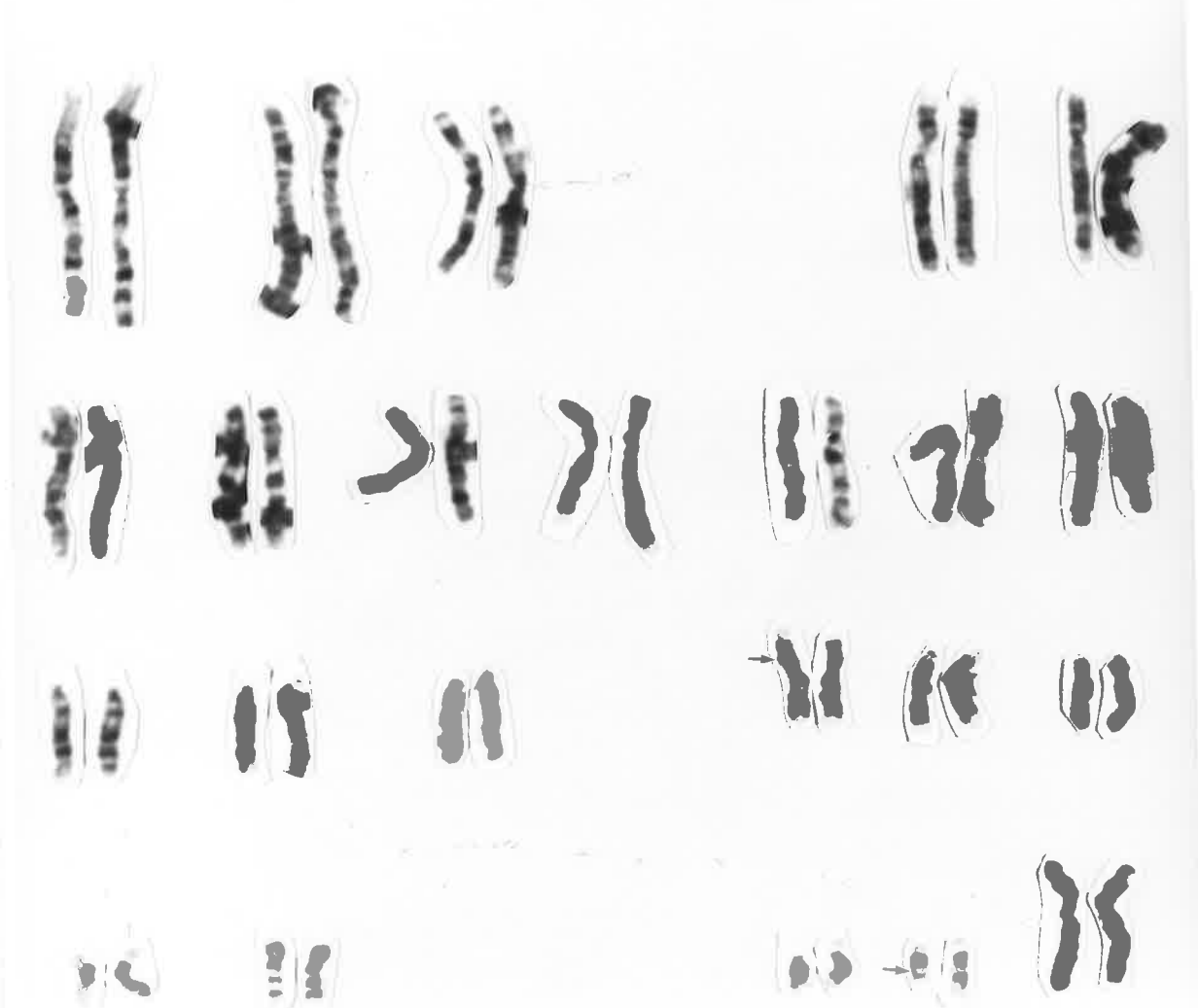
Figure 5.3.

(a) Karyotype of the cell line GM6227. (b) Karyotype of the cell line GM2324. Arrows indicate the sites of translocation.



a

46,XX,t(16;22)(p13.11;q11.21)



b

lines were photographed before *in situ* hybridization, and relocated for scoring after *in situ* hybridization.

HP. The *HP* probe (pULB1148) was hybridized to chromosomes expressing *FRA16B*, from two individuals, as described in appendix AIII.8. Nick translated pBR322 was hybridized to the same chromosomes, and at the same concentrations, as was pULB1148, as a control for hybridization with respect to the fragile site.

APRT. The *APRT* probe was hybridized initially to chromosomes expressing *FRA16B*, as indicated by previous reports localizing this gene to 16q (Barg *et al.*, 1982; Lavinha *et al.*, 1984). It was then also hybridized to chromosomes expressing fra(16)(q23), from two individuals. These experiments are outlined in appendix AIII.9.

MT. The three metallothionein gene probes were hybridized *in situ* to chromosomes expressing *FRA16B* and *FRA16C*, as described in appendix AIII.10. The *MT2A*r probe was hybridized to chromosomes from only one individual, in which expression of *FRA16B* had been induced. The two unique probes, *MT1B*u and *MT2A*u, were hybridized to chromosomes from fifteen unrelated individuals, which expressed this same fragile site, *FRA16B*. These two probes were also hybridized to chromosomes from one individual, in which treatment with FdC (see appendix AI.2) had induced the expression of the common fragile sites (Yunis and Soreng, 1984), including *FRA16C*.

Before *in situ* hybridization to chromosomes expressing *FRA16C*, the common fragile sites were induced in a number of individuals and examples of *FRA16C* examined, and compared to examples of *FRA16B*. In addition, chromosomes from an individual heterozygous for *FRA16B* (F41A, see appendix AII) were harvested after concurrent treatment with 50mg/l BrdU and either 0.005mM FdU, 0,05mM FdU, 0.005mM FdC or 0.05mM FdC, and caffeine (as described in appendix AI.2), and examined for the simultaneous expression of *FRA16B* and *FRA16C*..

As examples of *FRA16C* are difficult to locate and identify, slides spread with these chromosomes were examined before *in situ* hybridization, the co-ordinates of cells with one or two examples of *FRA16C* noted, and relevant details of the cell (the location of this chromosome in relation to others) drawn. Re-location was simple and where the morphology of the chromosomes had deteriorated, the identification of metaphases expressing *FRA16C* was not hampered.

A gap near the middle of band 16q21 was often seen in banded chromosomes where the common fragile sites have been induced with caffeine and either FdU or FdC (see fig. 5.10). This could be distinguished from *FRA16C* in the unbanded chromosomes used for *in situ* hybridization, as the chromosomes of the individual used had only a small amount of heterochromatin on both 16qs, making this gap distinctively higher on 16q than *FRA16C*. The region of 16q proximal to this gap was therefore noticeably shorter than that distal to the gap, in contrast to the regions of 16q either side of *FRA16C*, which were approximately equal in

this individual. Another gap was often observed above band 16q21, at 16q12-q13 (see figure 5.10).

ACH207 and ACH224. The two probes for RFLPs from chromosome 16, ACH207 and ACH224, were hybridized to chromosomes expressing *FRA16B* (appendix AIII.11).

All chromosomes used for *in situ* hybridization, in which fragile sites had been induced, were solid stained, both before (if pre-localizing) and after, to avoid distortion of the chromosome and obliteration of the fragile sites. Some fragile sites escape detection after trypsin G-banding, as this tends to swell the chromosomes, resulting in the closing of the gap. This is particularly important in the case of *FRA16C*, which in most cases was expressed as a narrow, single chromatid gap, which might easily be concealed. In addition, fragile sites can be made difficult to detect when flanked by one or two pale bands, as in the cases of *FRA16B* and *FRA16C* (see Hecht and Sutherland, 1984, for discussion).

5.3.RESULTS

The results of the above experiments are given in detail in appendix III: AIII.6-11. In the majority of cases, several slides were scored for grains with respect to the fragile site, as described in section 2.5. Thus, the results given in appendix AIII are more extensive than those published (Fratini *et al.*, 1986; Simmers *et al.*, 1986A; 1987A, C).

HBA gene complex. Both probes for the α-globin gene complex, JW101 (*HBA*) and pHPζ (*HBZ*), hybridized with a significant excess of silver grains distal to fra(16)(p12) (see appendix AIII.6). A significant excess of grains was revealed distal to fra(16)(p12) for the probe to the *HBA* genes (JW101), at concentrations from 0.1μg/ml to 0.8μg/ml (slides H23.5-H12.8). There was also a significant excess of silver grains distal to fra(16)(p12), over metaphases from individual F2ZA, probed for *HBZ*, with pHPζ, at concentrations from 0.8μg/ml to 2.1μg/ml (slides H16.12A-H16.15A). The slides which did not show a statistically significant excess of silver grains on either side of the fragile site did however reveal the same trend towards an excess on the distal side. (See fig. 5.4.)

The silver grains over the acentric fragment distal to fra(16)(p12) were scored according to their position with respect to the fragile site and the telomere. The results are given in Simmers *et al.* (1987C). A significant excess of grains was closer to 16pter than to fra(16)(p12).

Silver grains from 59 metaphases from the cell line GM6227, on slide H24.5, probed with 0.4μg/ml JW101, were scored. (See chapter 2 for an explanation of the following symbols.) For 16p: S=14=r, B=242, λ=1.53, $P<<10^{-5}$. For 1q32->1q44::16p13.11->16pter: S=11=r, B=245, λ=2.8, $P=2*10^{-4}$. There were no silver grains over the short arm of the der(16). Silver grains from 45 metaphases from the same cell line, on slide H24.6, probed with 0.8μg/ml JW101, were scored. For 16p: S=2=r, B=169, λ=1.07, P=0.35, n.s. For 1q32->1q44::16p13.11->16pter: S=16=r, B=155, λ=1.8, $P<<10^{-5}$.

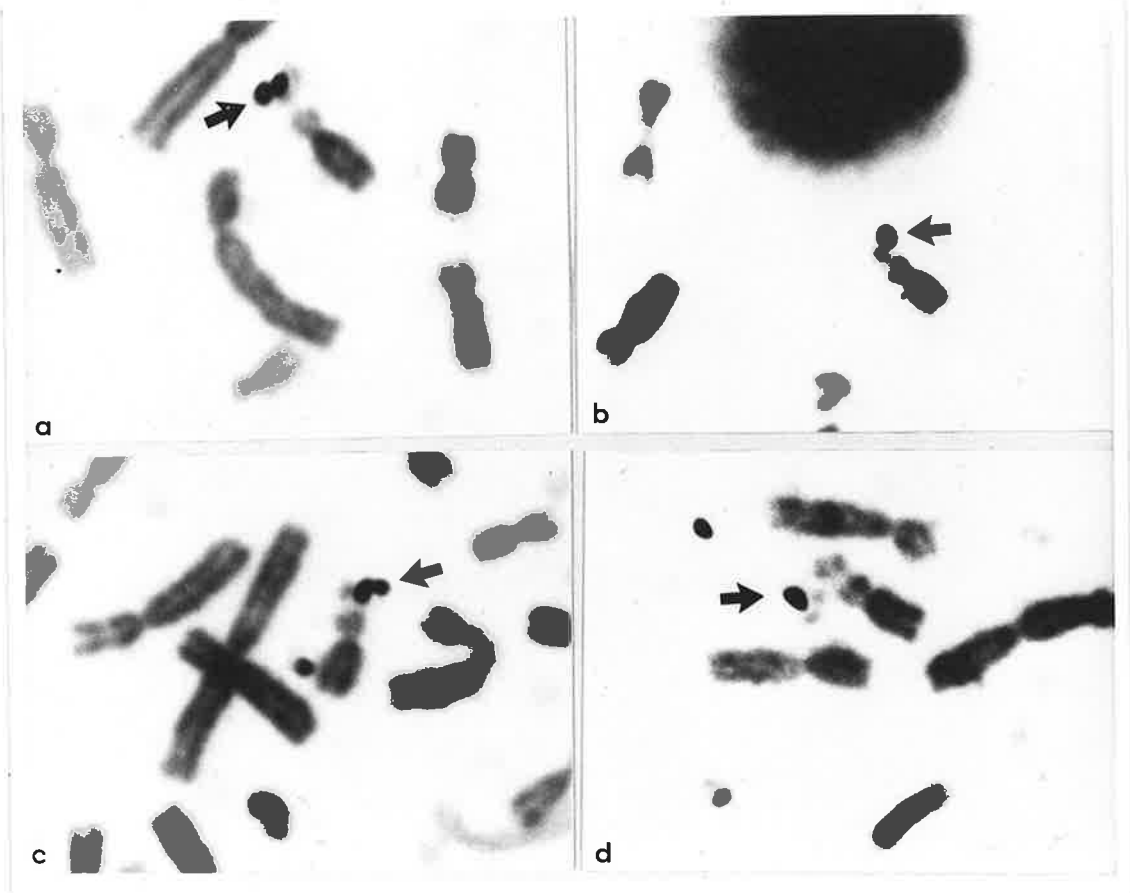


Figure 5.4.

Examples of fra(16)(p12) probed for *HBA* and *HBZ*, showing silver grains (arrowed) located distal to the fragile site.

There were no silver grains over the short arm of the der(16). (Relative length of 16p = ½*1.33; relative length of 1q32->1q44::16p13.11->16pter = 1.2)

The *HBA* probe hybridized to the normal 16p of GM6227, and to the derived 1q+, at 0.4μg/ml (fig. 5.5(a)), indicating that the two *HBA* genes are distal to the translocation breakpoint on 16p. Metaphases from this cell line probed with 0.8μg/ml of this probe also showed an excess of silver grains on 1q+, but no excess on 16p (fig. 5.5(b)).

Silver grains over chromosomes from the cell line GM2324, from 28 metaphases on slide H36.6 and 13 metaphases on slide H36.7, probed with 0.4μg/ml JW101, were scored. For 16p: S=11=r, B=381, λ=2.41, P=4*10^{-5}. For 16cen->16p13.11::22q11.21->22qter: S=10=r, B=382, λ=3.6, P=4*10^{-3}. For 22cen->22q11.21::16p13.11->16pter: S=12=r, B=380, λ=1.4, P<10^{-5}. Silver grains were scored from the same cell line, from 40 metaphases on slide H36.15, probed with 0.4μg/ml pHPζ. For 16p: S=15=r, B=531, λ=3.36, P<10^{-5}. For 16cen->16p13.11::22q11.21->22qter: S=6=r, B=540, λ=5.1, P=0.4, n.s. For 22cen->22q11.21::16p13.11->16pter: S=12=r, B=534, λ=2.0, P<10^{-5}. Silver grains from the same cell line, from 43 metaphases on slide H44.12, 25 metaphases on slide H44.13 and 34 metaphases on slide H44.14, probed with 0.2μg/ml pα3'HVR.64, were scored. For 16p: S=16=r, B=466, λ=2.94, P<10^{-5}. For 16cen->16p13.11::22q11.21->22qter: S=3=r, B=479, λ=4.6, P=0.8; n.s. For 22cen->22q11.21::16p13.11->16pter: S=8=r, B=474, λ=1.8, P=1*10^{-4}. (Relative length of 16p = ½*1.33; relative length

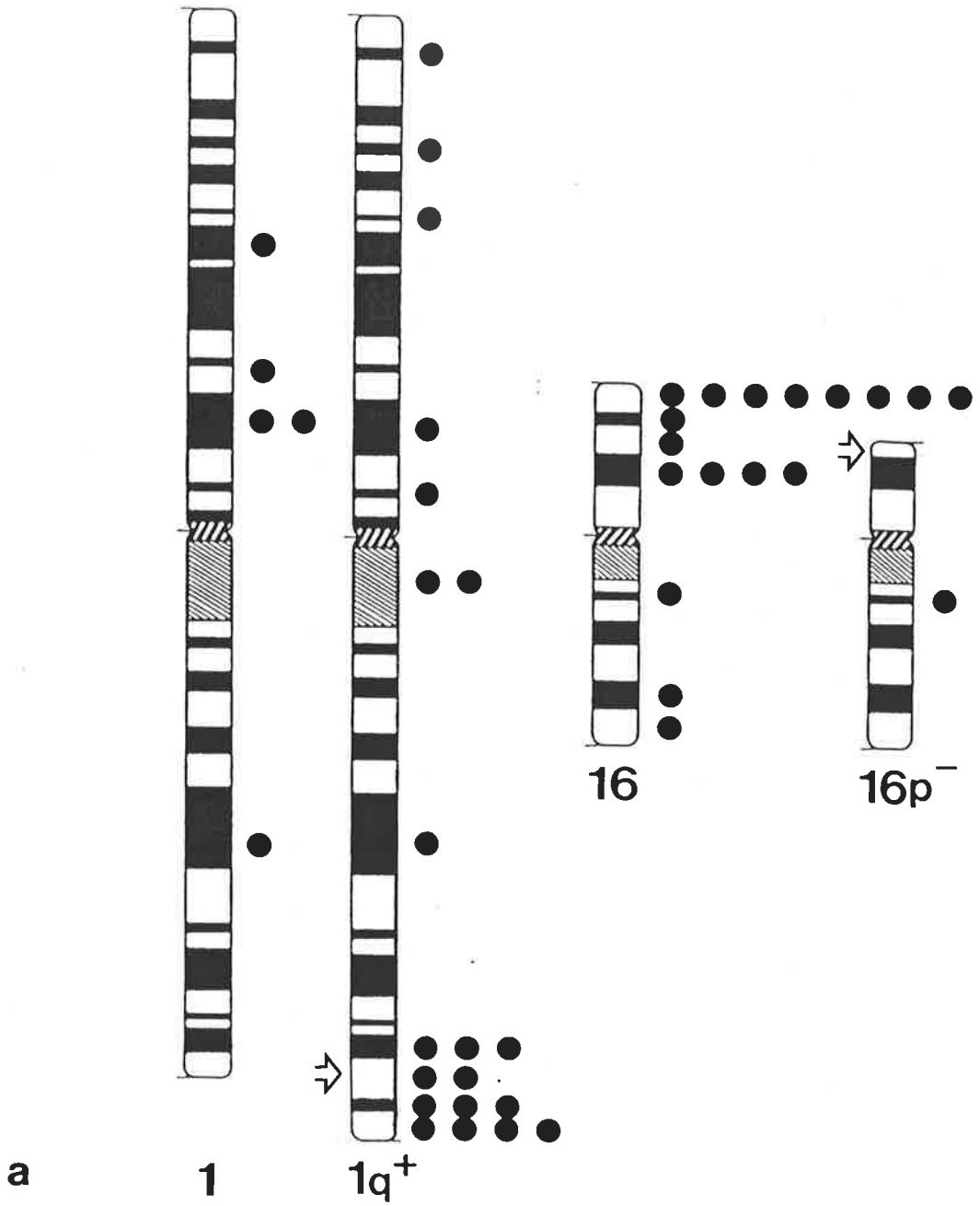
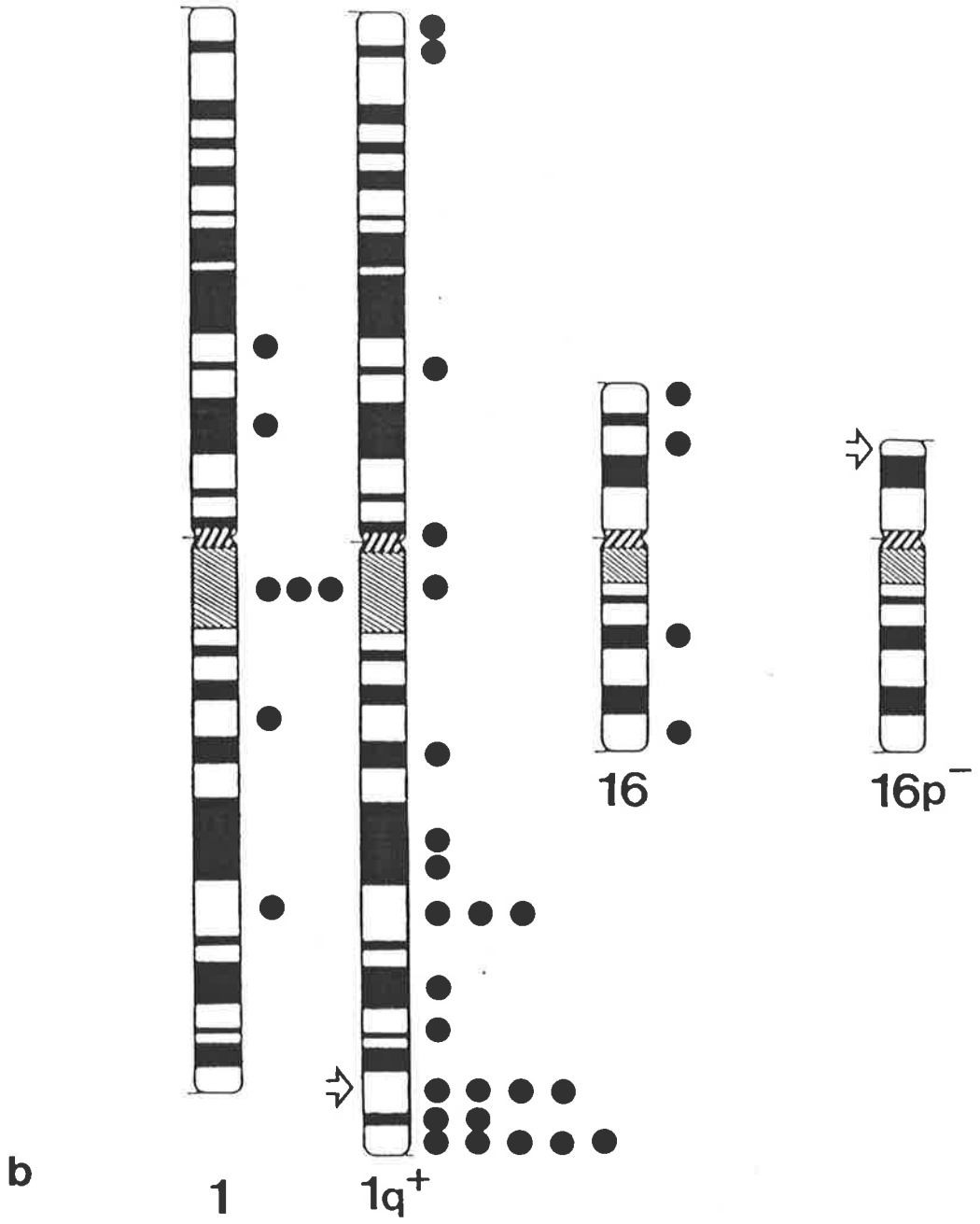


Figure 5.5.

(a)-(b) Silver grains scored over the normal and derived chromosomes 1 and 16 from the cell line GM6227, probed with (a) 0.4μg/ml JW101 and (b) 0.8μg/ml JW101.


b
1
1q+
16
16p−

of 16cen->16p13.11::22q11.21->22qter = 1.0; relative length of 22cen->22q11.21::16p13.11->16pter = 0.4.).

(JW101 and pHPζ respectively)

Both the *HBA* and *HBZ* probes were hybridized to metaphases from the cell line GM2324 (fig. 5.6(a) and (b)). Grain levels significantly above background were observed over 16p and 22q- for both probes, and also over the short arm of the der(16) for the slide probed for *HBA*. These results seemed to indicate that the breakpoint in the t(16;22) occurs within the region defined by the α-globin genes. If this is so, then probes from regions flanking the α-globin genes should hybridize to opposite sides of the breakpoint. pHPζ and pα3'HVR.64 are such probes (see fig. 5.7). pHPζ had already been localized distal to the breakpoint, so pα3'HVR.64, from the hypervariable region 3' to *HBA*, was hybridized to chromosomes from GM2324. A location proximal to the 16p breakpoint would confirm the location of this breakpoint within the region of the α-globin genes. However, the results of this hybridization (fig. 5.6(c)) indicate that the probe hybridized entirely distal to the breakpoint on 16p, the same result obtained for the *HBZ* probe.

Haptoglobin genes. The probe pULB1148 hybridized with a significant excess of silver grains distal to *FRA16B*, on metaphases expressing this fragile site (appendix AIII.8). Chromosomes from two individuals with this fragile site were used, and those from F22A were the most informative, more metaphases generally being available with *FRA16B* expressed, and the gaps generally being wider, offering greater

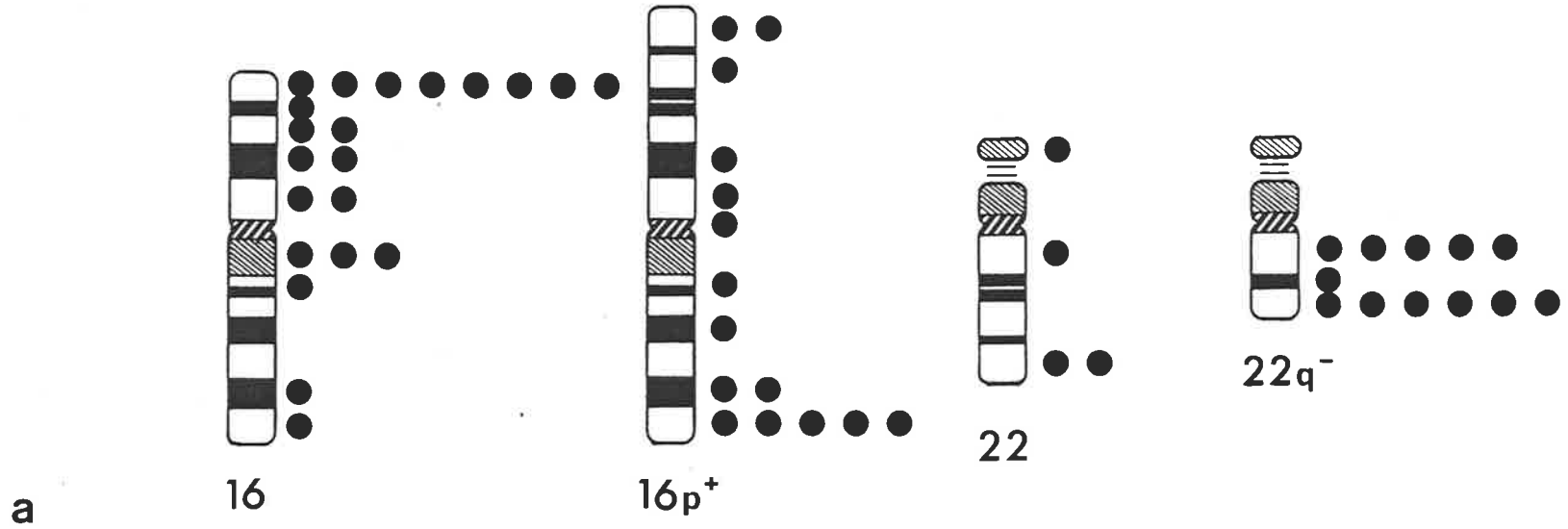
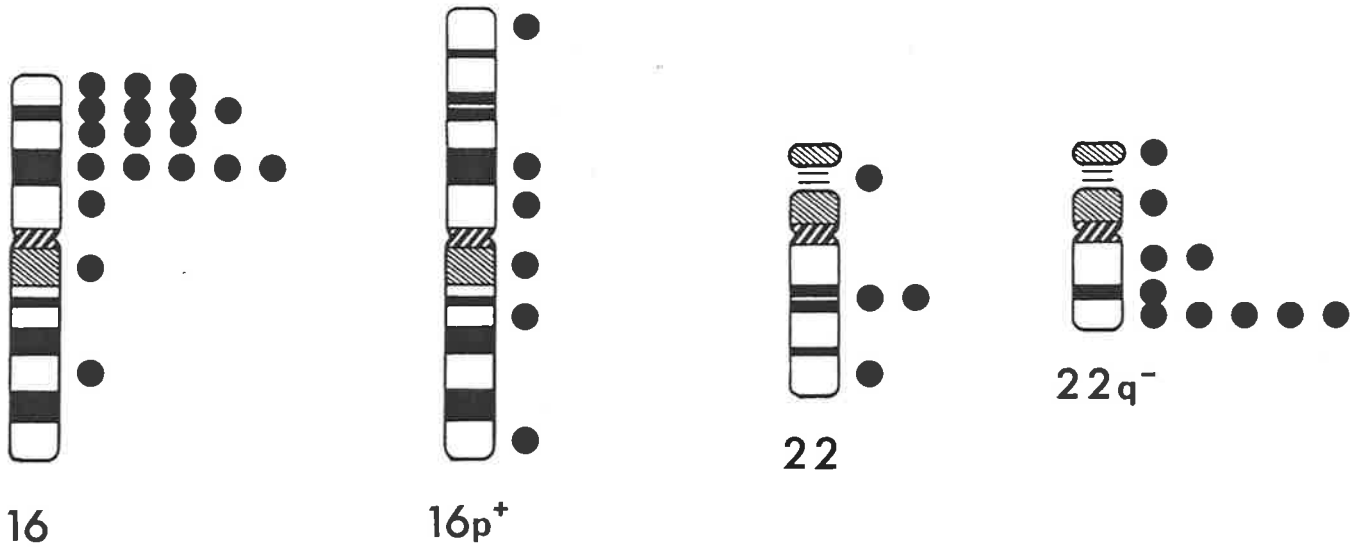
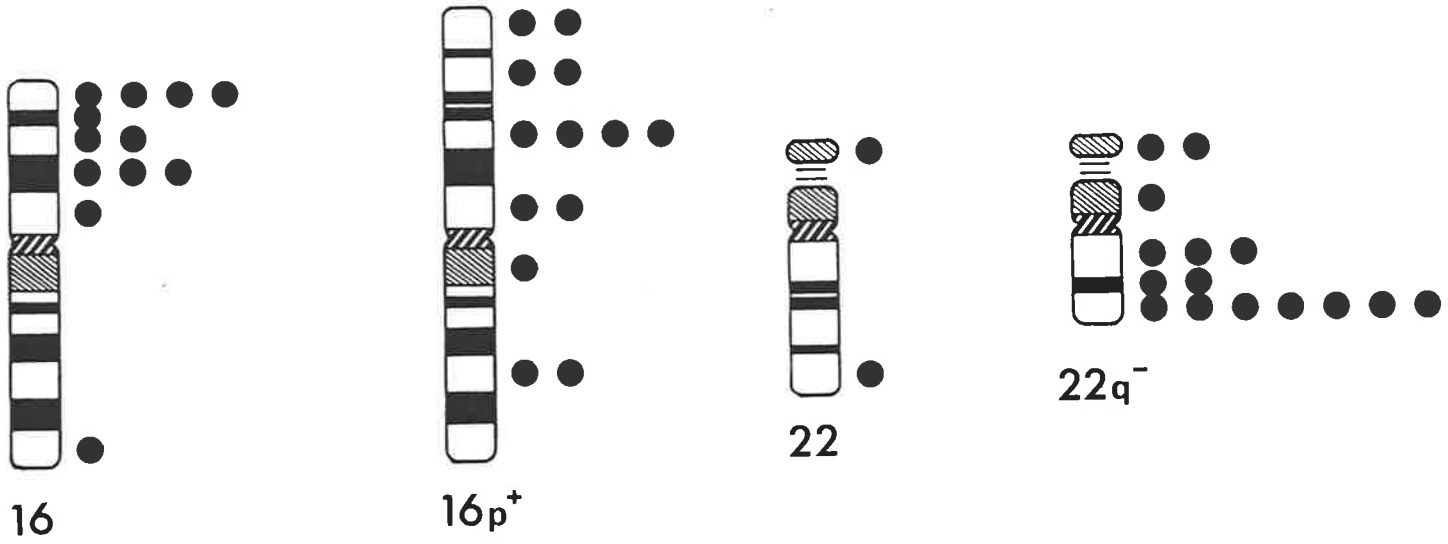


Figure 5.6.

(a)-(c) Silver grains scored over the normal and derived chromosomes 16 and 22 from the cell line GM23234, probed with (a) 0.4µg/ml JW101; (b) 0.4µg/ml pHPζ and (c) 0.2µg/ml pα3'HVR.64.



b



c

α-HAEMOGLOBIN GENE COMPLEX

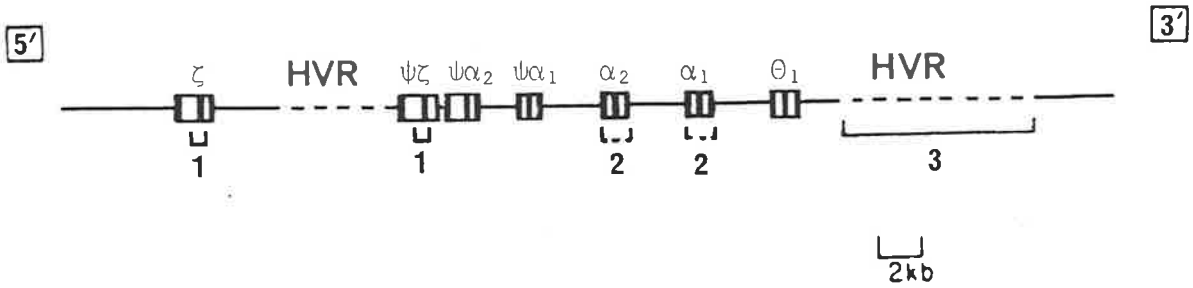


Figure 5.7.

A diagram showing the α-globin gene complex, with the region of homology to the probes described here indicated. ▮ = exon; ⌴ = region of homology to a probe used for this project. 1=pHPζ (*HBZ* probe); 2=JW101 (*HBA* probe); 3=pα3′HVR.64. (From Lauer *et al.*, 1980; Higgs *et al.*, 1981; Hardison *et al.*, 1986; Marks *et al.*, 1986.)

resolution. A metaphase showing an example of *FRA16B* labelled distal to the fragile site is illustrated in figure 2 in Simmers *et al.* (1986A).

Metaphases expressing *FRA16B*, probed with pBR322 as a control, showed only a low level of labelling of 16q. This level was not large enough to apply a chi-square test, except in the case of slide H9.28 (1.6μg/ml), but neither the region proximal nor that distal to *FRA16B* showed a consistent excess of silver grains, when all of these control slides were considered.

APRT gene. The results of hybridization of the *APRT* probe with respect to *FRA16B* and fra(16)(q23) are given in appendix AIII.9. Where grain numbers were sufficient for analysis, slides with metaphases expressing *FRA16B* showed an excess, usually significant, of hybridization distal to this fragile site, indicating that the *APRT* locus is distal to *FRA16B*.

Visual examination of the silver grains distal to the fragile site indicated that the majority occurred near or over 16qter (fig. 5.8). Thus, these silver grains were scored with respect to the fragile site and 16qter, as outlined in chapter 2. The results confirmed that *APRT* is closer to 16qter than it is to *FRA16B* (appendix AIII.9). *In situ* hybridization of the *APRT* probe to chromosomes expressing fra(16)(q23) resulted in significant excesses of silver grains distal to this fragile site, indicating that *APRT* is distal to *FRA16D*, (appendix AIII.9; see also Fratini *et al.*, 1986). Some of these metaphases illustrating silver grains distal to fra(16)(q23) can be found in Fratini *et al.* (1986), figure 4.

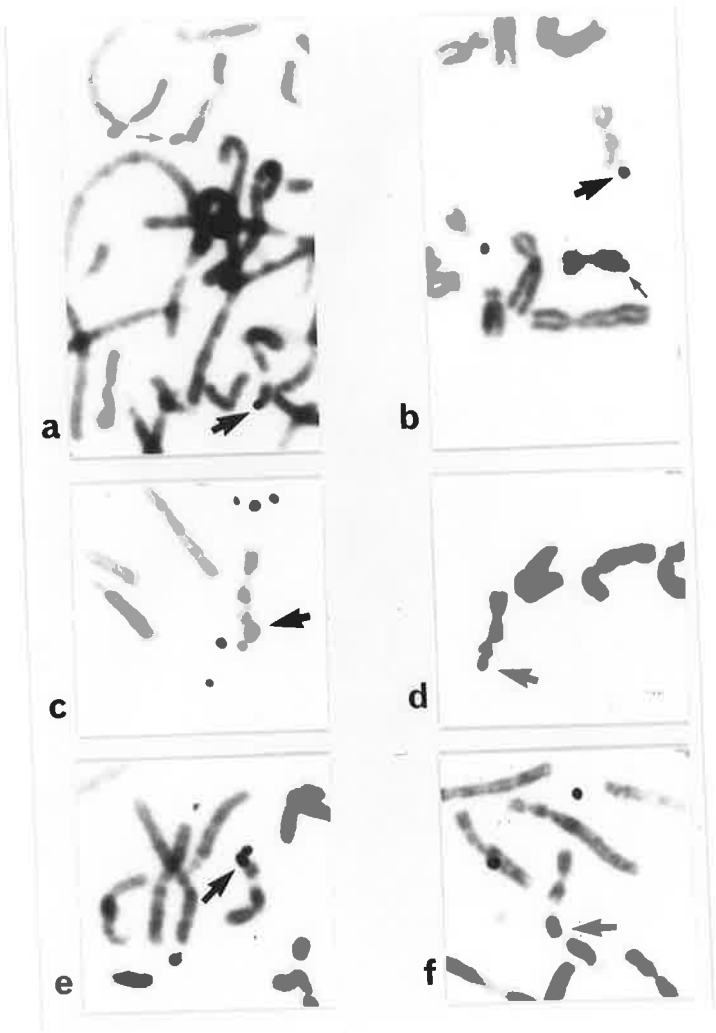


Figure 5.8.

(a)-(f) Examples of *FRA16B* probed for *APRT* showing silver grains distal to the fragile site (large arrows). (a) and (b) also show silver grains over the normal chromosome 16 homologue (small arrows).

Metallothionein gene cluster. The results of *in situ* hybridization of the *MT* probes to *FRA16B* and *FRA16C* are given in appendix AIII.10. These results, as summarized in Simmers *et al.* (1987A), indicate significant hybridization of all three probes proximal to *FRA16B*, and of the two unique probes, *MT2A*u and *MT1B*u, proximal to *FRA16C* (fig. 5.9).

For each of the fifteen *FRA16B* individuals studied, where grain numbers were large enough to apply a chi-square test (i.e. 10 grains proximal or distal to *FRA16B*), *MT2A*u and *MT1B*u localized proximal to *FRA16B*, with a significant excess of silver grains on the proximal side. The chromosomes of two patients (F7B, F38A), whose chromosomes expressed *FRA16B*, were used in addition to those mentioned in Simmers *et al.* (1987A), with the *MT1B*u and *MT2A*u probes. For each of these two individuals, the results for one of the probes provided no information (i.e. no silver grains observed on 16qs expressing *FRA16B*). For all other slides, however, there was an excess of silver grains proximal to *FRA16B*.

D16S4 and D16S5. The two probes had been hybridized at a range of concentrations to chromosomes expressing *FRA16B*. The slide with the optimal signal:background ratios (appendix AIV) was chosen for scoring with respect to the fragile site, for each probe. The two anonymous markers from chromosome 16 both resulted in significant excesses of silver grains distal to *FRA16B* (appendix AIII.11).

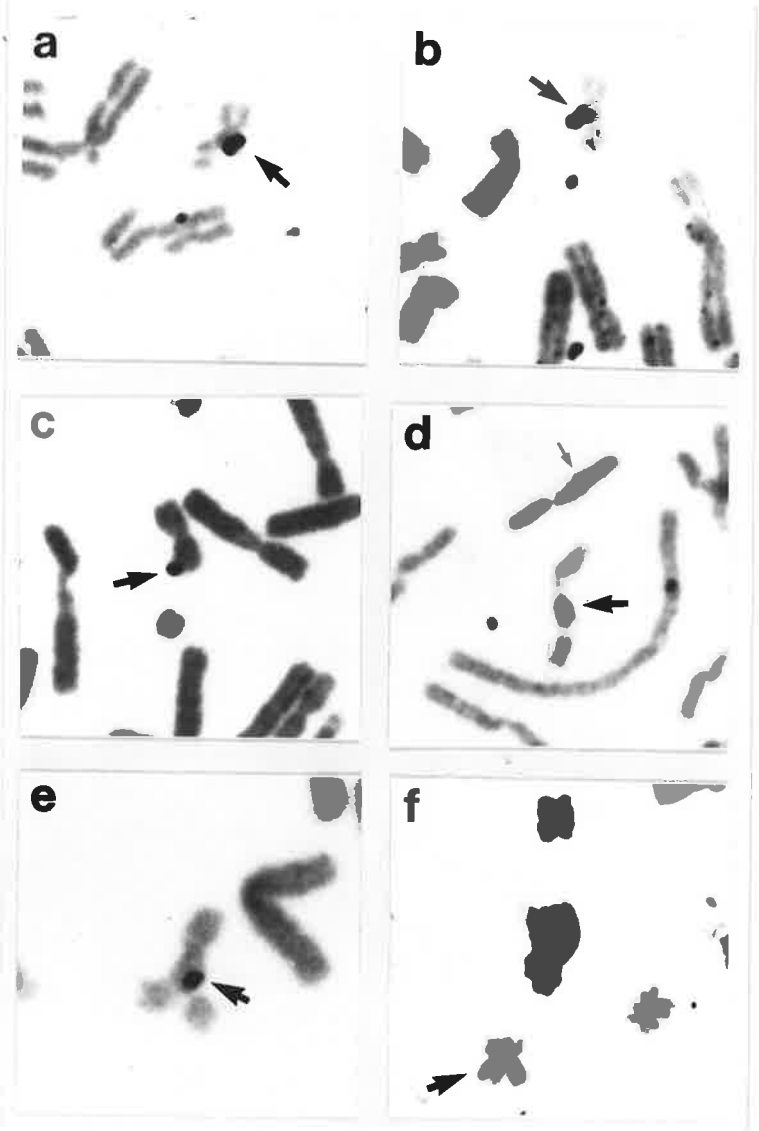


Figure 5.9.

(a-e) Examples of *FRA16B* and *FRA16C* showing silver grains from both probes, *MT2A*u and *MT1B*u, proximal to the fragile site (arrowed). (a), (c) were probed for *MT1B*; (b), (d-f) were probed for *MT2A*. (a-b) *FRA16C*; (c-d) *FRA16B*; (e) *FRA16B* expressed as a tri-radial figure. (f) silver grain over 16q in an endoreduplicated metaphase.

FRA16C. *FRA16B* and *FRA16C* were compared cytogenetically (see fig. 5.10). G-band analysis of examples of *FRA16B*, in metaphases from a carrier, treated with BrdU (but no FdU, FdC or caffeine) revealed a narrow dark region immediately distal to *FRA16B*. Although only one example of *FRA16C* with high resolution banding was found (first example), a similar dark region was not seen distal to this fragile site on this or other examples of *FRA16C*. *FRA16B* was typically expressed as an isochromatid gap, and *FRA16C* as a single chromatid gap. Fifty-eight examples of fragile sites at the interface of bands 16q21 and 16q22 (*FRA16B* and *FRA16C*) were found in metaphases from cultures treated with BrdU, FdU or FdC and caffeine for the induction of both fragile sites (see Materials and Methods, section 5.2.2). There was a small fragment of chromatin in the gap in two of these: one from a culture treated with 0.05mM FdC (plus caffeine and BrdU) and another from a culture treated with 0.005mM FdU (plus caffeine and BrdU). Figure 5.10C illustrates the latter, clearly banded example. The following interpretations were made. There is an isochromatid gap proximal to a single chromatid gap. There is a dark region distal to the gap in the left chromatid, and apparently also distal to the proximal gap in the right chromatid, on the small fragment. From the observations made above on *FRA16B* and *FRA16C*, this figure appears to represent the simultaneous expression of *FRA16B* and *FRA16C*, indicating that *FRA16B* is proximal to *FRA16C*.

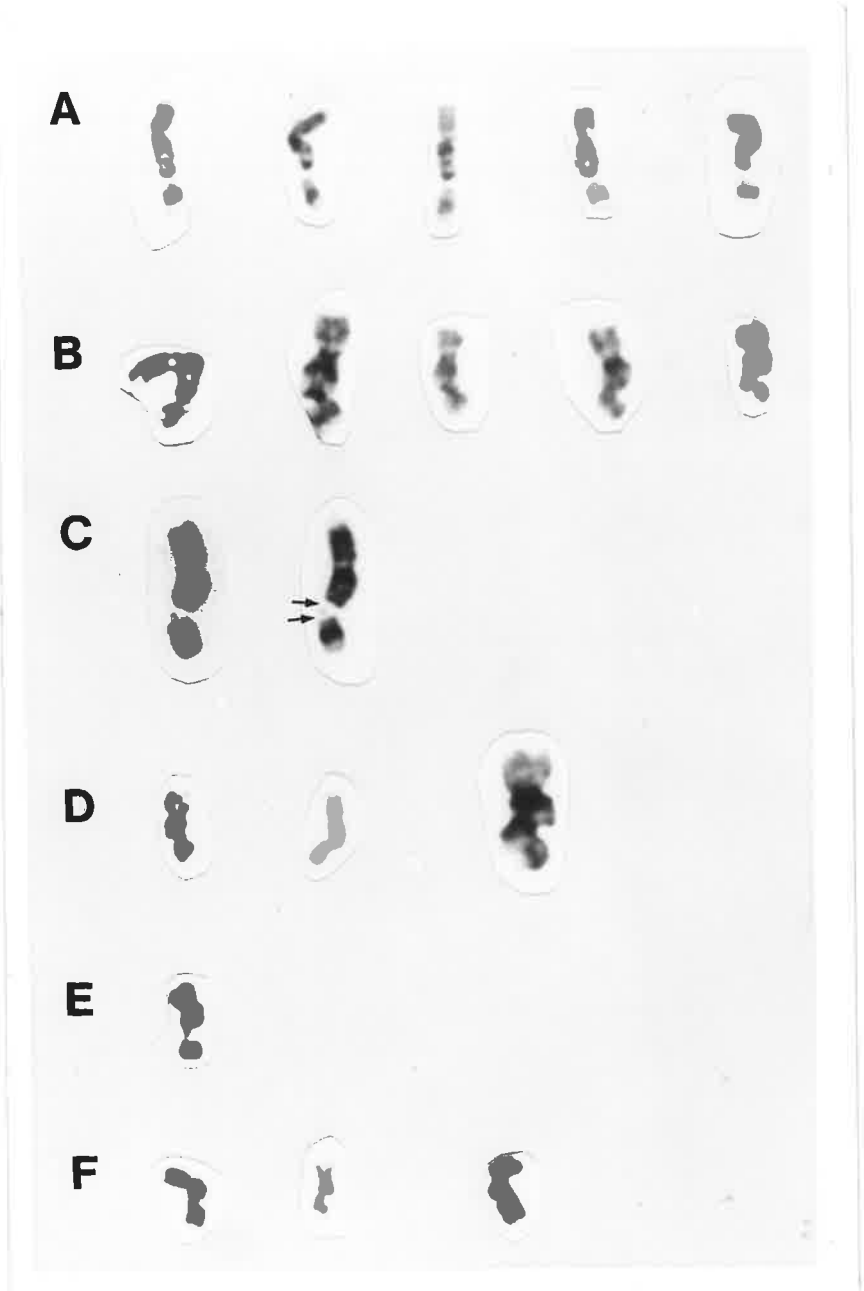


Figure 5.10.

Examples of (A) *FRA16B*; (B) *FRA16C*; (C) chromosome interpreted as expressing both *FRA16B* (upper arrow) and *FRA16C* (lower arrow) after treatment for induction of *FRA16B* and *FRA16C*; (D) gap seen within band 16q21; (E) chromosome interpreted as expressing the gap within band 16q21 and *FRA16C*; (F) a gap often observed at 16q12-q13, easily distinguished from *FRA16C*.

5.4.DISCUSSION

5.4.1.The α-globin gene complex

The α-like globin genes include the genes encoding one of the two chains in the haemoglobin molecules, of which there are foetal and adult forms, and their non-functional pseudogenes (Wood and Weatherall, 1983; Hill and Wainscoat, 1986). The ζ-globin gene (also known as ζ1, Lauer *et al.*, 1980) encodes the foetal α-like globin chain (Pressley *et al.*, 1980). Ψζ-globin gene (also known as ζ2, Lauer *et al.*, 1980) is a non-functional pseudogene (Pressley *et al.*, 1980), nearly identical to the ζ-globin gene (Proudfoot *et al.*, 1982). The two functional adult α-globin genes, α1 and α2, share extensive sequence homology, and usually encode identical polypeptide chains (Lauer *et al.*, 1980). Two non-functional pseudogenes, Ψα1 (Lauer *et al.*, 1980; Proudfoot and Maniatis, 1980) and Ψα2 (Hardison *et al.*, 1986), have been found 5′ to the functional α-globin genes, Ψα1 having closer homology to the functional α-globin genes than Ψα2 (Hardison *et al.*, 1986). Marks *et al.* (1986) have recently discovered a new α-like globin gene, Θ1, 3′ to the 1 gene. The α1 gene has characteristics of a functional gene (Marks *et al.*, 1986), although it may be a pseudogene (Hill and Wainscoat, 1986).

Figure 5.7 illustrates the organization of the α-globin gene complex, as known to date. Many variations in the numbers of these genes exist (Hill and Wainscoat, 1986). Six regions of length variation, or hypervariable regions (HVRs), have been found in the α-globin gene

complex, including the two highly polymorphic HVRs illustrated in figure 5.7, between the two ζ-globin genes, and at the 3′ end of the complex (Higgs *et al.*, 1981). HGM notation for the individual genes of this complex has varied (Ferguson-Smith and Cox, 1984; Cox and Gedde-Dahl, 1985). The groups of ζ- and α-globin genes will be referred to here as the *HBZ* and *HBA* loci respectively.

The molecular biology of the α-globin gene complex is important clinically, especially in research into and diagnosis of the α-thalassaemias and adult polycystic kidney disease (*PKD1*) (Weatherall, 1986). The finding that the *PKD1* gene is linked to *HBA* (Reeders *et al.*, 1985) has implied particular usefulness for the polymorphic markers from the α-globin gene complex, especially the highly polymorphic HVR 3′ to the α-globin genes (Reeders *et al.*, 1986A, B).

The progress made in mapping the α-globin gene complex up to the time this project started was followed in chapter 1. Using somatic cell hybrid analysis the genes were mapped to chromosome 16 (Deisseroth *et al.*, 1977; Deisseroth and Hendrick, 1978), and further to 16p (Koeffler *et al.*, 1981). Wainscoat *et al.* (1981) used evidence from dosage studies to localize *HBA* to 16p12->pter, and this was supported by Barg *et al.*'s (1982) localization using *in situ* hybridization. Malcolm *et al.* (1981) used the same method for regional localization of the β-globin genes, whereas subsequent reports indicated a more distal localization (see chapters 1 and 3). There was similarly room for some doubt about the regional localization for the α-globin gene complex to 16p12->qter. The results presented here confirm Barg *et*

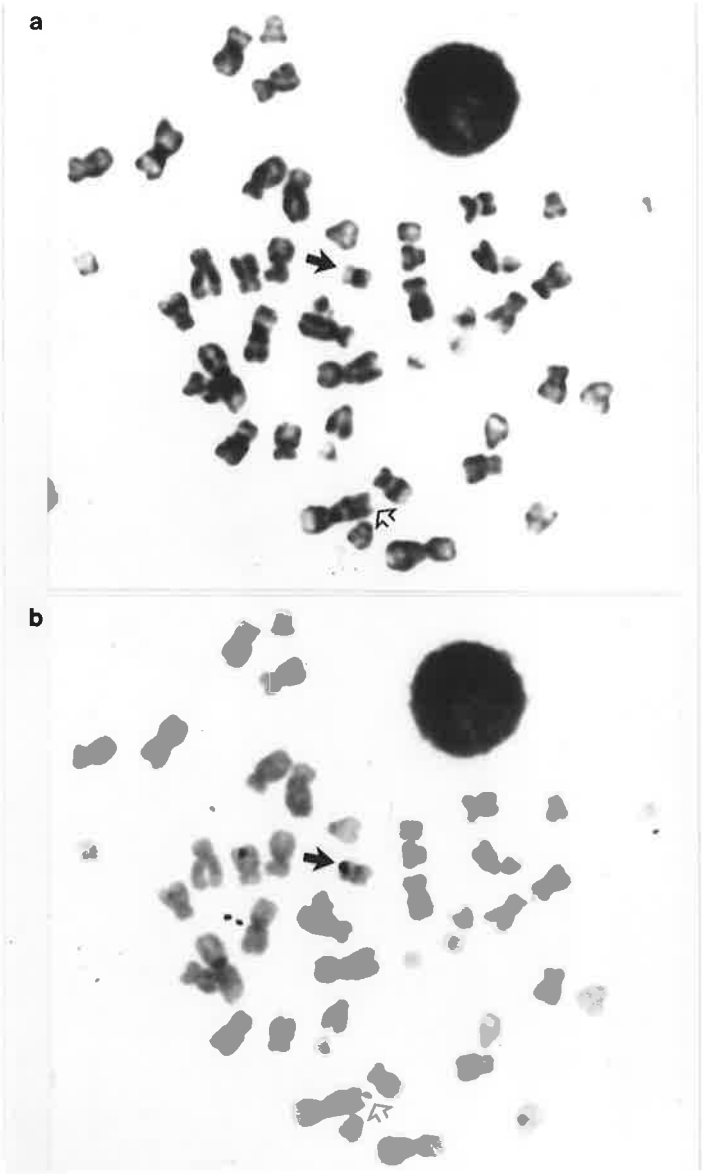


Figure 5.11.

A metaphase from the cell line GM6227, both (a) before and (b) after *in situ* hybridization, showing label over the normal 16p (closed arrow) and over 1q+ (open arrow), the sites of *HBA*.

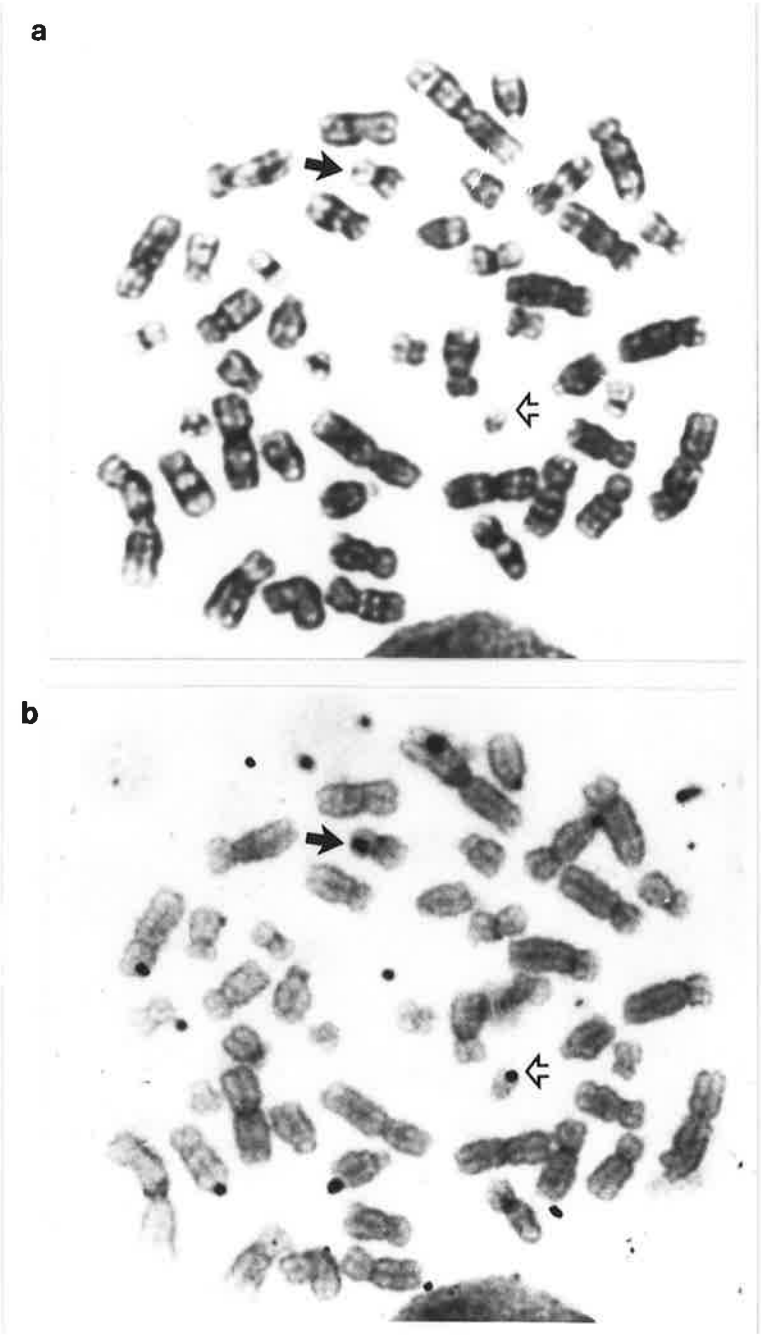


Figure 5.12.

A metaphase from the cell line GM2324, both (a) before and (b) after *in situ* hybridization, showing label over the normal 16p (closed arrow) and over 22q- (open arrow), the sites of the *HBA* gene cluster.

al.'s (1982) regional localization. The α-globin gene complex was localized distal to *FRA16A* (which is in sub-band 16p12.3, Sutherland and Hecht, 1985) and to the 16p13.11 breakpoint in the constitutional translocation, GM6227. (fig. 5.11) (The possibility of the breakpoint in GM6227 having occurred within the α-globin gene complex but outside the region defined by the probe JW101 has not been investigated by this study; however, a separate study (Nicholls *et al.*, 1987) has excluded this possibility.)

The results obtained from hybridization of the *HBA* probe, JW101, to chromosomes from GM2324 (fig. 5.6(a)) suggested that either the α1 or the α2 gene, or the intervening region (see fig. 5.7), had been split by the translocation. The *HBZ* probe, pHPζ, had been localized distal to the breakpoint (fig. 5.6(b)), and pα3'HVR.64 also localized entirely distal to the breakpoint, on the 22q- (fig. 5.6.(c)), placing the α-globin gene complex here. (fig. 5.12) The significant grain level (P=0.004) over the short arm of the der(16) obtained for chromosomes probed with JW101 is therefore due to a type I error. The silver grains were spread evenly along the short arm of the der(16), rather than occurring as a peak. This spurious result, and the lack of specific hybridization to 16p when probed with 0.8μg/ml JW101 (slide H24.6, see fig. 5.5(b)) (type II error), illustrate the possibility of obtaining such errors with *in situ* hybridization, especially when the number of metaphases scored gives a relatively low expected grain level over the site of hybridization. One should be aware of such possibilities. This is also demonstrated by the low level of hybridization obtained in this

laboratory over the normal 17q for chromosomes with the APL-specific t(15;17) probed for *G-CSF* (figs. 4.4 and 4.5).

The translocation breakpoint on 16p in the cell line GM6227 (fig. 5.3(a)) was defined at 16p13.11 (Catalog of Cell Lines, 1985). The karyotype of the cell line GM2324 (fig. 5.3(b))was originally defined as 46,XX,t(16;22)(p13;q12) (Catalog of Cell Lines, 1985). Nicholls *et al.* (1987) reported revised breakpoints, on evidence from high resolution banding analysis, and redefined the karyotype t(16;22)(p23.11;q11.21). However, the breakpoints may be interpreted as 16p13.3 and 22q12.2 (D.F. Callen, personal communication) (refer to fig. 5.13).

The α-globin gene complex was localized to 16p13.2->pter by classifying silver grains on the acentric fragment distal to fra(16)(p12), resulting from hybridization of the *HBA* and *HBZ* probes, according to their position closer to *FRA16A* or 16pter, where possible. A significant excess of silver grains occurred closer to 16pter, this being the crucial result in localizing the α-globin gene complex to 16p13.2->pter.

Two conflicting localizations have recently been made for the α-globin gene complex, that of Nicholls *et al.* (1987), to 16p13.1 and that of Breuning *et al.* (1987), to 16p13.3 (see fig. 5.14). Both used constitutional rearrangements involving 16p. Nicholls *et al.* (1987) also employed *in situ* hybridization, using a biotinylated probe.

The relative resolution obtainable by these methods, and by the method reported here (scoring on the acentric fragment,

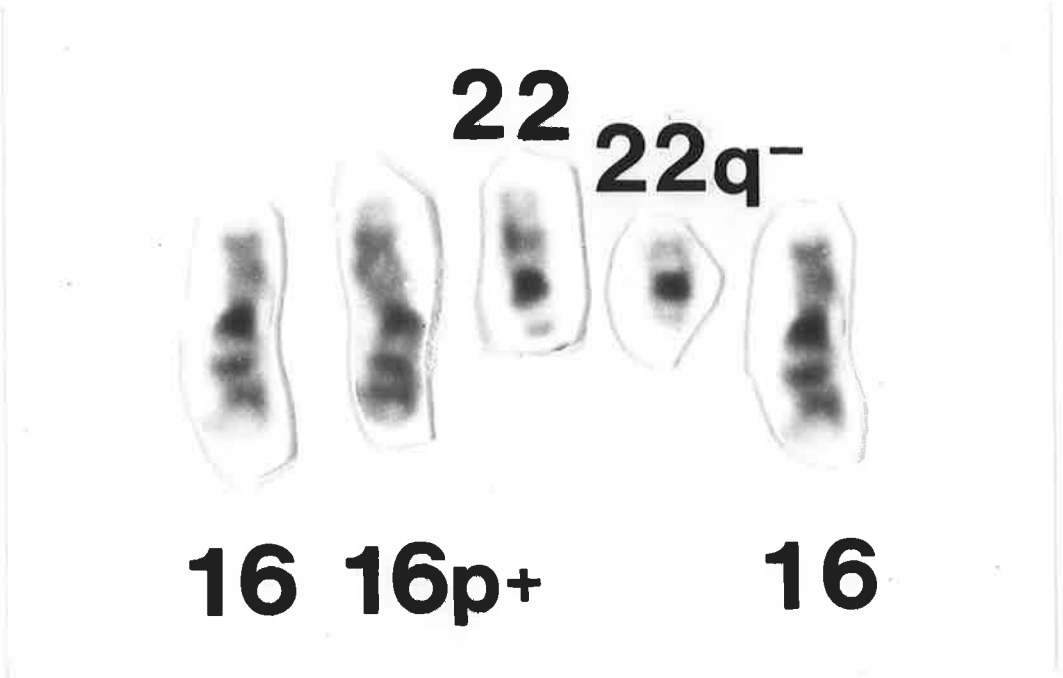


Figure 5.13.

Normal and derived chromosomes 16 and 22 from the cell line GM2324, with homologous regions aligned (chromosome 16 is shown twice). Analysis of rearrangements involving chromosome 16 is difficult, and two conflicting interpretations of this translocation have been made (see text).

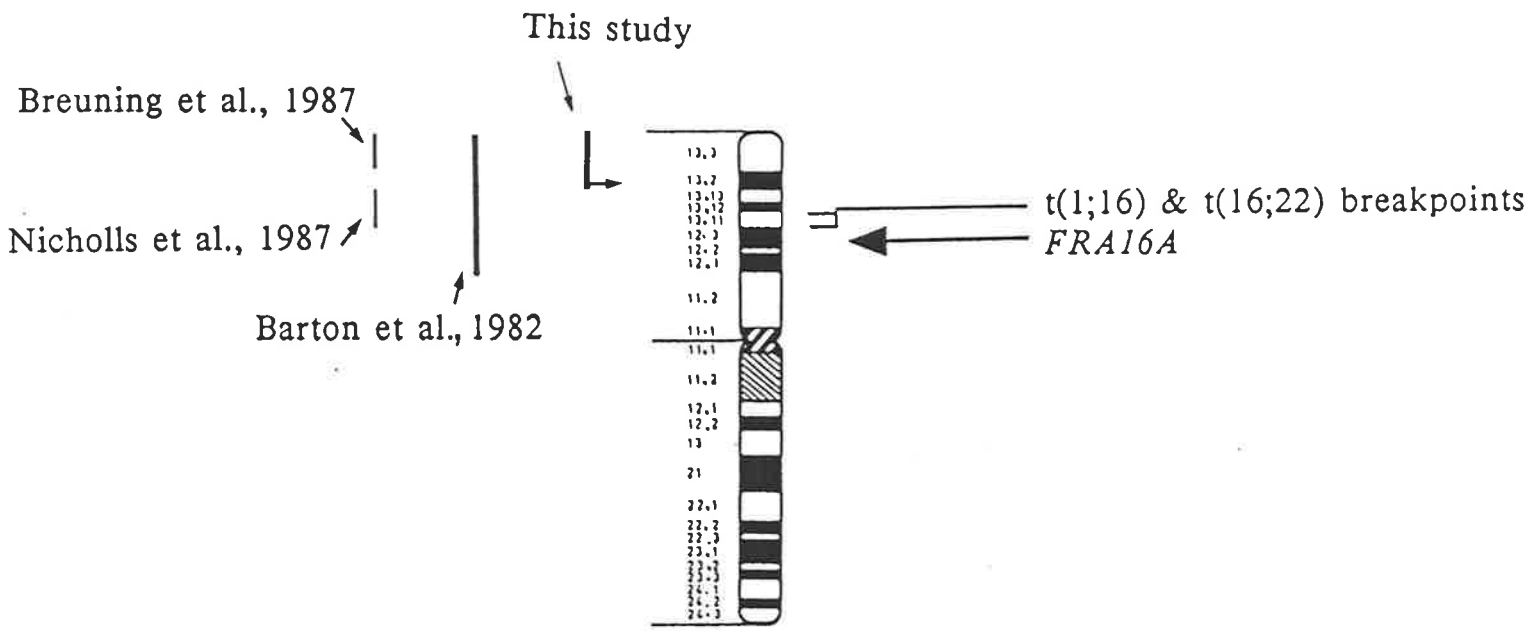


Figure 5.14.

An idiogram of chromosome 16, showing the *HBA* localizations discussed in this chapter, and the chromosome markers used here.

which is short) is in question, and is discussed in Simmers *et al.* (1987C). The results of Nicholls *et al.* (1987) and Breuning *et al.* (1987) mainly depend on the accuracy of localization of the translocation breakpoints which they used for their mapping studies. The localization of *HBA* and *HBZ* reported here, distal to the breakpoints of the two cell lines, is in agreement with the results of Nicholls *et al.* (1987), who used the same translocations. Their definition of the breakpoints is not in conflict with any of the localizations (Nicholls *et al.*, 1987; Breuning *et al.*, 1987; Simmers *et al.*, 1987C). However, they also localized the α-globin gene complex proximal to the breakpoint of an inversion, localized to within sub-band 16p13.1.

Breuning *et al.* (1987) discussed the discrepancy between their result and that of Nicholls *et al.* (1987), carefully examining and favouring their accuracy in defining their own translocation breakpoint analysis, which is also supported by the present result. An advantage of the method of localization reported here (using fra(16)(p12)) is that it does not rely on accurate breakpoint analysis, which is particularly difficult when 16p is involved, because of the indistinct banding pattern (Breuning *et al.*, 1987), but on the clearly defined location of *FRA16A*. The validity of this localization, compared to those of Nicholls *et al.* (1987) and Breuning *et al.* (1987) is discussed in detail in Simmers *et al.* (1987C).

Nicholls *et al.* (1987) observed an apparent meiotic crossover between the α-globin gene complex and the translocation breakpoint of GM6227, in the single offspring examined. The location of the α-globin gene

complex at 16p13.3, as supported by the present data and Breuning *et al.* (1987), makes this crossover less unlikely than would a location within band 16p13.1.

Localization by the method of scoring with respect to the two ends of a clearly defined chromosome segment, as described above, has been shown capable of giving an accurate result: *APRT* was correctly localized in this way to the distal portion of the acentric fragment from *FRA16B* to 16qter (see sections 5.3 and 5.4.3). The resolution for this fragment on 16q was potentially better, however, as it is longer than that from *FRA16A* to 16pter (compare fig. 5.8 with fig. 5 in Simmers *et al.*, 1987C and fig. 5.4).

Although there was a significant difference between the number of silver grains closer to fra(16)(p12) and those closer to 16pter, observer bias and/or chance could have played a part in this result. As regards the first possibility, the classification of silver grains was carefully checked, and any which were of ambiguous location excluded from the analysis. The resolution obtainable using *in situ* hybridization with a tritium labelled probe was discussed in chapter 3 (section 3.4.2.1). The acentric fragment distal to fra(16)(p12) was usually longer than the width of a chromatid, so the resolution obtained from a locus at either end of this fragment should be great enough for localization by this method.

The shorter the acentric fragment (*FRA16A*->16pter), the shorter is the distance of scatter required for a silver grain to occur over the

opposite half of the chromosome fragment to that from which the β-particle has been emitted. Interestingly, upon examination of examples of silver grains observed over the distal acentric fragment (see fig. 5 in Simmers *et al.*, 1987C), it can be seen that those examples of silver grains closer to the fragile site (fig. 5(c) in Simmers *et al.*, 1987C) (chosen for the figure on the basis of clarity alone) are on chromosomes which are generally shorter than the others illustrated (all at the same scale). This observation appears to reflect the conclusion that the α-globin gene complex is closer to 16pter than to *FRA16A*.

The results of linkage analysis between the α3'HVR and *FRA16A* (Simmers *et al.*, 1987C) are also in agreement with the localization reported here, although the confidence limits are wide (J.C. Mulley in Simmers *et al.*, 1987C).

The regional localization 16p13.2->pter, obtained as a result of scoring on the acentric fragment distal to fra(16)(p12), is conservative. This region includes the point half-way between *FRA16A* and 16pter. Although this mid-point might vary at the chromosome band level as the relative lengths of the bands vary with the degree of chromosome contraction (Münke *et al.*, 1984), the data indicate that the α-globin gene complex is close enough to 16pter to give a difference in signal between the two halves of the acentric fragment, and is therefore not close to this mid-point.

5.4.2.The haptoglobin locus

Haptoglobin is a haemoglobin-binding serum glycoprotein (Bowman and Kurosky, 1982), consisting of two α- and two β-chains. Three common α chain types, α1S, α1F and α2FS, define a polymorphism. A partial gene duplication of the sequence for the α-chain (*HPA*), probably involving the alleles for α1F and α1S, resulted in the allele for the α2FS haptoglobin chain (Maeda *et al.*, 1984).

As foreshadowed by studies of haptoglobin mRNA in other mammals (Haugen *et al.*, 1981; Chow *et al.*, 1983), sequence analysis of cDNA clones encoding the human haptoglobin α- and β-chains indicated that these chains are cleaved from a single polypeptide precursor; this implies that they are encoded by a single open reading frame (Raugei *et al.*, 1983; van der Straten *et al.*, 1983; Yang *et al.*, 1983). There is a second *HP* gene detected by Southern analysis with an *HP* cDNA clone (Raugei *et al.*, 1983; Maeda *et al.*, 1984), named *HPR*, or haptoglobin related (Maeda *et al.*, 1984). It is the result of a duplication event, and is located 2.2kb 3′ to the *HP* gene, in the same orientation (Maeda, 1985). The *HPR* gene may give rise to a functional product, differing from Hp by only 27 or 28 amino acids (Maeda, 1985; Bensi *et al.*, 1985).

By virtue of the polymorphism in the Hpα chain, haptoglobin has been one of the most frequently studied genetic markers (Hill *et al.*, 1986), and mapping studies have been possible from an early stage, long before the cloning of the *HP* gene. Early marker studies of chromosome rearrangements, both by linkage analysis and family typing, suggested

that the *HPA* locus was on a D group chromosome (Hustinx *et al.*, 1965; Gerald *et al.*, 1967; Bloom *et al.*, 1967), although linkage studies were inconclusive (Bloom *et al.*, 1967; Robson *et al.*, 1969). Amos *et al.* (1970) also published data in support of linkage between the *HLA* locus (which was unassigned) and *HPA*, information used to incorrectly assign *HLA* to chromosome 16 (Magenis *et al.*, 1970).

Successful localization of *HPA* to chromosome 16 was first achieved by Robson *et al.* (1969) by linkage analysis using translocation breakpoints as markers. It was further found to be linked to the fragile site on 16q (*FRA16B*) by Magenis *et al.* (1970). Theirs was the first report of linkage analysis using a fragile site (Mulley, 1985). Their report was also the first assignment of a human locus to a single chromosome arm (Renwick *et al.*, 1970). The results of the present study clarify the longstanding uncertainty as to the order of *HPA* and *FRA16B* on chromosome 16 and the consequent location of *HPA* on 16q.

Renwick (1972), using the data of Magenis *et al.* (1970) found the most likely site of *HPA* to be in the middle of 16q (proximal to the fragile site), or at an equal distance distal to *FRA16B*. Robson *et al.*'s (1969) data also supported the proximal location (Renwick, 1971). These data favoured the order 16cen-*HPA*-*FRA16B* over 16cen-*FRA16B*-*HPA* with 9:1 odds (Renwick, 1971).

The *HPA* locus was excluded from 16q22->qter by deletion mapping (Ferguson-Smith and Aitken, 1978). On the basis of this exclusion and the location of *FRA16B* at the junction of bands 16q21 and 16q22, Magenis

and Chamberlin (1979) suggested that *HPA* is just proximal to band 16q22 (and therefore also to *FRA16B*), within band 16q21. However, given that the precise breakpoint within 16q22 of the deletion had not been determined, *HPA* could not be excluded from band 16q22 (Mulley, 1985). Correspondingly, Povey *et al.* (1980), in consideration of Ferguson-Smith and Aitken's (1978) data and their own linkage analysis between *HPA* and the centromeric heterochromatin, favoured a localization for *HPA* in 16q21 or the proximal part of 16q22. Mulley (1985), using the data of Magenis *et al.* (1970), calculated a linkage distance for *HPA* about 9cM from *FRA16B*, and using the combined data above, found the most likely position of *HPA* to be within band 16q21. Thus, the prevailing opinion placed *HPA* within band 16q21, proximal to *FRA16B*.

Using the newly available probe for both *HPA* and *HPB* (the β-chain coding region), McGill *et al.* (1984) localized *HP* to band 16q22, using *in situ* hybridization. This result contradicts the above localization of *HP* proximal to *FRA16B* and within band 16q21. There is room for error, however, in the spread of silver grains over 16q. The localization of *HP* distal to *FRA16B*, reported here, confirms McGill *et al.*'s (1984) localization to 16q22. The data reported above therefore place *HP*, and the adjacent *HPR* gene, 9cM distal to *FRA16B*, in the central or distal region of band 16q22, the location considered by Mulley (1985) if *HP* was distal to *FRA16B*. Callen *et al.* (1987) have now described a much narrower regional localization for *HP*, from 16q22.15->q22.18, with the aid of translocation breakpoints at these boundaries, which is consistent with the above conclusions.

5.4.3.The *APRT* locus

Adenine phosphoribosyltransferase (APRT), one of the two human purine phosphoribosyltransferases, is an enzyme which catalyses the reaction of adenine with 5-phosphoribosyl-pyrophosphate to form adenosine-5′-monophosphate and pyrophosphate (Mowbray *et al.*, 1972).

Only one DNA region is recognised by Southern analysis with the *APRT*-specific probe (Stambrook *et al.*, 1984). However, pseudogenes have also been detected in the mouse genome (Dush *et al.*, 1986), including a processed pseudogene syntenic with the functional *APRT* locus (Dush *et al.*, 1986; Farber and Zielinski, 1986).

Henderson *et al.* (1969) found *APRT* to be autosomally inherited. Kusano *et al.* (1971) developed a system for the selection of cells containing the functional *APRT* gene. Using this system, human-mouse somatic cell hybrids can be made, containing an $APRT^-$ mouse genome and various human chromosomes. The presence of the human *APRT* gene may be correlated with the consistent presence of a certain chromosomal region, and assigned to this region (Kusano *et al.*, 1971). As no bi-armed chromosome was observed in the $APRT^+$ cell lines, it was concluded that the gene resided on one of the human acrocentric chromosomes (Kusano *et al.*, 1971), an incorrect localization probably attributable to an undetected rearrangement (Tischfield and Ruddle, 1974). The locus was however, later correctly assigned by two independent laboratories to chromosome 16 by this method (Kahan *et al.*, 1974; Tischfield *et al.*, 1974;

Tischfield and Ruddle, 1974). Kahan *et al.* (1974) localized APRT to the long arm of this chromosome.

The subsequent mapping data for *APRT* have been conflicting. Barg *et al.* (1982) mapped this locus to 16q12->q22, a localization supported by Lavinha *et al.* (1984). Rethoré *et al.* (1982) localized *APRT* to 16q22.2->qter. The shortest region of overlap (SRO) was 16q22.2->q22.3 (Ferguson-Smith and Cox, 1984); yet no evidence of linkage has been found between *APRT* and *HP* (Castiglione *et al.*, 1986), or between *APRT* and *FRA16B* (J.C. Mulley, personal communication). (Refer to fig. 5.15).

The results reported here, and further results reported by Fratini *et al.* (1986), clarify the location of *APRT*. The *APRT* probe hybridized distal to *FRA16B*, in accordance with the SRO given at HGM7 (Ferguson-Smith and Cox, 1984). Further analysis of the silver grain distribution, however, indicated a location closer to 16qter than to *FRA16B* (fig. 5.8), i.e., from about 16q23.2->16qter (fig. 5.15). This result was confirmed by subsequent hybridization of the probe to chromosomes expressing fra(16)(q23), and by Southern analysis using a cell line with a breakpoint in 16q24, determined to be proximal to the *APRT* locus (Fratini *et al.*, 1986). The new localization of *APRT* to 16q24 by this report (Fratini *et al.*, 1986) is consistent with the findings of Rethoré *et al.* (1982), but contrary to those of Barg *et al.* (1982) and of Lavinha *et al.* (1984) (refer to fig. 5.15).

By themselves, the inconsistencies between the *APRT* localization studies, where translocation breakpoints were

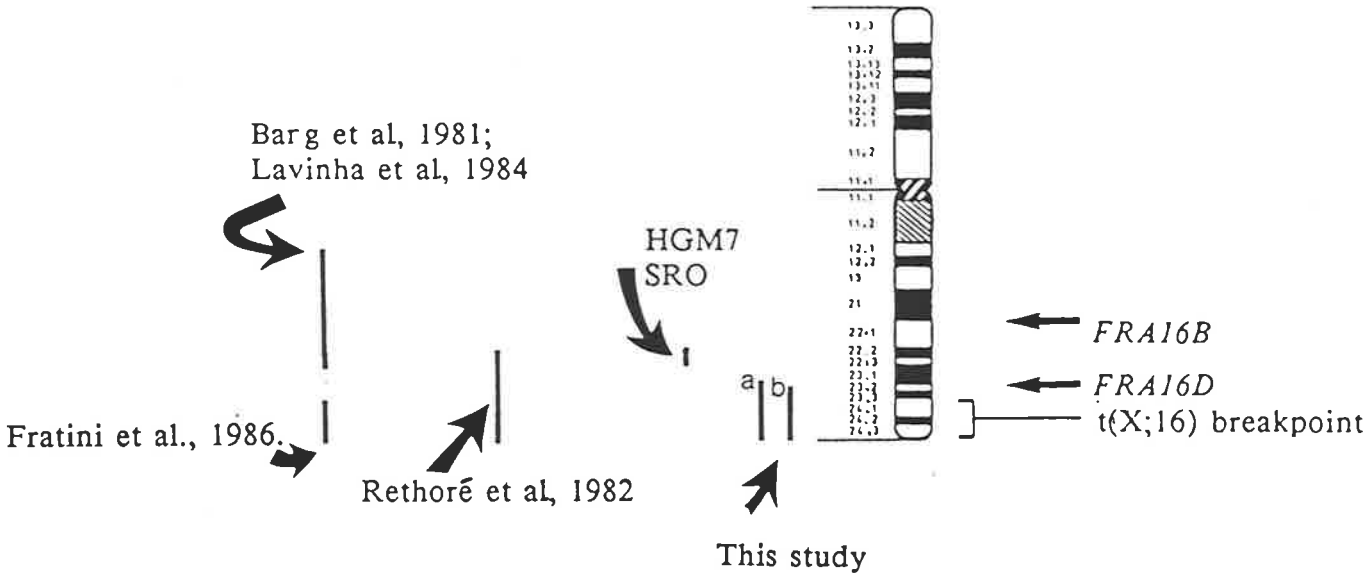


Figure 5.15.

An idiogram of chromosome 16, showing the *APRT* localization results of various authors, and the SRO given at HGM7 (Ferguson-Smith and Cox, 1984) (left). The locations of the two fragile sites (this project), the mid-point between *FRA16B* and 16qter, and the breakpoint (Fratini *et al.*, 1986) used for the localization of *APRT* contrary to this SRO are illustrated to the right.

used (the somatic cell hybrid analysis studies reported in Fratini *et al.*, 1986; Barg *et al.*, 1982 and Lavinha *et al.*, 1984; and the dosage studies of Rethoré *et al.*, 1982) can be explained by the incorrect assignment of at least one set of breakpoints, but no clue is given as to which are correct. The *in situ* hybridization results independently indicate that the assignments of Barg *et al.* (1982) and of Lavinha *et al.* (1984) are incorrect. Furthermore, Callen (1986) has induced the common fragile site in band 16q23, proximal to the breakpoint in the cell line used for localization in Fratini *et al.* (1986), confirming that this breakpoint is within band 16q24. In the absence of supporting data, it could equally well be assigned to band 16q22 by cytogenetic analysis of the derivative chromosomes (Callen, 1986), consistent with the localization obtained using other breakpoints (Barg *et al.*, 1982; Rethoré *et al.*,1982; Lavinha *et al.*, 1984).

A posteriori there is no reason to doubt the assignment of the breakpoint reported by Rethoré *et al.* (1982), to band 16q22.2, or the validity of their APRT dosage studies. Marimo and Giannelli (1975) found APRT levels to reflect the number of copies of the gene.

The *in situ* hybridization results presented here localize *APRT* distal to *FRA16D* (which is in sub-band 16q23.2, Yunis and Soreng, 1984; Berger *et al.*, 1985), to 16q23.2->qter. This result was corroborated, and further refined, by localization of *APRT* to the terminal band 16q24 (Fratini *et al.*, 1986). These results also explain the absence of linkage between *APRT* and *HP* (Castiglione *et al.*, 1985), and between *FRA16B* and *HP* (J.C. Mulley, personal communication), and confirm the observations of

Jeremiah *et al.* (1982) and Povey *et al.* (1982), placing *APRT* distal to *HP*, by linkage analysis.

5.4.4.The metallothionein gene cluster

The metallothioneins (MT) are ubiquitous, low molecular weight proteins, rich in cysteine, which bind heavy metals (Karin, 1985). There are two electrophoretically separable forms, MT-I and MT-II, and in the mouse each is encoded by a single gene. In man however, several functional MT-Is exist, each encoded by a different gene. In all, there are at least 20 *MT* genes, eight of these being pseudogenes (R. I. Richards, personal communication).

All of the functional *MT* genes are clustered on chromosome 16 (Karin *et al.*, 1984A): these comprise the *MT2A* gene and the *MT1* genes (fig. 5.2). A polymorphic processed pseudogene, *MT2B*, has been identified (Karin and Richards, 1982A) at 4p11->q21 (Lieberman *et al.*, 1985): this is intronless, and assumed to have been incorporated into chromosome 4 by reverse transcription of an *MT2A* mRNA (Karin and Richards, 1982A). At least four other *MT* genes are dispersed on other chromosomes, probably on chromosomes 1 (two loci), 18 and 20 (Schmidt *et al.*, 1984). These are also non-functional, and at least two of the genes not on chromosome 16 are processed pseudogenes (Karin *et al.*, 1984A). Le Beau *et al.* (1985C) also localized one of the pseudogenes to 1p34->p36.

The *MT2A* gene is 900 base pairs long, including two introns (Karin and Richards, 1982A). The coding regions of the *MT* genes are highly

homologous, and hence the *MT2A*r cDNA probe (see Materials and Methods) cross-reacts with all members of the gene cluster at 16q and the dispersed pseudogenes. Although the proteins are therefore very similar, with no apparent functional differences, these metallothioneins are differentially induced, by virtue of different promoter and regulatory regions 5′ to the coding sequences (Richards *et al.*, 1984). These regions can be complex, including separate regions for regulation by different substances, and for a basal level of expression (Karin *et al.*, 1984B).

The specificity of the 5′ flanking regions allows these regions adjacent to the single genes to be studied using probes containing DNA from these regions. For example, Lieberman *et al.*(1985) localized the processed *MT2* pseudogene on chromosome 4 by *in situ* hybridization, using a probe containing DNA flanking this gene. The same principal applied in the use of the probes *MT2A*u and *MT1B*u here. They gave specific signal from the 5′ flanking regions of the *MT2A* and *MT1B* genes respectively. The use of these specific probes was important to the localization of the region between these genes, at or away from *FRA16B* and *FRA16C* (see chapter 6).

The results presented here for *in situ* hybridization of the three probes for the metallothionein gene cluster indicate that all or most of the cluster is proximal to both *FRA16B* and *FRA16C*, particularly the region between the *MT2A* and *MT1B* (see discussion in Simmers *et al.*, 1987A). In the absence of any independent data separating the entire *MT* cluster from *FRA16B* or *FRA16C*, however, the possibility of either fragile site

splitting the *MT* cluster near its distal end, although remote, cannot be entirely excluded. Although such splitting of the *MT* cluster was suggested by Le Beau *et al.* (1985C), this is now unlikely, as the cluster would have been split between *MT2A* and *MT1B* according the reasons given by Le Beau *et al.* (1985C) (see chapter 6).

Le Beau *et al.* (1985C) localized the *MT* cluster to band 16q22 by *in situ* hybridization. As both *FRA16B* and *FRA16C* have been assigned to the junction of bands 16q21 and 16q22 (Magenis and Chamberlin, 1979; Croci, 1983; Yunis and Soreng, 1984), the localization of the *MT* cluster reported here, proximal to these fragile sites, and therefore probably in band 16q21, was surprising, as it contradicted their data. Le Beau *et al.*'s (1985C) localization to band 16q22 was made by *in situ* hybridization, and might be explained by grain scatter; a more cautious assignment from their data would include the two surrounding bands. However, their localization also corresponded to the localization of a translocation breakpoint specific to acute myelomonocytic leukaemia (AMMoL), in band 16q22 (Arthur and Bloomfield, 1983A, B; Le Beau *et al.*, 1983; see chapter 6), which they found to occur within the *MT* cluster (Le Beau *et al.*, 1985C). The breakpoint and the *MT* locus should coincide cytogenetically. The data presented here therefore imply that the AMMoL-specific breakpoint is also within band 16q21. Examination of published photographs of this inv(16)(p13q22) (Yunis *et al.*, 1981; Yunis, 1984A; Dachary *et al.*, 1986; Moir *et al.*, 1984; and Maseki *et al.*, 1984) suggests that if the 16q breakpoint is within band 16q21, this is not readily detectable. Band 16q21 is of darker staining intensity than any band on the normal 16p, and a region with such

staining intensity is not seen on the derived 16p proximal to the translocated region of 16q. The breakpoint would therefore be towards the distal end of 16q21. Translocation breakpoints are usually assigned to G-negative bands (see Sutherland and Simmers, 1987), as has been the case with the inv(16); this is usual with the use of G-banding for breakpoint analysis (Savage, 1977).

Further studies clarifying the localization of the *MT* cluster will be interesting, in particular, linkage analysis with *FRA16B*, to determine how close *MT* is to *FRA16B* and to the junction of bands 16q21 and 16q22. Such linkage analysis has not yet been possible, due to the lack of sufficient informative meioses (V.J. Hyland, J.C. Mulley, personal communication). The detection of recombination between *FRA16B* and a polymorphic *MT* locus (e.g. Hyland *et al.*, 1987A) would further substantiate the conclusion that *FRA16B* and *MT* do not coincide.

5.4.5.The *D16S4* and *D16S5* loci

ACH224 detects a restriction fragment length polymorphism (RFLP), whereas ACH207 detects two RFLPs (Hyland *et al.*, 1987C). The two probes, ACH207 and ACH224, specific for the *D16S4* and *D16S5* loci respectively, were originally regionally localized using a somatic cell hybrid panel containing regions of chromosome 16, described by Callen (1986). *D16S4* mapped to the region 16q13->q22.1, and *D16S5* to the region 16q22.1->q24 (Hyland *et al.*, 1987B). These were two of five anonymous clones from the same flow sorted library to be localized to a region including 16q22 (Hyland *et al.*, 1987B). As they were assigned to

regions which included *FRA16B*, and are polymorphic (Hyland *et al.*, 1987C), they were of potential value in protocols aimed at cloning *FRA16B*.

The experiments discussed here were therefore aimed at determining to which side of *FRA16B* these two loci, *D16S4* and *D16S5* lie, and in doing so, to more finely localize them.

Both probes were localized distal to *FRA16B*.

D16S5 was initially localized distal to the 16q breakpoint of CY5 (Hyland *et al.*, 1987), which was assigned to band 16q22 (Callen, 1986). Thus, its proximity to *FRA16B*, which is at or near the interface of bands 16q21 and 16q22 (Magenis and Chamberlin, 1979; Croci, 1983), and their order, were not known, given the possibility of imprecise breakpoint localization. Finer analysis of the translocation breakpoint, and the combined evidence from other nearby breakpoints, including high resolution banding analysis and localizations using all of these markers, support a localization of the CY5 breakpoint at 16q22.15 (Callen *et al.*, 1987). Thus, *D16S5*, which is distal to this breakpoint (Hyland *et al.*, 1987B), is not close to *FRA16B* at a molecular level. The probe in fact appeared to hybridize near the terminal end of 16q (see fig. 5.16), suggesting that it is closer to its distal boundary of 16q24 (Hyland *et al.*, 1987B), than to *FRA16B*.

D16S4 also localized distal to *FRA16B*. It has subsequently been localized proximal to a breakpoint in 16q22.12 (Callen

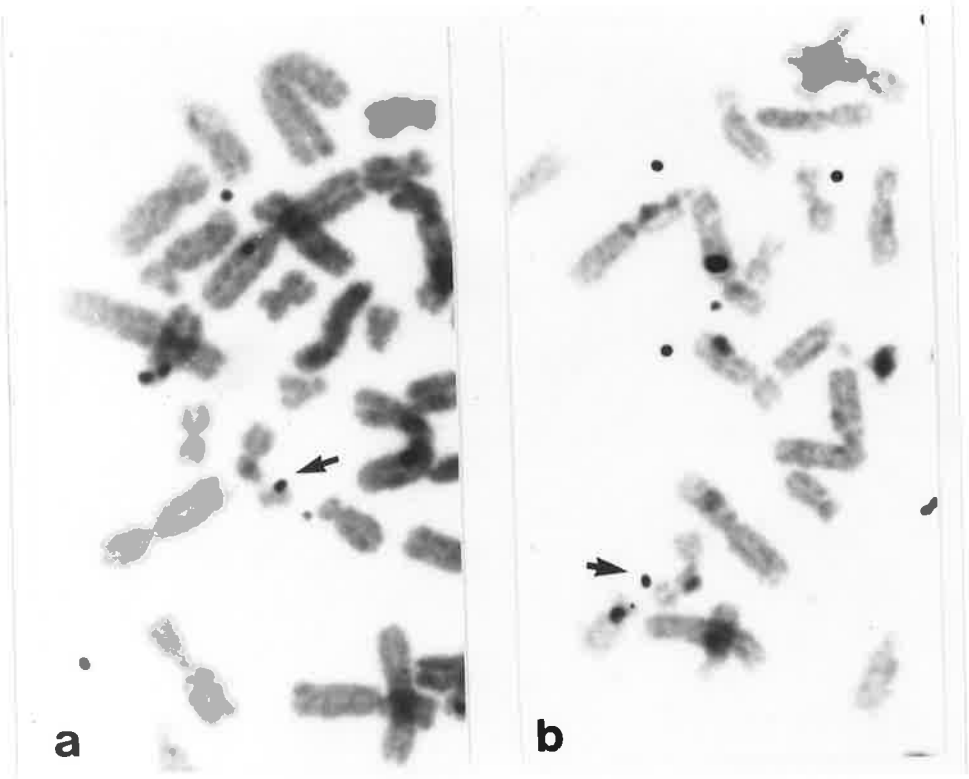


Figure 5.16.

Partial metaphases showing the locations of the two anonymous fragments from chromosome 16, as representative silver grains distal to *FRA16B* (arrow): (a) the probe ACH207, representing the *D16S4* locus, and (b) the probe ACH224, representing the *D16S5* locus.

et al., 1987), and therefore has a narrow regional localization of 16q22.10->q22.12 (Callen *et al.*, 1987). This region has been estimated to contain about 1 000 to 2 000 base pairs of DNA (Callen *et al.*, 1987), which would place *D16S4* at most this far away from *FRA16B*.

Preliminary linkage analysis between *FRA16B* and *D16S4* revealed a peak lod score of 1.7 at Θ=0.07 (V.J. Hyland, J.C. Mulley, personal communication). One possible recombinant was observed between *FRA16B* and *D16S4* in 23 informative meioses, but the individual was negative for *FRA16B*, and may have been a non-penetrant carrier (J.C. Mulley, personal communication).

Preliminary linkage analysis between *HP* and *D16S4* has shown a peak lod score of about 4.2 in the vicinity of Θ=0.05 (J.C. Mulley, personal communication), consistent with their relative locations. A peak lod score of 4.19 at Θ=0.05 was found from preliminary linkage analysis between *HP* and *FRA16B* (V.J. Hyland, J.C. Mulley, personal communication). Although these data have not yet been thoroughly analysed (J.C. Mulley, personal communication), they also suggest that *D16S4*, which is between *FRA16B* and *HP* (Callen *et al.*, 1987), is very close to *FRA16B*.

In summary, localization of the two anonymous DNA fragments with respect to *FRA16B* has provided further information on their position on chromosome 16. *FRA16B* provided a proximal boundary for the narrow regional localization of *D16S4* arrived at by Callen *et al.* (1987). The distal boundary of *D16S4*, at 16q22.12, indicates that it is

cytogenetically close to *FRA16B*. This is as yet the closest known location of a DNA sequence to *FRA16B*.

5.4.6.Overview: mapping chromosome 16

The data presented in this chapter provide a more detailed map of chromosome 16 than that which previously existed. More loci in addition to those investigated here have been mapped on chromosome 16. HGM8 reviewed the chromosome 16 map (Cox and Gedde-Dahl, 1985). This will be examined below in relation to those loci on chromosome 16 relevant to the localizations discussed above.

PGP (phosphoglycolate phosphatase) is closely linked to the α-globin gene complex, and to *PKD1* (Reeders *et al.*, 1986A). It (as well as *HAGH* (hydroxyacyl glutathione hydrolase)) was also localized to band 16p13, distal to the breakpoint in the cell line GM6227 (Mulley and Callen, 1986), the location of the α-globin gene complex has also been distally localized (this report). Breuning *et al.* (1987), using another translocation breakpoint which separates the α-globin gene complex and *PGP*, found *PGP* to be proximal to the α-globin gene complex.

TAT (tyrosine aminotransferase) was localized to chromosome 16 (Barton *et al.*, 1986; Natt *et al.*, 1986), and has been localized to 16q22.12->q22.15, distal to *FRA16B* and *D16S4*, and proximal to *HP* (Callen *et al.*, 1987).

Barg *et al.* (1982) and Lavinha *et al.* (1984), using the same chromosome rearrangements that were used for the erroneous localization of *APRT*, mapped *DIA4* (diaphorase-4) to within the same limits, 16q12->q22. *DIA4* therefore should be given a wider regional localization than given previously (Barg *et al.*, 1982; Lavinha *et al.*, Cox and Gedde-Dahl, 1985). As *DIA4* was determined to be proximal to *APRT* (Jeremiah *et al.*, 1982), and only tentatively distal to *HP* (Povey *et al.*, 1982), but distal to *MT* (Schmidt *et al.*, 1984), a new regional localization for *DIA4* of 16q21->q24 should apply.

The use of many defined markers along chromosome 16 can provide a powerful tool for the regional localization and ordering of genes and anonymous DNA segments, and much progress has been made in this direction (this report; Hyland *et al.*, 1987B; Callen *et al*, 1987).

In this chapter the inconsistencies between the various localization studies have been discussed. They illustrate the inaccurate conclusions which may be drawn from the use of incorrectly localized breakpoints of chromosomal rearrangements. Savage (1977) suggested that any breakpoint localization has an inherent uncertainty of three bands, including the two bands either side of the band to which the breakpoint has been assigned, because of the tendency to assign breakpoints to pale bands. Depending on the similarity of bands from the chromosome regions involved, however, this uncertainty may be even wider (e.g. Barg *et al.*, 1982 and Lavinha *et al.*, 1984; Callen, 1986; the uncertainty of the location of the APL-specific t(15;17) breakpoints).

When using fragile sites for gene mapping studies (see also section 1.6), their precise localization is of distinct benefit. One disadvantage, however, is that labelling of the region on the opposite side of the fragile site to that to which the probe hybridizes will generally be seen. So far there has been, for each localization reported here (except that of *D16S4*), additional evidence that the DNA sequence of interest is not split by the fragile site (see discussion in Simmers *et al.*, 1987A; and the evidence that the α-globin gene complex is in band 16p13, and that *D16S5* is distal to the breakpoint in 16q22.15, all away from the respective fragile sites). In the case of *D16S4*, a possible recombinant between *FRA16B* and *D16S4* has been found, but there is no strong evidence separating the two loci. Unless there is evidence to the contrary, there is always a possibility that the fragile site splits a DNA sequence or gene of interest.

Callen *et al.* (1987) described a novel method for determining whether scatter across a fragile site represents splitting of the DNA region homologous to the probe. The levels of scatter across the fragile site were compared, for GSRs both narrowly and widely separated by the fragile site. A decrease in the amount of scatter with increased separation can be equated with hybridization to only one side of the fragile site (Callen *et al.*, 1987). The relative levels of scatter across the fragile site for two probes were also used to suggest an order for the loci (Callen *et al.*, 1987).

In addition to the studies reported here, *in situ* hybridization has been employed to localize DNA sequences on chromosomes expressing fra(X)(q27)

(Szabo *et al.*, 1984; Mattei *et al.*, 1985B; Purrello *et al.*, 1985A, B; Oberlé *et al.*, 1986; Patterson *et al.*, 1987). The identification of flanking markers in such a way can contribute greatly to carrier detection and prenatal diagnosis of the fragile X by family studies (Oberle *et al.*, 1986;Mulley *et al*, 1987), in the absence of a direct fragile X probe.

As gene mapping advances, regional localizations and chromosome maps will ideally be compiled on a body of evidence which gives independent confirmation and consistency of results using different approaches.

5.4.7.The fragile sites on chromosome 16

The *in situ* hybridization studies reported here included the use of each of the fragile sites on chromosome 16. This chromosome can therefore be divided into four main regions, separated by *FRA16A*, *FRA16B/FRA16C* and *FRA16D*. The use of breakpoints of chromosome rearrangements can further divide chromosome 16 into smaller regions for localization (Callen, 1986; Callen *et al.*, 1987).

The rare fragile sites (by definition, Sutherland and Hecht, 1985) occur only in some individuals. The fact that the set of rare fragile sites occurs at very exact positions, with specific conditions required for their expression, indicates that they probably result from the mutation of a few characteristic types of DNA structure.

Yunis and Soreng (1984) noted that some of the fragile sites they described are assigned to the same bands as rare fragile sites (4 of 16 rare and 51 common fragile sites in the same band). An updated comparison from HGM8 (Berger *et al.*, 1985) reveals 6 of the 18 rare fragile sites in the same band as 6 of the 71 common fragile sites. Ledbetter *et al.* (1986; 1987) have also described a common fragile site at the same location as fra(X)(q27), inducible in normal human metaphases with a high concentration of aphidicolin (1.5μM) (Ledbetter *et al.*, 1987). It has therefore been suggested that rare fragile sites result from the mutation of common fragile sites (Yunis and Soreng, 1984; Ledbetter *et al.*, 1986).

Figure 5.10C illustrates what is possibly the simultaneous expression of *FRA16B* and *FRA16C*, seen in chromosomes from a *FRA16B* individual in which both fragile sites had been induced, using BrdU and FdU/caffeine. Another, unclear example was also seen (see section 5.3). Such a figure was not seen in any individual without *FRA16B*, but fig. 5.10E illustrates a similar, but relatively larger, fragment, infrequently observed in the chromosomes of individuals without *FRA16B*, in which the common fragile sites had been induced with FdU or FdC, and caffeine. It probably represents *FRA16C* and the gap seen higher on 16q, in the middle of band 16q21 (see Materials and Methods). In fig. 5.10C, there is apparently an isochromatid gap proximal to a single chromatid gap. If this figure illustrates the simultaneous expression of *FRA16B* and *FRA16C*, then this mode of expression suggests that *FRA16B* is the more proximal of the two (see Results). This is consistent with the observation (see Results) of a dark region (a small portion of band

16q21?) immediately distal to *FRA16B*, but not *FRA16C*: this appears to be present distal to the gap, on the chromatid on the left, in which the distal fragile site is not expressed. Confirmation that *FRA16B* is proximal to *FRA16C* would depend on more cases of such apparent simultaneous expression being observed, only in the chromosomes of individuals who carry *FRA16B*. Alternatively, the localization of a DNA sequence between *FRA16B* and *FRA16C* by *in situ* hybridization would prove that they do not coincide, and that in this case, the rare fragile site is not a mutation of the common fragile site. The probe ACH207, which defines a locus closely distal to *FRA16B* (see section 5.4.5), might lie between *FRA16B* and *FRA16C*.

This chapter described part of a larger study, of which one aim is to characterize the fragile sites. The nature of the DNA at fragile sites is unknown, and many different hypotheses have been advanced to explain their expression and unusual inheritance, mostly based on fragile X research: Nussbaum and Ledbetter (1986) have provided an excellent critical review of research in this area and of these hypotheses.

Isolation of the DNA at a fragile site should ultimately bring its structure to light. If this can be achieved for the DNA of fra(16)(p12), a folate sensitive fragile site, the structure of the DNA at fra(X)(q27) (*FRAXA*) might be educed. The *FRA16A* DNA might be used as a direct probe for fra(X)(q27), if sufficiently homologous, or its features used to determine a method for cloning *FRAXA*. Although the extent of structural similarity between the folate-sensitive and other classes of fragile sites is not known, the cloning of one of these

(*FRA16B*, *FRA16C* or *FRA16D*) should shed some light on the general nature of fragile sites.

Eventual identification of the fragile site DNA might again involve *in situ* hybridization to metaphase chromosomes in which they have been induced. Such experiments would be extremely useful if proceeding in a direction towards the fragile site (e.g. Davies, 1986), indicating when the fragile site has been passed. If the DNA at the fragile site is single stranded, as is suggested by Sutherland *et al.* (1985), then *in situ* hybridization or Southern analsis of a candidate fragile site probe might be successful without prior denaturation of the target DNA. This would in turn support the notion of single-strandedness at the fragile site.

In the case of *in situ* hybridization of a putative fragile site probe to chromosomes expressing this fragile site, label would appear directly over the gap, if this is where its DNA lies when the fragile site is expressed. Such a location would confirm the identity of the probe.

5.5.SUMMARY AND CONCLUSIONS

Mapping studies on a number of loci on chromosome 16 were described in this chapter. With the use of fragile sites, and two chromosome rearrangements, the map of chromosome was refined considerably. The order of these chromosome 16 loci (underlined) and the other loci discussed is: 16pter - α-globin gene complex - *FRA16A* - *PGP* - 16cen - *MT* - *FRA16B* - (?*FRA16C*), *D16S4* - *TAT* - *HP* - *D16S5*, *FRA16D* - *APRT* - 16qter.

The use of fragile sites for gene mapping aided in the redefinition of the regional localizations for some loci, which were shown to have been previously incorrectly localized.

CHAPTER 6

INVESTIGATING THE PROPOSED RELATIONSHIP BETWEEN FRAGILE SITES AND CANCER

6.1. INTRODUCTION

Yunis (1984B) and LeBeau and Rowley (1984) were the first to make a comparison of fragile site and cancer breakpoint locations on an idiogram of the human chromosomes, after finding some patients with cancer breakpoints in their malignant cells in the same bands as rare autosomal fragile sites. Hecht and Hecht (1984A) and Hecht and Sutherland (1984), using larger sets of known cancer breakpoints and fragile sites, found the relationship to be significant. Significant correspondence has also been found between the common fragile sites and cancer breakpoints (Hecht and Hecht, 1984A; Hecht and Glover, 1984). It became apparent that a possible relationship, both positional and functional, existed. Cancer chromosome rearrangements were hypothesized to occur at fragile site loci, and be facilitated by their presence (Yunis, 1983; LeBeau and Rowley, 1984; Hecht and Hecht, 1984A).

This chapter consists of two sets of experiments designed to test for this proposed correspondence between fragile sites and cancer breakpoints. They are the first reported attempts at testing for such correspondence at the actual level of the DNA.

6.2.MATERIALS AND METHODS

The MT probes and in situ hybridization experiments performed with these *MT* probes are detailed in section 5.2.

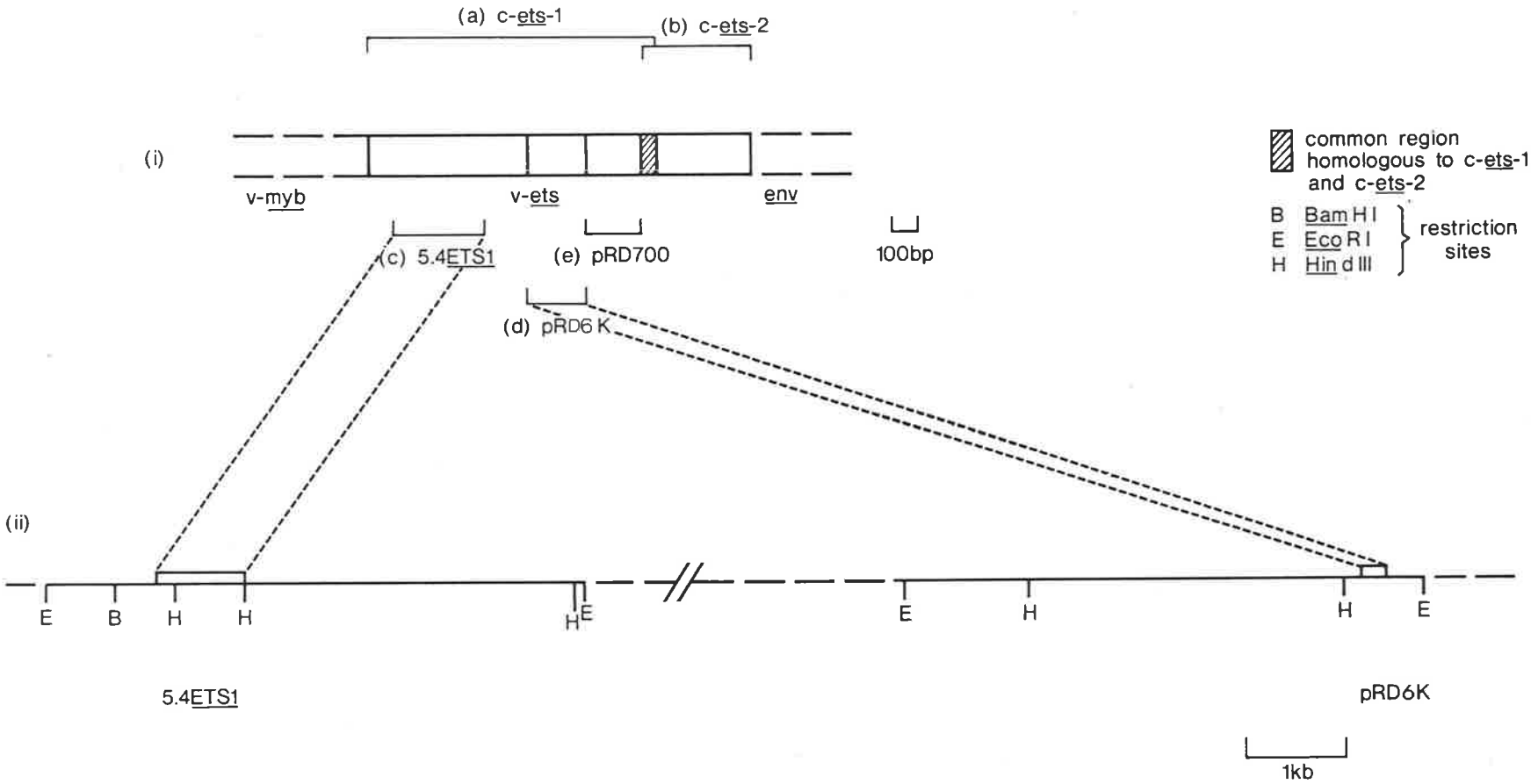
The c-ets-1 gene probe used for these investigations (hereafter termed 5.4*ETS1*) is described by de Taisne *et al.* (1984). These authors cloned a 5.4kb genomic *Eco*RI fragment into the plasmid pKH47. The 5.4kb repeat-free insert hybridizes to another, 8.6kb genomic fragment which itself has homology to v-*ets* (see figure 6.1).

In situ hybridization of the c-ets-1 probe. These experiments were carried out in two parts. The probe 5.4*ETS1* was hybridized to chromosomes expressing the rare fragile site, fra(11)(q23) (see appendix AIII.12).

There is a consistent chromosome gap which can be induced in lymphoblastoid culture by induction with BrdU. This is located in sub-band 11q23.1, just above 11q23.2 (E. Baker, personal communication). The 5.4*ETS1* probe was also hybridized *in situ* to pre-photographed metaphases expressing this gap. Location and photography before *in situ* hybridization ensured that metaphases expressing this gap could be recognized if the morphology was worsened by the *in situ* hybridization process. As the gap is often small, these photographs provided a record of the metaphase in the event that the gap was partially or totally covered by a silver grain.

Figure 6.1.

A diagram illustrating the regions of the (i) E26 and (ii) human genomes homologous to the probes 5.4*ETS1* (de Taisne *et al.*, 1984; D. Stehelin, personal communication) and pRD6K (Watson *et al.*, 1985; Watson *et al.*, 1986A). The probe p-ets-BB contains most of v-*ets*, as a 1.2kb insert from E26, with homology to both c-*ets*-1 (including the regions recognized by both 5.4*ETS1* and pRD6K) and c-*ets*-2. The v-*ets* sequence (e.g. p-ets-BB) recognizes 8.6, 6.2 and 0.83kb *Eco*RI fragments from c-*ets*-1, and a 3.6kb *Eco*RI fragment from c-*ets*-2 (Watson *et al.*, 1985; Watson *et al.*, 1986A). 5.4*ETS1* recognizes the 8.6kb human *Eco*RI fragment (de Taisne *et al.*, 1984) and pRD6K recognizes the 6.2kb *Eco*RI fragment ((Watson *et al.*, 1985).


(a) c-ets-1
(b) c-ets-2
(i)
v-myb
v-ets
env
(c) 5.4ETS1
(e) pRD700
100bp
(d) pRD6 K
common region homologous to c-ets-1 and c-ets-2
B Bam H I
E Eco R I
H Hin d III
restriction sites
(ii)
E B H H H E E H H E
5.4ETS1
pRD6K
1kb

6.3.RESULTS

6.3.1.Metallothionein gene probes

The results of hybridization of the *MT*-specific probes to chromosomes expressing *FRA16B* and *FRA16C* have been detailed in section 5.3. All results statistically testable by chi-square analysis showed a significant difference between the level of grains on either side of the fragile site, with the excess proximal to this fragile site. Other results, although not in sufficient numbers for chi-square analysis, showed either no grains on the 16q expressing the fragile site, or an excess proximal to the fragile site.

6.3.2.c-*ets*-1 gene probe

Results of hybridization of 5.4*ETS1* to chromosomes expressing fra(11)(q23) and the LCL-specific gap at 11q23.1 are given in appendix AIII.12. A significant excess of silver grains was observed distally in both cases, indicating that the probe has hybridized distal to both fra(11)(q23) and the LCL-specific gap.

Eighty-three prelocated metaphases expressing the LCL-specific gap at 11q23.1 on 104 chromosomes 11 (i.e. in one or both chromosomes 11 of each metaphase) were scored after *in situ* hybridization with 5.4*ETS1*.

Silver grains were further scored on the distal acentric fragments of both the fragile site and the gap induced in the LCL. Any grain

touching this fragment was scored, including "central" grains which touched the acentric fragment. These silver grains were classified as closer to the distal or proximal end of the acentric fragment, or not unambiguously closer to either end (as described in Simmers *et al.*, 1987C, for sub-localization of the α-globin gene cluster).

The result of scoring distal to fra(11)(q23) is equivocal: there is not enough evidence to determine if *ETS1* is closer to either fra(11)(q23) or 11qter (table 6.1). However, the result of scoring distal to the gap induced in the LCL at 11q23.1 indicates that *ETS1* is probably closer to 11qter than to the gap (table 6.1; detailed more fully in appendix AIII.12).

6.4.DISCUSSION

6.4.1.Chromosome rearrangements in cancer

As the chromosomes of malignant cells continue to be examined in detail, using high-resolution banded chromosomes, most are found to have chromosome rearrangements (e.g. Yunis *et al.*, 1981; 1984). Certain rearrangements occur consistently in the cells of one or more types of cancer (Yunis, 1983; Mitelman, 1983; 1985; Berger *et al.*, 1985). The bringing together of certain DNA regions in this way has provided a proliferative advantage for the cell (Rowley, 1984).

As a result of some translocations, a gene encoding a protein related to the control of proliferation, probably a

Table 6.1.

Sub-localization of *ETS1* by scoring of the grains over the acentric fragment distal to (i) fra(11)(q23) and (ii) the LCL-specific gap at 11q23.1.

	distal grains	proximal grains	X^2 (*probability*)	ambiguous or central grains
(i)	41	35	0.47 (*n.s.*)	19
(ii)	23	11	4.24 (*P<0.05*)	13

proto-oncogene, may come under cis transcriptional regulation by a sequence normally regulating (and upstream to) a gene functioning in the cell at its current stage of differentiation (Rowley, 1984; Heim and Mitelman, 1986). Alternatively, part of the transcribed region of a proto-oncogene may be joined to and transcribed with part of another sequence, resulting in a novel gene product with altered structure and function (Bishop, 1987). This latter mechanism is exemplified by the Philadelphia translocation of chronic myelogenous leukaemia, in which the c-*abl* proto-oncogene on chromosome 9 has been relocated adjacent to a sequence on chromosome 22, identified by the clustering of the chromosome 22 breakpoints within a limited region as the breakpoint cluster region (Groffen *et al.*, 1984), producing an abnormal *bcr-abl* protein (Heisterkamp *et al.*, 1985).

Yunis *et al.*(1981) provided the first report of an abnormality of chromosome 16 in a case of acute myelomonocytic leukaemia (AMMoL or ANLL-M4). This was defined as an inv(16)(p13q22), using methotrexate synchronization for high resolution chromosome banding. Arthur and Bloomfield (1983A; 1983B) noted the specificity of the chromosome 16 abnormality, and Le Beau *et al.*(1983) noted a very strong correlation between the inv(16) karyotype in M4 and an abnormal but distinctive eosinophil morphology and cytochemistry. This new category of M4, termed M4-EO, strongly correlated with the presence of the del(16) or inv(16), has been added to the FAB classification of leukaemias (Bennett *et al.*, 1985). Bone marrow eosinophilia is a common but not universal feature. No significant morphological differences have been found

between cases with the del(16) and those with the inv(16) (IV IWCL, 1984B).

A translocation between the two chromosome 16 homologues, with what appear to be the same breakpoints, has also been reported in this leukaemia (Testa *et al.*, 1984). The metallothionein gene cluster (*MT*; see chapter 5) is split by this translocation, and by the inv(16) (Le Beau *et al.*, 1985C). This gene cluster and its localization have been discussed in chapter 5.

Other variant rearrangements involving band 16q22 in M4-EO have been noted in association with M4-EO: e.g. a t(16;17)(q22;q25) (Tsai *et al* (1986)) and a t(5;16)(q33;q22)(Bhambhani *et al.* (1986)). However, the identity of the del(16)(q22) has been questioned (Yunis, 1984A). Poor chromosome morphology may have in such cases resulted in misclassification of the inv(16)(p13q22), as is evidenced by the inherent difficulty in detecting and evaluating the abnormal 16 (e.g. LeBeau *et al.*, 1983; IV IWCL, 1984D), especially in distinguishing between the inv(16) and the del(16)(e.g. Harth *et al.*, 1986; Bernard *et al*,. 1986). Examination of published photographs of the "del(16)" (Arthur and Bloomfield, 1983B; Harth *et al.*, 1986) supports the suggestion that they are actually the inv(16). Supposing that the inv(16) (or t(16;16)) brings an oncogene under the control of an *MT* promoter (Le Beau *et al.*, 1985C; Haslinger and Karin, 1985), it is difficult to predict how a deletion (presumably at the same breakpoint within the *MT* cluster) could bring about the same clinical phenotype.

Rearrangements involving 11q, most being at 11q23, have been strongly associated with the M4 (acute myelomonocytic leukaemia, AMMoL) and M5 (pure acute monocytic leukaemia, AMoL) forms of ANLL (IV IWCL, 1984C), especially M5a, with poorly differentiated blast cells (IV IWCL, 1984B); and also with the M2 (acute myeloblastic leukaemia, AML, with maturation) form with abnormal monocytic proliferation (Yunis, 1984A). The other chromosome region involved in these translocations is variable, but is most frequently 9p22 (IV IWCL, 1984C). Hagemeijer *et al.* (1982) found the t(9;11) to be the most frequent abnormality in a series of ANLL patients, recognizing its specific association with M5 leukaemia.

A t(4;11)(q21;q23) (Oshimura *et al.*, 1977), has been specifically associated with acute lymphocytic leukaemia (ALL) (Van den Berghe *et al.*, 1979).

The acute leukaemia with the t(4;11) has characteristics of both myeloid and lymphoid leukaemias, and is therefore of uncertain origin (Parkin *et al.*, 1982; Nagasaka *et al.*, 1983; Strong *et al.*, 1985; Strong and Kersey, 1984; Daeschner *et al.*, 1985; Campos *et al.*, 1986; De Braekeleer, 1986B; Zaccaria *et al.*, 1986). Kaneko *et al.* (1986) have also reported differing lymphoid and myeloid characteristics in those cases of ANLL with translocations involving 11q23. These two categories of acute leukaemia (ANLL and the t(4;11) acute leukaemia) with involvement of 11q23 are also similar in being highly age-specific (Abe *et al.*, 1983; Prigogina *et al.*, 1986; Arthur *et al.*, 1982).

A consistent t(11;22), along with a variety of other multiple chromosome abnormalities, has been reported in association with three solid tumours: Ewing sarcoma of the bone (ES) (Aurias *et al.*, 1983; Turc-Carel *et al.*, 1983); and the peripheral neuroectodermal tumours, peripheral neuroepithelioma (NE) (Whang-Peng et al., 1984; Whang-Peng *et al.*, 1986) and Askin tumour (AT) (Whang-Peng *et al.*, 1986). The breakpoints of the translocation were localized to the interfaces of bands 11q23 and q24, and 22q11 and q12, and the translocation thus termed t(11;22)(q24;q12) (Turc-Carel *et al.*, 1983). These breaks may however be within the surrounding sub-bands 11q23.3 and 22q11.23, or bands 11q24 and 22q12 (Turc-Carel *et al.*, 1984).

The c-*sis* oncogene is distal to the breakpoints on chromosome 22 of both ES and NE (Whang-Peng *et al.*, 1984; Geurts van Kessel *et al.*, 1985), and Whang-Peng *et al.* (1984;1986) concluded, from this and cytogenetic evidence, that the ES (of unknown histiogenesis) and NE and AS (both with evidence of neural origin) may have a common histiogenesis (Kaplan *et al*, 1987 stated that all three have a common neuronal origin) or a common basis for malignant transformation. The specific translocations of ES and NE have also been studied at the molecular level using other markers, by *in situ* hybridization: the translocation breakpoints of both have the same location with respect to the Ig lambda light chain locus on chromosome 22 (Emanuel *et al.*, 1986) and to part of the c-*ets*-1 gene on chromosome 11 (Griffin *et al*, 1986). This additional molecular data further supports the identity of the ES and NE translocations; that of Askin tumour is likely to give identical results.

There have been other reports of non-random involvement of band 11q23 in chromosomal abnormalities,including chromosome rearrangements involving band 11q23 among a number of specific abnormalities in various myelodysplastic syndromes (Raskind *et al.*, 1984; Feder *et al.*, 1985; Ohyashiki *et al.*, 1985B; 1986).

6.4.2.The c-*ets*-1 oncogene

The v-*ets* oncogene was found in the replication defective avian retrovirus E26, and occurs in this virus adjacent to another oncogene, v-*myb* (Leprince *et al.*, 1983). Transcripts of these two oncogenes are included in a hybrid protein, $P135^{gag\text{-}myb\text{-}ets}$ (Nunn *et al.*, 1983). The chicken proto-*ets*, or c-*ets* locus is probably over 50kb long, with an exon complexity of about 7.5kb (Nunn *et al.*, 1984). The intron-less v-*ets* of E26 encodes a 1.5kb mRNA (Watson *et al.*, 1986), homologous to the 3′ end of the chicken gene (Nunn *et al.*, 1984). The mammalian counterpart of this oncogene is present as two distinct genes: c-*ets*-1 and c-*ets*-2, at the *ETS1* and *ETS2* loci respectively. In humans c-*ets*-1 is on chromosome 11, and c-*ets*-2 on chromosome 22 (Watson *et al.*, 1985).

The *ETS1* probe, 5.4*ETS1*, was used for the experiments reported here (see Materials and Methods). The region of homology with *ETS1* is shown in figure 6.1. *ETS1* has been localized to 11q23->q24 (de Taisne *et al.*, 1984), and a number of authors have tested for involvement of the c-*ets*-1 oncogene in the cancer chromosome rearrangements with breakpoints at 11q23, and localized *ETS1* with respect to these breakpoints. These studies are summarized in table 6.2.

Table 6.2.

Summary of the results of localization of *ETS1* probes with respect to cancer breakpoints and tests for rearrangement of the c-*ets*-1 oncogene.

Source	probe	rearrangement and results
Sacchi et al., 1986	pRD6K	t(4;11) in AL; distal; no rearrangement detected in four cases with "several restriction enzymes"; reduced level of c-*ets*-1 transcription compared to lymphocytes. t(9;11) in ANLL; no rearrangement detected with "several restriction enzymes".
Diaz et al., 1986	p-ets-BB	t(9;11) in ANLL; distal; no rearrangement dectected in two cases with *Bam*HI or *Hin*dIII.
Caubet et al., 1986	5.4*ETS1*	t(1;11)(q21;q23) in M5; distal.
Morris and Fitzgerald, 1987	(?)	t(11;19)(q23;p13) in blast crisis of chronic myeloid leukaemia; distal; no rearrangement detected with "several restriction enzymes".
Rovigatti *et al.*, 1986	pRD6K	HSR[1] 11q23 in ANLL-M4; c-*ets*-1 rearranged and amplified. inv ins(21;11)(q22.3;q23q14.2) in small lymphocytic cell lymphoma; c-*ets*-1 rearranged and amplified; c-*ets*-2 rearranged.
Griffin et al., 1986	5.4*ETS1*	t(11;22) in ES and NE; proximal; no rearrangement detected in thirteen cell lines with four restriction enzymes, for a distance of 25kb including the region homologous to. 5.4*ETS1*.

1. HSR: homogeneously staining region.

Rearrangement and amplification of c-*ets*-1 was observed in two chromosome abnormalities involving band 11q23 (Rovigatti *et al.*, 1986), presumably with associated increase in the c-*ets*-1 product. However, no rearrangement of c-*ets*-1 was detected in any of the translocations with breakpoints at band 11q23 (see table 6.2). The c-*ets*-1 oncogene, however, is of unknown length, but probably larger than 25kb (Griffin *et al.*, 1986). All of those probes used for the experiments listed in table 6.2 covered only regions with homology to v-*ets*, whereas three of the ten peptides the encoded by the cellular (chicken) *ets* are not encoded by v-*ets* (Ghysdael *et al.*, 1986). Although the functions of the c-*ets*-1 and c-*ets*-2 products are not known, they appear to play a role in haematopoiesis (Nunn *et al.*, 1984; Ghysdael *et al.*, 1986; Bhat *et al.*, 1987). These observations, and those of alteration of the c-*ets*-1 DNA (Rovigatti *et al.*, 1986) or the level of c-*ets*-1 expression (Sacchi *et al.*, 1986) are not inconsistent with the possibility that the translocations at 11q23 in acute leukaemia occur through a region of the gene or its regulatory region, as yet undetected by Southern analysis.

The predicted involvement of the c-*ets*-1 gene or some other gene involved in myeloid differentiation on chromosome 11 (Yunis, 1983; Rowley, 1984; Strong *et al.*, 1985), has yet to be demonstrated in the rearrangements in acute leukaemia at 11q23. Only the specificity of the breakpoint at 9p22 in the t(9;11) of AMoL has been explained so far: it splits the interferon gene cluster (Diaz *et al.*, 1986). A *cis*-regulatory sequence for one of the interferon genes may control the expression of a gene at 11q23, possibly c-*ets*-1, in this case (Diaz *et*

al., 1986). Chromosome 11 is involved in many translocations with other chromosomes, at band 11q23, in leukaemias with at least some myeloid characteristics. It has not been demonstrated that these breakpoints coincide, nor has it been determined in what way all of these rearrangements contribute to carcinogenesis.

6.4.3.Finer localization of *ETS1*

Two *in situ* hybridization distributions from *ETS1* localization have been published: that of de Taisne *et al.* (1984) shows a peak over band 11q24; and that of Rovigatti *et al.* (1986) shows a peak over sub-band 11q23.3, of both a normal chromosome 11 and one with a homogeneously staining region (HSR) at 11q23. De Taisne *et al.* (1984) gave a regional localization for *ETS1* including band 11q23 (11q23->q24) to account for the possibility of observer bias towards the pale R-band, 11q24. This precaution is in line with the lack of mapping resolution to within one band by *in situ* hybridization alone. Griffin *et al.* (1986) have sub-localized *ETS1* to 11q23.3->q24 using a constitutional t(11;22) with a breakpoint at 11q23.3. Localization of *ETS1* proximal to the breakpoint on 11q of ES and NE (Griffin *et al.*, 1986) does not lead to further mapping refinement, as the breakpoint(s) may be in band 11q24 (see above). Sacchi *et al.* (1986B) have incorrectly cited Watson *et al.* (1985) for a localization for *ETS1* within band 11q23: Watson *et al.* (1985) only cited that *ETS1* is on chromosome 11.

The results of the *in situ* hybridization studies reported here provide further information on the whereabouts of *ETS1*.

The c-*ets*-1-specific probe hybridized distal to fra(11)(q23). Even if the *ETS1* locus does span the fragile site (see Simmers *et al.*, 1987A; section 5.4.6), the location of *ETS1* can be further defined, from the position of the fragile site, *FRA11B*, to the end of 11q24 (as determined by de Taisne *et al*, 1984) (fig. 6.2). As *FRA11B* is within band 11q23.3, just distal to the midpoint of this band, this is a narrower region than that defined by Griffin *et al.* (1986).

By examining the locations of silver grains on the acentric fragments distal to fra(11)(q23) and to the LCL-specific gap at 11q23.1 (see table 6.1; results), more information on the location of *ETS1* was sought. *ETS1* is not close enough to either *FRA11B* or to 11qter to give a significant excess of grains in either direction at this level of resolution. The midpoint of the region *FRA11B*->11qter is in the distal half of band 11q24 (fig. 6.2). This suggests that *ETS1* might be within band 11q24. Results of scoring on the distal half of the acentric fragment distal to the LCL-specific gap show that *ETS1* is closer to 11qter than to this gap in sub-band 11q23.1. The midpoint of this fragment is near the distal boundary of 11q23.3 (fig 6.2). These data also point to the location of *ETS1* within band 11q24. This is not strong evidence however, as it is not known at what distance from this mid-point a locus may give sufficient difference in signal to enable the resolution of its localization to one or the other side of this point.

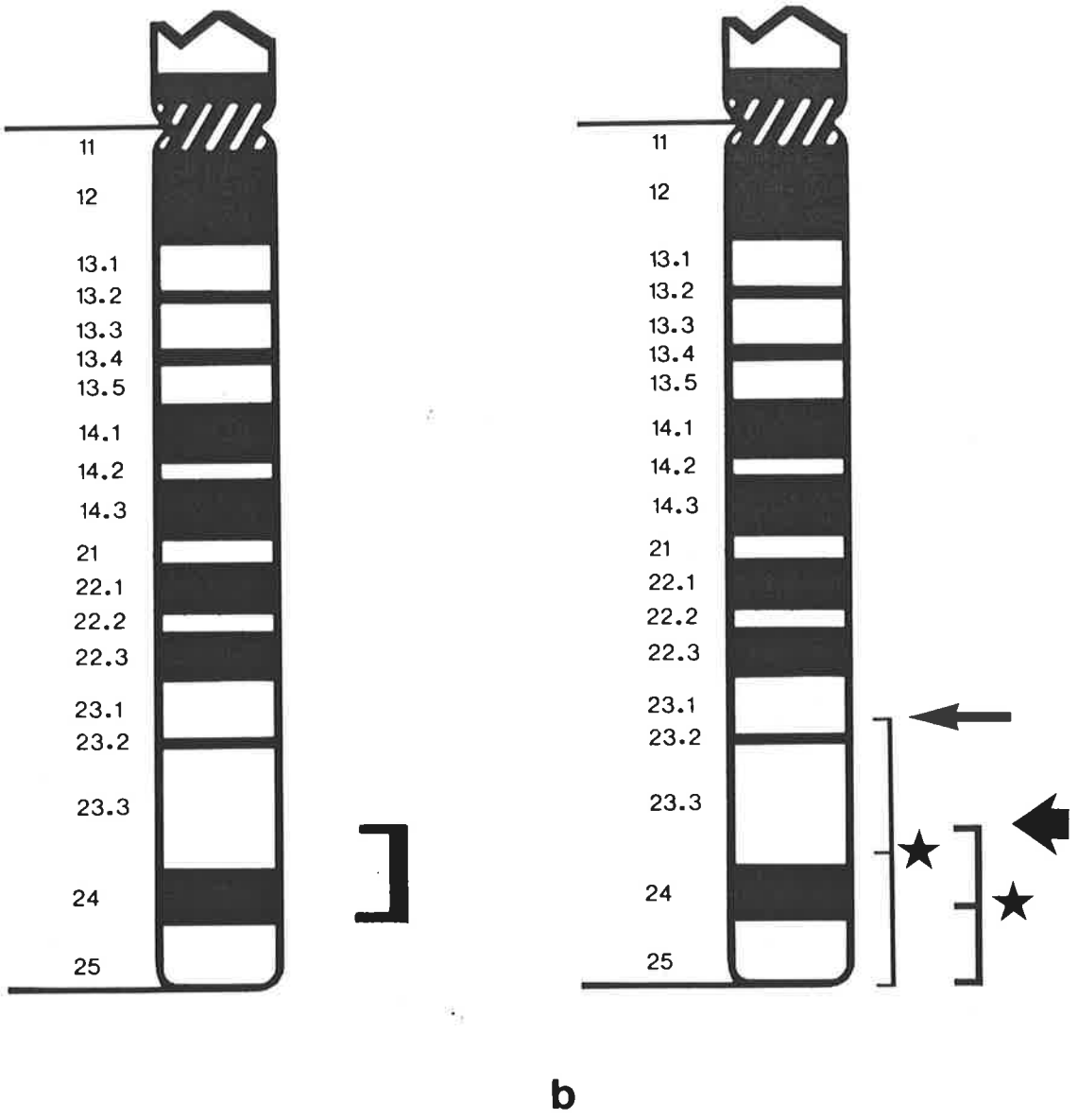


Figure 6.2.

Idiograms of the long arm of chromosome 11, showing (a) the regional localization for *ETS1* determined in this chapter, from *FRA11B* to 11q24, and (b) the site of the LCL-specific gap at 11q23.1 (upper arrow) and *FRA11B* (lower arrow), and the points mid-way from these markers to 11qter (*), are indicated.

6.4.4.Fragile sites and cancer breakpoints

Non-homologous recombination, the basis for acquired chromosome rearrangements, was thought to occur entirely at random within the genome (Rowley, 1984). However, mechanisms of non-random recombination, from which oncogenesis may result, have been discovered. Misdirected recombination may occur in B-cell and T-cell lineages as follows. Immunoglobulin and T-cell receptor genes may sometimes incorrectly recombine with sequences similar to those recognized by the respective recombination enzymes; and some of these non-random rearrangements may provide a selective, proliferative advantage to the cell (Haluska *et al.*, 1987).

In other cell types, where no normal recombination mechanism occurs, although rearrangement might arise from illegitimate recombination between other repeated sequences found in the genome (Rogers, 1985; Heisterkamp and Groffen, 1985), no other mechanisms for chromosome rearrangement, which can lead to malignancy, have been shown (Haluska, 1987). However, chromosome breakage and rearrangement at fragile sites has become a popular, and much touted notion (see Introduction). The fragile site divides the chromosome into two distinct portions, and sometimes deleted and tri-radial figures are seen, evidence of actual breakage at the fragile site in vitro (Sutherland, 1979A).

The major area of search for evidence for chromosome rearrangement at fragile sites is that relating to the non-random breakpoints seen in cancer chromosome rearrangements ("cancer breakpoints"). Two lines of

evidence have been presented by various authors for a positive correlation between fragile sites and cancer breakpoints. The first involves placing the known fragile sites and the known chromosome breakpoints in cancer on an idiogram of the human chromosomes, and testing for significant correspondence (see Introduction). The second involves the finding of patients with a cancer breakpoint and a fragile site in the same chromosome band (see later).

The experiments reported here investigate the relationship between three fragile sites, *FRA16B*, *FRA16C* and *FRA11B*, and the cancer breakpoints in the same bands, 16q22 and 11q23, described above. Probes homologous to portions of the metallothionein gene cluster (*MT*) were hybridized to chromosomes expressing *FRA16B*, and another fragile site in this region, *FRA16C*, to test for correspondence with the specific breakpoint in AMMoL at 16q22. *In situ* hybridization of a probe homologous to c-*ets*-1 to chromosomes expressing fra(11)(q23) was also carried out in order to reveal something about the relationship between the fragile site fra(11)(q23) and the cancer breakpoints at 11q23, by comparing the results to previously reported relationships between c-*ets*-1 and these breakpoints. The probe was also hybridized *in situ* to chromosomes expressing the LCL-specific gap at 11q23.1, because of the possibility that it was involved in the rearrangements at 11q23, and/or would provide further information about the location of *ETS1*.

If fragile sites do predispose an individual to developing cancer, one would find a greater than expected frequency of a certain type of cancer or group of cancers with a specific breakpoint in individuals with the

corresponding rare fragile site in their normal cells, and conversely, in groups of individuals suffering from a particular type of cancer, with a specific cancer breakpoint, one would find a greater than expected frequency of carriers of the corresponding fragile site. The latter would indeed seem to be the case for M4-EO with the inv(16), as evidenced by the number of these patients reported to be heterozygous for *FRA16B*. Table 6.3 describes the reported cases where M4-EO patients with the inv(16) have been tested for carrier status with respect to *FRA16B*. Fourteen of the 29 patients are reported to carry *FRA16B*, but this figure is difficult to evaluate. Only four of the positive cases reported are well documented, with conditions of induction and percentage expression mentioned, and the percentages indicate that these are probably *FRA16B*. The methotrexate synchronization method of Yunis (1984A) should not induce expression of this fragile site, but it may have been spontaneously expressed in these cases.

Studies have shown that the frequency of *FRA16B* in the Australian population is about 1% (Sutherland, 1985B), or more recently, 5% in the German population using the inducing agent berenil (Schmid *et al.*, 1986). Thus, the proportion of cases of AMMoL claimed to have *FRA16B* would appear to be higher than in the general population, supporting a possible causative effect.

This relationship was investigated by some of the experiments reported in this chapter. LeBeau *et al.* (1985C) reported the inv(16) and the t(16;16) in AMMoL to be split by the *MT* cluster. The probe used by these authors cross-

Table 6.3.

Cases in the literature of M4-EO[2] patients with inv(16) or del(16), who were tested for the presence of *FRA16B*.

Source	No. of patients tested for *FRA16B*	No. with *FRA16B* (% expression)	agents used for induction of *FRA16B* in lymphocytes
Yunis, 1984A	2[3]	2[4] (13%; 36%)	none mentioned
Berger et al., 1985	6[5]	0	none mentioned
Arthur and Bloomfield, 1983C	3[6]	1 (0-14%)	þBrdU
LeBeau and Rowley, 1984	5[7]	4 (?)	not mentioned
LeBeau, 1986	6	6 (?)	not mentioned
Harth *et al.*, 1986	1	0 ($^0/_{200}$)	BrdU; Hoechst 33258
Glover *et al.*, 1986	3	0	BrdU; distamycin A
Murata *et al.*, 1987	3	1 (0-8%)	BrdU; distamycin A; Hoechst 33258
Totals:	29	14	

2. Some of these cases were not classified M4-EO, but it will be assumed that this is because of the difficulty of classification to the M4 category and the ready confusion of M4 with M2 and M5 (Bennett et al., 1985). For example, malignant cells of some cases of Yunis (1984A) had morphological characteristics of M4-EO, but were classified as M2 under older FAB rules (Bennett et al., 1976).
3. plus 2 "not tested".
4. in both cases the chromosome carrying FRA16B was inverted.
5. plus 3 "not tested", and one negative control.
6. plus 2 controls, found negative.
7. plus 5 controls, found negative.

hybridizes with all the members of the *MT* gene cluster. Thus, it hybridizes to an overall region similar to that homologous with the *MT2A*r probe. The *MT2A*r probe was therefore first used to test the hypothesis that the AMMoL breakpoints at 16q22 coincide with *FRA16B*. The probe hybridized distal to *FRA16B*. As it hybridized to a large region containing the *MT* genes on 16q, a small part of this region distal to the fragile site would not have been detected. The rearrangements reported in LeBeau *et al*. (1985C) were sufficiently close to the mid-region of the cluster to give a significant *in situ* hybridization signal on both sides of the breakpoints. Chromosomes expressing *FRA16B* from only one individual were tested in this report, however, and it was conceivable that the fragile site could be the mutation of one of a series of reiterated sequences, which might therefore occur in different parts of the *MT* cluster of different individuals.

For these reasons the two probes specific to regions either side of the AMMoL breakpoints on 16q (as determined by M.O. Diaz, M.M. LeBeau and M. Karin, personal communication) were hybridized to chromosomes from several individuals with *FRA16B* inherited from different sources. As discussed in Simmers *et al*. (1987A), the results indicate that the *MT* cluster is proximal to *FRA16B*, and is not split by either fragile site, at least at the site of rearrangement in AMMoL. Therefore *FRA16B* does not coincide with the breakpoint at or near 16q22 in AMMoL, and the rearrangement leading to this leukaemia does not occur by breakage at *FRA16B*.

A further approach to the suspected relationship between fragile sites and cancer breakpoints was provided by Yunis and Soreng (1984), who correlated the presence of specific cancer breakpoints with an increase of expression of corresponding common fragile sites, suggesting that the cancers were the result of a greater risk attached to the increased frequency of expression. As it is likely that all individuals are homozygous for all fragile sites (Sutherland and Hecht, 1985), the correlation between common fragile sites and cancer breakpoints might otherwise be meaningless in terms of predicting individuals predisposed to developing cancer: if all individuals have all the common fragile sites as an integral part of the genome, none is more likely than any other to develop cancer by breakage and rearrangement facilitated by these fragile sites. However, Yunis and Soreng (1984) reported an increased frequency of expression of the common fragile site, *FRA16C*, in a patient with AMMoL and the inv(16)(p13q22), and other similar cases, suggesting that a greater than normal frequency of expression of this and other common fragile sites in certain individuals may also predispose the chromosomes to rearrangement here, leading to cancer.

For completeness, the common fragile site *FRA16C* was tested as a candidate for predisposition to breakage and rearrangement leading to AMMoL. Hybridization of the two probes from either side of the breakpoint in AMMoL, *MT1B*u and *MT2A*u, to chromosomes expressing *FRA16C*, did not reveal splitting of the *MT* cluster by this fragile site. Again, there is further discussion in Simmers *et al.* (1987A).

Further specific instances of patients with a fragile site and a cancer breakpoint in the same band have been reported. Yunis (1983) provided the first of such reports. His cases consist of two patients with small lymphocytic cell lymphoma, found to carry the rare fragile site, fra(11)(q13) (also Yunis *et al.*, 1984); one with a malignant lymphoma and fra(12)(q13); and the previously mentioned cases with *FRA16B* and AMMoL. Other reports include a patient with Ewing sarcoma and fra(11)(q23) (Gollin *et al.*, 1986 - see later), and a patient with myelofibrosis and fra(11)(q13) (Sessarego *et al.*, 1983). In these cases, induction of the fragile site is well documented.

The report of Gollin *et al.* (1986) is particularly relevant to this discussion. They reported fra(11)(q23) in a patient with ES with the t(11;22), suggesting that the translocation was a result of breakage at the fragile site. The result of *in situ* hybridization of the 5.4*ETS1* probe distal to fra(11)(q23) shows that this fragile site does not coincide with the breakpoint in ES, nor with that in NE, to which the same probe hybridized proximally (Griffin *et al.*, 1986 - see table 6.2). Similarly, fra(11)(q23) probably does not coincide with the 11q breakpoint in AT, which appears to be identical to those of ES and NE (see section 6.4.1). Therefore, in the case reported by Gollin *et al.* (1986), the translocation leading to ES did not occur as a result of breakage and rearrangement at fra(11)(q23).

The 11q23 breakpoints of the translocations in AL and ANLL could not be distinguished on the basis of the *in situ* hybridization results, all being proximal to the site of

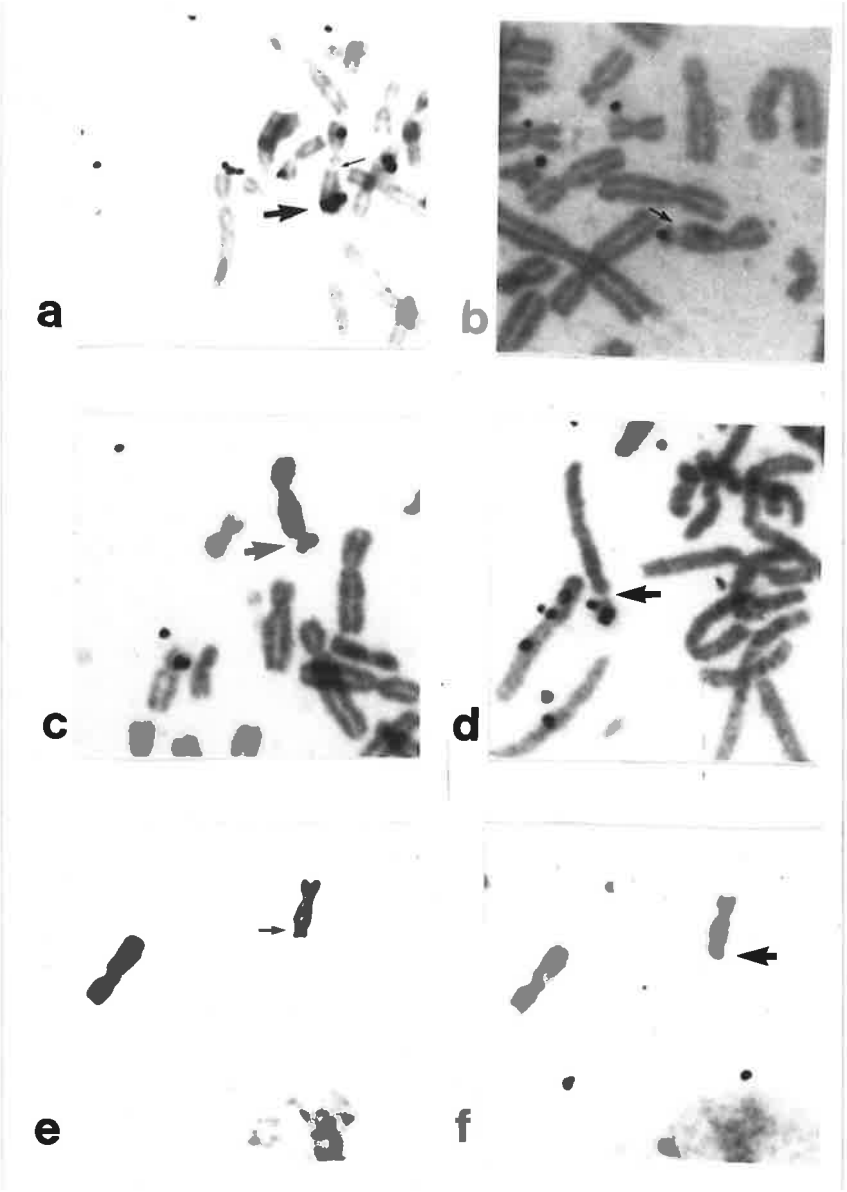


Figure 6.3.

(a) Partial metaphase showing label (large arrow) over *ETS1* on a chromosome expressing fra(11)(q13) (small arrow). (b-d) Partial metaphases showing fra(11)(q23) probed for c-*ets*-1, with silver grains distal to the fragile site (arrows). (e-f) A metaphase expresssing the LCL-specific gap at 11q23.1 (arrow in (e)), both before (e) and after (f) *in situ* hybridization with 5.4*ETS1*. A silver grain distal to the gap is arrowed in (f).

probe hybridization (see table 6.2; fig. 3 in Simmers and Sutherland, 1987). On the basis of the results presented here, neither can they be distinguished from *FRA11B*, which is also proximal to the site of hybridization of the probe, 5.4*ETS1*, used for *in situ* hybridization. However, localization results from the use of pRD6K and 5.4*ETS1*, probes homologous to different regions of c-*ets*-1, as indicated in figure 3 of Simmers and Sutherland (1987), are not strictly comparable: the possibility that the breakpoint at 11q23 in the t(4;11) (Sacchi *et al.*, 1986) or the t(1;11) (Caubet *et al.*, 1986), or *FRA11B*, occurs between the regions of homology to both probes has not been entirely excluded.

The grain distribution from 5.4*ETS1* with respect to fra(11)(q23) (appendix AIII.12; see results) does not suggest splitting of the c-*ets*-1 oncogene by fra(11)(q23). However, as the probe only covers 5.4kb of the much larger gene, these data do not exclude such a possibility. While there is a small amount of evidence for a positional relationship between fragile sites and oncogenes (Yunis and Soreng, 1984; Daniel, 1986), it is probably a secondary effect of the correspondence of each with the locations of cancer breakpoints (Daniel, 1986). The sub-localization results reported here for *ETS1* (see table 6.1 and section 6.4.3) suggest that the oncogene is not close to fra(11)(q23) at the DNA level. If the c-*ets*-1 oncogene is involved in some of the translocations with breakpoints at 11q23 (see section 6.4.2), then these breakpoints would also map away from *FRA11B*.

There is also a common fragile site reported at 11q23.3 (Yunis and Soreng, 1984), but this was not examined in this project with the

5.4*ETS1* probe, primarily because of the difficulty in inducing large numbers of such infrequently expressed fragile sites. Three patients with acute leukaemia with translocations at 11q23 were reported to carry this common fragile site, although elevation of its level of expression was not observed (van den Berghe *et al.*, 1987). Furthermore, Sutherland and Simmers (1987) have presented evidence that there is no statistical association between common fragile sites and cancer breakpoints.

As mentioned earlier, the notion that fragile sites are prone to breakage, leading to chromosome rearrangement, is an attractive one. However, it does not suggest a mechanism for selection for the cells with the rearranged chromosomes (LeBeau and Rowley, 1984). From what is known about fragile sites, there is no reason (apart from the correlations between fragile sites and cancer breakpoints being discussed) to suspect that genes important to the neoplastic process are positioned such that rearrangement at fragile sites would provide a proliferative advantage to the cell.

The experiments presented here provide direct evidence against the specific possibility of non-random rearrangements in malignant cells having occurred by breakage at the fragile site. It could still be argued that the reported correlations between fragile sites and cancer breakpoints are evidence of a more indirect mechanism whereby fragile sites cause cancer, acting at a distance from the resulting breakpoints. Some of the fragile site/cancer breakpoint associations may still be accounted for without resorting to vague and complex alternative mechanisms which ignore the original attractiveness of breakage at

fragile sites. For example, it remains to be explained why such a high proportion of AMMoL patients were reported to carry *FRA16B*. It is possible that *FRA16C* was in fact seen in many of these cases rather than *FRA16B*, especially where the method of fragile site detection and frequency of expression have not been documented (table 6.3). The cases of increased frequency of expression of *FRA16C* in a patient with AMMoL and the inv(16) and of the ES patient with fra(11)(q23) are isolated reports, which might be explained by chance and biassed ascertainment and reporting. The careful re-examination of some of the cancer breakpoints assigned to the same band as a fragile site might lead to the conclusion that some of these assignments were in error (Dutrillaux et al., 1985).

In addition, there is an as yet unexplained significant correlation between the bands containing non-random breakpoints in cancer chromosome rearrangements and the bands containing known fragile sites, both rare and common. Sutherland and Simmers (1987) have addressed this question. To summarize this paper, previous statistical tests for such correspondence did not take into account the phenomenon of most known fragile sites and non-random cancer breakpoints being assigned to light G-bands. Taking only the light G-bands into account, there is no longer significant correspondence with cancer breakpoints for common fragile sites, or for combined rare and common fragile sites.

There still remains a significant correspondence between bands containing rare fragile sites and those containing cancer breakpoints, however. Other factors which might have influenced this phenomenon are

chance, and other features of the chromosome bands, for example band size (De Braekeleer *et al.*, 1985), resulting in different likelihoods that each possesses a fragile site or a cancer breakpoint. Knowledge that a fragile site exists in a certain band may influence the assignment of nearby breakpoints in cancer chromosome rearrangements to the same band (B. Dutrillaux, EMBO Fragile Site Workshop, September, 1986). For example, a chromosome deletion in small lymphocytic cell lymphoma was designated del(11)(q11.5q23) (Yunis *et al.*, 1982) or del(11)(q14.2q23) (Yunis *et al.*, 1984). The breakpoint on 11q in a t(11;14) in the same disease was designated 11q13.3, however, in three patients, two of whom had fra(11)(q13) in their normal karyotype (Yunis *et al.*, 1984). It may be that the breakpoint is identical to that in the del(11), but that the existence of the fragile site at 11q13.3 has influenced its assignment to the same sub-band.

Various papers inferring a relationship between the presences of any rare fragile site and of cancer, without the chromosome band containing the fragile site being involved in the cancer karyotype (e.g. Haas *et al.*, 1985; Shabtai *et al.*, 1985) are more dubious in nature. Shabtai *et al.*(1985) predicted that fragile sites are the sites of viral modification, or the sites of cellular oncogenes, and that the mechanism for development of cancer in patients with fragile sites does not involve breakage at the fragile site, but some other unknown mechanism. Such conclusions are based on a number of observations of cancer patients and families with multiple occurrences of cancer, where fragile sites are also found. It should be noted in the case of these authors, that the common fragile sites are apparently treated as if they were

rare fragile sites. This, together with the likelihood of biassed reporting and ascertainment in these cases, provide little support for a relationship between fragile sites and cancer breakpoints. Certain genetic defects, which might otherwise have been attributed to the concurring fragile sites, have been found in two instances to have been inherited independently of the fragile sites (Tommerup *et al.*, 1986B; Kumar *et al.*, 1986).

Fragile sites have also been statistically associated with consitutional chromosome rearrangements (Hecht and Hecht, 1984B; C). In two reported cases (García-Sagredo *et al.*, 1983; Webb *et al.*, 1987), a child has been found a *de novo* constitutional deletion or rearrangement, the chromosome having apparently broken at the fragile site in its parent. In other reports,the presence of a chromosomal abnormality in a newborn has been attributed to a fragile site in its parent, and an increased risk of chromosomal abnormalities in children of parents with fragile sites inferred (e.g. Ventruto *et al*, 1984; García-Sagredo and San Román, 1984). It may be that breakage has occurred at the fragile sites in the cases reported by García-Sagredo *et al.*(1983) and Webb *et al.*(1987), where the fragile site and translocation breakpoint are within the same band. Unlike cancer chromosome rearrangements, selection of the clone with the chromosome rearrangement is not required. The only requirement would be that the chromosome rearrangement is compatible with life. However, with such anecdotal reports, it is necessary to keep in mind that the breakpoints which appear to be in the same band as the fragile site do not necessarily coincide (Hecht and Hecht, 1984D), and that the data are probably biassed towards reporting of the abnormal cases,

especially where the evidence presented by the case is in line with a particular point of view. Davis and Hagaman (1987) have reanalyzed the association between constitutional rearrangements and fragile sites, finding it to be non-significant.

If the fragile site-cancer breakpoint relationship exists, one would expect to find carriers of rare fragile sites who suffer from cancer, with a cancer breakpoint in the malignant cells in the same band as the fragile site. There should also exist families segregating for rare fragile sites and for a cancer or a group of cancers with specific breakpoints here. The recent literature reports unfruitful searches for cancer chromosome rearrangements within the same band as the fragile sites in fragile site carriers with cancer, and for fragile sites in the normal cells of cancer patients,at the same site as the cancer breakpoint (e.g. Muleris *et al.*, 1987; Dal Cin *et al.*, 1986; Glover *et al.*, 1986). In the reports detailed in table 6.3, those of Harth *et al* (1986), Glover *et al.* (1986) and Murata *et al.* (1987) describe thorough testing for *FRA16B* in patients with AMMoL and the inv(16), with only one positive individual in seven found.

These more recent reports of negative findings, especially those with well documented tests for expression of fragile sites, perhaps balance those initial positive reports.

In short, there is very little solid evidence for a relationship between fragile sites and cancer.

6.5.SUMMARY AND CONCLUSIONS

The results of experiments reported in this chapter provide evidence against three specific examples by which breakage at fragile sites has been suggested to cause chromosome rearrangement leading to cancer. *FRA16B* has been reported in a large proportion of patients with AMMoL and malignant cell rearrangements at 16q22, but this breakpoint does not coincide with the fragile site, nor with the common fragile site in the same vicinity, *FRA16C*, which has been reported to be expressed at an increased frequency in a patient with this leukaemia. Fra(11)(q23) has been reported in a patient with the t(11;22) in ES, but maps away from the breakpoint on 11q. The t(11;22) specific to ES, NE, and probably AS, therefore cannot occur by breakage and rearrangement at fra(11)(q23).

The results of this chapter show that the cancer patients reported in the literature to carry rare fragile sites and have cancer breakpoints in the same bands, 16q22 and 11q23, did not have an increased chance of developing these cancers by the presence of the fragile sites.

APPENDIX AI

MATERIALS AND METHODS

AI.1.Tissue Culture

All tissue culture was at 37°C.

Peripheral blood lymphocytes. Up to eight drops of heparinized venous blood were grown in 5ml culture medium MEM-FA (Commonwealth Serum Laboratories) (Sutherland *et al.*, 1979) or RPMI 1640 (Gibco) containing 10% foetal calf serum (FCS) (Flow), 0.5 units/ml heparin (David Bull Laboratories) and 2% (v/v) phytohaemagglutinin (M form, Gibco) for three days. (Adapted from Moorhead *et al.*, 1960.)

Lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 with 10% FCS, changed two or three times weekly and aerated with 5% CO_2. Medium was changed one day before harvest. (Catalog of Cell Lines, 1985.)

Cell synchronization. Twenty-two hours before harvest, cells were blocked in the S phase by the addition of (i) 0.3mg/ml thymidine (for the "TBu" method) or (ii) 0.2mg/ml bromodeoxyuridine (BrdU) (for the "BuT" method). After washing the cells twice in Dulbecco's phosphate buffered saline (calcium ion- and magnesium ion-free), the block was released by the addition of fresh medium to the original volume (in the case of lymphocyte culture) or 125% of the original volume (in the case

of LCL), with 0.01μg/ml BrdU (for (TBu) or 2.4μg/ml thymidine (for BuT), $5^3/_4$hr (lymphocyte culture) or $5^1/_2$hr (LCL) before harvest. For "BuBu" synchronization, cells were blocked with BrdU as for BuT and released with BrdU as for TbU. (Adapted from Dutrillaux, 1975.)

AI.2.Fragile site induction

Folate sensitive rare fragile sites were induced in lymphocytes by culturing in the folate-free medium MEM-FA (Sutherland, 1979).

Distamycin A-inducible and BrdU-requiring rare fragile sites were induced in lymphocytes by the addition of 50μg/ml BrdU, 6-8hr before harvest (Sutherland *et al.*, 1984).

Aphidicolin-inducible common fragile sites were induced by the addition of 0.2μM (0.07μg/ml) aphidicolin, 24hr before harvest. The aphidicolin was first dissolved in dimethyl sulphoxide at not more than 0.2% of the final culture volume. (Glover *et al.*, 1984.)

FUdR-inducible common fragile sites were induced by the addition of 0.005-0.1mM (1.2-25μg/ml) fluorodeoxyuridine (FdU) or fluorodeoxycytidine (FdC) 24hr before harvest, and 2.2mM (0.43mg/ml) caffeine 6hr before harvest. (Yunis and Soreng, 1984.)

BrdU-inducible gap at 11q23.1 was induced in lymphoblastoid culture by cell synchronization and harvest of lymphoblastoid culture by the BuT method (E. Baker, personal communication).

AI.3.Chromosome harvest and spreading

One drop of 0.1mg/ml colchicine (Sigma) was added to unsynchronized lymphocyte cultures 1hr before harvest, and to synchronized lymphocyte cultures 20min before harvest; and to LCL 15min - 1hr before harvest.

Cells were centrifuged at 1 000rpm, or 2 000rpm (lymphocytes) for 5min, the supernatant removed, and the pellet resuspended in 0.075M KCl to 9½ml.

After 20min the suspensions were mixed with 1ml fixative (3:1 methanol:acetic acid), centrifuged at 1 000rpm for 5min, the supernatant removed and the pellet resuspended. At least four changes of fixative were then made, with the cells spun down (1 000rpm, 5min) and supernatant removed between changes.

Harvested cells were stored at an appropriate concentration in fixative at -20^{o}C or -70^{o}C until spread. If unused within three to seven days after spreading, slides were stored sealed at -20^{o}C with desiccant.

A sample from each harvest was spread onto a dry microscope slide, and one onto a cold wet slide, to determine which, if either, method gave chromosome spreads of a better quality. (Adapted from Moorhead *et al.*, 1960.)

AI.4.Nick translation

Nick translation was performed using an Amersham N.5500 nick translation kit, as recommended by the manufacturers. Tritiated deoxyribonucleotides were used: deoxy [1′,2′,2,8-^{3}H] adenosine 5′-triphosphate (^{3}H-dATP), deoxy [1′,2′-^{3}H] guanosine 5′-triphosphate (^{3}H-dGTP), and deoxy [1′,2′,5^{-3}H] cytidine 5′-triphosphate (^{3}H-dCTP) (Amersham). The enzyme solution and deoxythymidine 5′-triphosphate (dTTP)/buffer solutions from the Amersham N.5500 nick translation kit were used. As an alternative to this dTTP/buffer solution, a dTTP/buffer solution was made with 50 parts nick translation buffer (10 parts = 0.5M Tris Cl (pH7.2), 0.1M $MgSO_4$, 1mM dithiothreitol, 500µg/ml bovine serum albumin), one part 10mM dTTP (Boehringer Mannheim) and 49 parts water (Maniatis *et al.*, 1982).

To label 1µg of DNA, 1 000pmol each of ^{3}H-dGTP, ^{3}H-dCTP and ^{3}H-dATP in ethanol:water (1:1) were desiccated, and to these, a volume of water was added to make a final volume of 100µl, then 20µl of dTTP/buffer and the DNA in water or in T/E (10mM tris[hydroxymethyl] nitromethane/0.1mM ethylene diaminetetraacetic acid [disodium type] [EDTA]). After mixing, 20µl of enzyme solution was added and gently mixed, and the nick translation was allowed to proceed for 2hr at 14-15^{o}C. The reaction was stopped with 10µl EDTA and 10µl 10% sodium dodecyl sulphate (SDS).

The reaction products were phenol extracted, back-extracted with T/E, and chloroform extracted. The volumes of reaction products, and thus DNA lost, were noted and accounted for in the final calculation of

specific activity. These extraction steps were later omitted from the labelling procedure.

Labelled DNA was separated from unincorporated nucleotides by running the mixture with 0.04% (w/v) bromophenol blue through a Sephadex G50 medium grade or G50 fine grade (Pharmacia) column with T/E. Fractions were collected and an appropriate volume from each (e.g. 2µl) counted in scintillation fluid (Beckman Ready-Solv EP). If this 2µl constituted a sufficient proportion of the fraction, the amount of DNA used for scintillation was taken account of and deducted from the final amount. The fractions from the first labelled peak, containing incorporated nucleotides, were pooled. Five hundred-fold sheared, denatured salmon sperm DNA, in water, was added, and the DNA precipitated with 0.1 volume of 1M NaCl, pH8.0, 3M ammonium acetate or 3M sodium acetate, pH5.2, and 2-2½ volumes of ethanol, at -20°C or -70°C, and resuspended in water.

AI.5.*In situ* hybridization

The method used was adapted from those of Harper and Saunders (1981), Trent *et al.* (1982), Donlon *et al.* (1983) and Zabel *et al.* (1983).

The slides were treated with 200µl of 100µg/ml RNase A in 2*SSC (pH7.0) (1*SSC = 0.15M NaCl, 0.015M tri sodium citrate) at 37°C for 1hr in a 2*SSC-saturated environment, then washed in four changes of 2*SSC (pH7.0) for 2min each, dehydrated in a graded ethanol series, and air dried. Chromosomes were denatured at 70°C in 70% deionized formamide (Sigma; deionized with Bio-Rad analytical grade mixed bed resin), 2*SSC

(pH7.0) for 2min, then dipped into 70% and 95% ethanol for 1min each at -20°C, and air dried. Labelled and carrier DNA (see section AI.4) at various concentrations in 50% deionized formamide, 10% dextran sulphate (Pharmacia) and 2*SSCP (1*SSCP = 0.12M NaCl, 0.015M trisodium citrate, 0.1M sodium phosphate) (10*SSCP, pH6.0, was made using $^1/_5$ volume of 1M sodium phosphate, pH6.0, so that the hybridization mix when made up with DNA, SSCP, formamide and dextran sulphate was pH7.0 (from M.E. Harper, protocol for *in situ* hybridization, 1980), was denatured at 100°C for 5min, at the same time as, or shortly before, chromosome denaturation, then put immediately on ice for about 5min. This hybridization mix was again thoroughly mixed immediately prior to adding 20µl to each slide. The slides were then covered with a siliconized coverslip (siliconized with Prosil 28 organosilane concentrate), and sealed with rubber cement (Earth brand).

After hybridization overnight in a 2*SSC-saturated environment at 37°C (or another specified temperature), the coverslips were removed, and the slides were washed for 10min in each washing solution, then dehydrated for 2min in each of 95% and 70% ethanol solutions, and air dried. The washing solutions, unless otherwise specified, were: three of 50% deionized formamide, 2*SSC, pH7.0, 39°C; three of 2*SSC, pH7.0, 39°C; and three of 2*SSC, pH7.0, at room temperature. Slides hybridized with different probes were washed in separate solutions for at least the first wash.

For each new probe used, a range of concentrations was generally chosen for hybridization to different slides. Initially this range of probe

concentrations was applied to the test slides (those to be used for localization). In later experiments a series of slides with normal chromosomes was included for testing the various probe concentrations, and test slides were made using the concentration predicted to be optimal, based on experience with other probes.

AI.6.Autoradiography

All steps were carried out in a dark room under a yellow safelight.

Kodak NTB-2 nuclear research emulsion was used, diluted 2:1 or 1:1 with water, and melted at 43°C, or at 44°C if 300mM (final concentration) ammonium acetate was included. Glycerol (final concentration 1%) was also included.

The slides were dipped in molten emulsion, drained horizontally on cold metal plates for at least 20min, then dried at room temperature for 1hr 40min. They were exposed at 4°C with desiccant. Test slides were included with each experiment and developed at intervals in order to determine an appropriate exposure time for the remaining slides. Slides were developed in Kodak D19 as specified, fixed for 6 min in Agfa Gevaert G334c Rapid X-Ray Fixer with constant agitation, and soaked in 10% Na_2SO_4 for 5min, which was gradually replaced with water; and air dried. (Adapted from Rogers, 1973; 1979.)

For each experiment, slides were included for development after certain periods of exposure, in order to assess an appropriate time for

development of the experimental slides. Two slides, with metaphases but no radioactive probe, were included with most experiments. One was developed immediately after dipping, to ascertain whether there was an unduly high level of exposed silver grains in the emulsion before exposure. The other was developed with the test slides, to ascertain whether there was any major source of background not due to probe, on the test slides, from either environmental or developmental conditions.

AI.7.Staining of chromosomes

Permanent slides were mounted in DPX. Chromosomes were observed under oil immersion at 1 000x magnification. Slides were destained by passing through absolute alcohol, then methanol:acetic acid, 1:3, and air dried.

G-banded chromosomes. The slides were first aged at 37½C for at least 4hr, then treated with H_2O_2:H_2O, 2:9 for 3½min, rinsed with saline (0.9% NaCl) and air dried; treated with trypsin:saline, 1:40 for an appropriate length of time (5sec or more), rinsed with saline and air dried; and stained for 6min in Leishman's stain: phosphate buffer, 1:4, pH6.8 (Gurr 065568), rinsed in water and air dried. Destaining of chromosomes was achieved by immersing the slides in two changes of fixative (methanol:acetic acid, 3:1), for 10-20 seconds each. (Seabright, 1971; 1973.)

Solid staining was obtained by staining with Leishman's stain (see *G-banded chromsomes*). Unbanded chromosomes were used for locating fragile sites.

Solid staining after in situ hybridization. The slides were stained for 20min with either: Leishman's stain:Gurr's phospate buffer, pH6.8, 1:4; Giemsa:Gurr's phosphate buffer, pH6.8, 1:10; or Wright's stain: 0.06M phosphate buffer, pH6.8, 1:3. Some of the excess stain was rinsed from the emulsion with Gurr's phosphate buffer, and the slides were rinsed with water and air dried. (Modified from Harper and Saunders, 1981.)

G-banding after in situ hybridization. Chromosomes were banded after *in situ* hybridization using a modification of the method of Zabel *et al.* (1983). The chromosomes had been synchronized by the BuT method (see AI.1). The slides were treated with 1μg/ml Hoechst 33258 in 2*SSC, pH7.0, for 15min, rinsed with water and air dried. The slides were then covered with 2*SSC (pH7.0) and exposed to UV light (300W Wotan Ultra-Vitalux with interference reflector, Breville/Tyquin), 20cm from the source for 15min, rinsed with water and air dried. They were then stained with Leishman's stain, as above.

Approach to banding/staining for in situ hybridization. Chromosomes were often banded, photographed and destained before *in situ* hybridization, for the experiments described in this project. This was the case in the initial absense of a suitable post-hybridization banding technique, and also for chromosomes obtained from lymphoblast culture, as the banding method of Zabel *et al.* (1983) was not found to be readily successful using these chromosomes. A trypsin G-banding technique was used (except in the case of the chromosomes from acute promyelocytic leukaemia, chapter 4; see appendix AII). G-banding after *in situ*

hybridization was performed using the method of Zabel *et al.* (1983). This method involved cell synchronization with BrdU and release with medium supplemented with thymidine. Colchicine was not necessary to obtain chromosomes in mitosis, but was added before harvest, in order to break down the spindle fibres and obtain better spreading (c.f. Zabel *et al.*, 1983).

In this project the initial experiments were carried out with probes to chromosomes recognisable without chromosome banding, so a banding technique was not necessary. When fragile sites were used for localization, chromosome preparations were prepared using culture methods with which induction of the fragile site was of primary importance.

APPENDIX AII

DETAILS OF CHROMOSOMES USED FOR *IN SITU* HYBRIDIZATION

Name	Cell type[1]	Karyotype	Freq. of fs	Culture conditions[2]	Slide nos.
C208/79	LCL	46,XY,t(2;16)(p13;q22)	60%[3]	BuBu	H41
C379/86	PBL	46,XX		TBu	H41
C451/86	PBL	46,XY		TBu	H43
C494/86	PBL	46,XY			H44
C495/86	PBL	46,XY			H44
C563/84	PBL	46,XY		TBu	H6
CF3	PBL	46,XY; common fs induced for fra(16)(q23)	12%[4]	aphidicolin	H26
CF4	PBL	46,XX; common fs induced for fra(16)(q23)	15%[4]	aphidicolin	H26
CF6	PBL	46,XX, common fs induced for *FRA16C*	5%[5]	FdC and caffeine	H42
F2B	PBL	46,XX,fra(16)(p12)	42%	MEM-FA	H16

1. LCL: lymphoblastoid cell line; PBL: lymphocyte (from venous blood); BM: bone marrow.
2. given where relevant to fragile site induction or cell synchronization and/or subsequent banding method.
3. 60% of metaphases with a gap at 11q23.1 in one or both chromosomes 11.
4. represents percentage of chromatids 16 expressing fra(16)(q23).
5. represents percentage of chromosomes 16 expressing fra(16)(q22).

F2O	PBL	46.XY			H6
F2P	PBL	46.XY			H16
F2R	PBL	46,XX,fra(10)(q25)		MEM-FA[6]	H16
		fra(16)(p12)	23%		
F2ZA	PBL	46,XY,fra(16)(p12)	60%	MEM-FA	H12
F4F	PBL	46,XY,fra(16)(q22)	26%	H33258[7]	H35
					H40
F7B	PBL	46,XY,fra(16)(q22)	7%	H33258[8]	H35
					H40
F7F	PBL	46,XY,fra(8)(q22),		BrdU	H9
		fra(16)(q22)	22%		
F11C	PBL	46,XY			H24
					H25
F15F	PBL	46,XY			H12
F18B	PBL	46,XY,fra(10)(q25),		BrdU	H35
		fra(16)(q22)	28%		H40
F22A	PBL	46,XX,fra(16)(q22)	2%	-	H9.1-5
					H14.1-7
			63%	BrdU	H9.6-16,
					18-28
					H14.8-25
					H35
					H40
F25A	PBL	46,XY,fra(16)(q22)	16%	BrdU	H35
					H40

6. BrdU also added for induction of fra(10)(q25); expression frequency without BrdU: 60%.
7. 100µg/ml Hoechst 33258, 24hr before harvest.
8. 50µg/ml Hoechst 33258, 24hr before harvest.

F35G	PBL	46,XY			H20
F36A	PBL	46,XX,fra(16)(q22)	18%	BrdU	H35
					H40
F38A	PBL	46,XY,fra(16)(q22)	22%	BrdU	H20
					H35
					H40
F41A	PBL	46,XX,fra(16)(q22)	47%	BrdU	H20.10-15
					H35
					H40
			28%	TBu	H20.16-18
F42B	PBL	46,XX			H51
F42E	PBL	46,XY,fra(10)(q25),			H35
		fra(16)(q22)	28%	BrdU	H40
F42G[9]	PBL	46,XY,fra(10)(q25),			H35
		fra(16)(q22)	44%	BrdU	H40
F50C	PBL	46,XX,fra(16)(q22)	16%	BrdU	H35
					H40
F50I	PBL	46,XY			H40
					H42
F52A	PBL	46,XY,fra(16)(q22)	38%	BrdU	H35
					H40
F52B	PBL	46,XX			H26
F53C	PBL	46,XX,fra(16)(q22)	52%	BrdU	H35
					H40
					H51

9. related to F42E by marriage.

F56B	PBL	46,XX,fra(16)(q22)	23%	BrdU	H35
					H40
F59B2	PBL	46,XY,fra(11)(q13)	32%	MEM-FA	H41
					H52
F63A	PBL	46,XX,fra(16)(q22)	39%	BrdU	H35
					H40
F65A	PBL	46,XY,fra(11)(q23)	13%	MEM-FA	H41
					H52
GM2324	LCL	46,XX,t(16;22)(p13.11;q11.21)[10]			H36
GM6227	LCL	46,XX,t(1;16)(q44;p13.11)		BuT	H24
ML	BM	46,XX,t(15;17)(q22;q21)[11]		FuBu[12]	H49
SD	BM	complex[13]		FuBu[12]	H49
TB3	PBL	46,XY			H7
TB8	PBL	46,XY			H35
TB9	PBL	46,XY		BuT	H29
					H36
TB10	PBL	46,XY		BuT	H29
					H43
					H51
					H52
TB11	PBL	46,XY		BuT	H49

10. Nicholls et al., 1987.
11. from a patient with APL.
12. FdU block, BrdU release (Webber and Garson,. 1983). Cells cultured and analysed, and chromosomes spread and pre-banded for in situ hybridization using an alkaline phosphatase buffered Giemsa (0.25M, pH10.5), in another laboratory.
13. from a patient with APL, at time of second relapse; karyotype 47,XY,-7,
-7,t(7;16)(q11;q11),t(15;17)(q22;q21)i(17q-),-16+M(der7)+2 F-like markers.

APPENDIX AIII

CONDITIONS AND RESULTS OF *IN SITU* HYBRIDIZATION

AIII.1.Hybridization of repeat sequence probes to normal chromosomes

Specific activity of pHY2.1 = $3.0*10^7$cpm/µg.

Specific activity of pX1-r101-12 = $3.2*10^7$cpm/µg.

Slide	Probe Conc. (µg/ml)	Exposure time (days)	Individual and relevant details of chromosomes
pHY2.1:			
H6.1	0.35	8	F2O; chromosomes from storage at -20°C in fixative; wash (a).
H6.2	1.4	8	F2O; chromosomes from storage at -20°C in fixative; wash (a).
H6.3	0.7	8	F2O; chromosomes from storage at -20°C in fixative; wash (a).
H6.4	0.7	8	C563/84; TBu; fresh chromosomes; wash (a).
H6.5	0.7	8	F2O; chromosomes from storage at -20°C in fixative; wash (b).
H6.6	0.7	100	F2O; chromosomes from storage at -20°C in fixative; wash (a).

H6.7	0.7	5	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.8	0.7	8	F20; chromosomes from storage at -20°C in fixative; wash (a).

pX1-r101-12:

H6.9	0.35	100	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.10	1.4	100	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.11	0.7	100	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.12	0.7	100	C563/84; TBu; fresh chromosomes; wash (a).
H6.13	0.7	100	F20; chromosomes from storage at -20°C in fixative; wash (b).
H6.14	0.7	100	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.15	0.7	21	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.16	0.7	8	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.17	-	5	F20; chromosomes from storage at -20°C in fixative; wash (a).
H6.18	-	18	F20; chromosomes from storage at -20°C in fixative; wash (a).

NOTES: Emulsion Ilford K2:H_2O = 1:1;

Development D19, 5min, with agitation;

Wash (a): standard (appendix AI);

Wash (b): first 3 washes standard; followed by one wash in 2*SSC, pH7.0, 39^{o}C, 10min; two washes in 2*SSC, pH7.0, 65^{o}C, 10min each; one wash 0.5*SSC, room temp., 10min; one wash 0.2*SSC, pH7.0, room temp., 10min; one wash 0.1*SSC, pH7.0, room temp., 10min.

HYBRIDIZATION RESULTS[1]**:**

Slide H6.1: S=56=r; B=210; 40 cells; λ=1.72; P<<<10^{-5}.

Slide H6.7: S=113=r; B=187; 50 cells; λ=1.53; P<<<10^{-5}.

AIII.2.Hybridization of pHPζ (zeta-globin gene probe) to normal chromosomes

Specific activity of probe = 3.5*10^{7}cpm/μg.

1. S, signal; B, background; r, observed grain number {=S}; λ, expected grain number {=B*rel. length of signal's GSR/(total rel. length-rel. length of signal's GSR)}

Slide	Probe Conc. (ug/ml)	Exposure time (days)	Individual
H7.1	0.2	111	TB3.
H7.2	0.2	111	TB3.
H7.3	0.2	26	TB3.
H7.4	0.2	111	TB3.
H7.5	0.05	37	TB3.
H7.6	0.1	37	TB3.
H7.7	0.2	37	TB3.
H7.8	0.4	37	TB3.
H7.9	0.8	37	TB3.
H7.10	1.6	37	TB3.

NOTES: Emulsion NTB-2:H_2O = 1:1;

Development D19, 5 min, with agitation.

HYBRIDIZATION RESULTS:

Slide H7.1: S=13½=r; B=261½; 25 cells; λ=3.37; P≈2*10^{-5}.

Slide H7.3: S=14=r; B=628; 55 cells; λ=8.10; P=0.04.

Slide H7.10: S=30½=r; B=802½; 58 cells; λ=10.35; P<10^{-5}.

AIII.3.Hybridization of *TNF* gene probe to normal chromosomes

Specific activity of probe = 3.1*10^7cpm/ug.

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual and relevant details of chromosomes (before and *after in situ* hybridization).
H29.1	0.4	14	TB9; 46,XY; BuT; *G-banding unsuccessful.*
H29.2	0.4	38	TB9; 46,XY; BuT; *G-banded.*
H29.3	0.4	38	TB9; 46,XY; BuT; *G-banded.*
H29.4	0.1	38	TB9; 46,XY; BuT; *G-banded.*
H29.5	1.2	38	TB9; 46,XY; BuT; *G-banded.*
H29.6A	-	1	TB9; 46,XY; BuT; for initial background level.
H29.6B	-	26	TB9; 46,XY; BuT; *G-banded*; for final background level.
H29.7	0.05	26	TB10; 46,XY; BuT; *G-banded.*
H29.8	0.1	26	TB10; 46,XY; BuT; *G-banded.*
H29.9	0.2	26	TB10; 46,XY; BuT; *G-banded.*
H29.10	0.2	26	TB9; 46,XY; BuT; *G-banded.*
H29.11	0.4	26	TB10; 46,XY; BuT; *G-banded.*
H29.12	0.4	26	TB9; 46,XY; BuT; *G-banded.*
H29.3	0.8	26	TB10; 46,XY; BuT; *G-banded.*
H29.14	0.8	26	TB9; 46,XY; BuT; *G-banded.*
H29.15	1.6	26	TB10; 46,XY; BuT; *G-banded.*

NOTES: Emulsion NTB-2:H_2O = 2:1;

Development D19 3 min, with agitation

LOCALIZATION RESULTS: See chapter 4.

AIII.4.Hybridization of *G-CSF* gene probe to normal chromosomes

Specific activity of probe = $1.7*10^7$cpm/µg.

Slide	Probe Conc. (µg/ml)	Exposure time (days)	Individual and relevant details of chromosomes (before and *after in situ* hybridization).
H43.1	0.4	19	C451/86; TBu; 46,XY; G-banded with trypsin and pre-photographed.
H43.2	0.4	39	C451/86; TBu; 46,XY; G-banded with trypsin and pre-photographed.
H43.3	0.4	44	TB10; BuT; 46,XY; *G-banded*.
H43.4	0.4	44	TB10; BuT; 46,XY; *G-banded*.
H43.5	-	1	TB10; BuT; for initial background level; 46,XY; *G-banded*.
H43.7	-	44	TB10; BuT; for final background level; 46,XY; *G-banded*.
H43.9	0.05	44	TB10; BuT; 46,XY; *G-banded*.
H43.10	0.1	44	TB10; BuT; 46,XY; *G-banded*.
H43.11	0.2	44	TB10; BuT; 46,XY; *G-banded*.
H43.12	0.4	44	TB10; BuT; 46,XY; *G-banded*.
H43.13	0.8	44	TB10; BuT; 46,XY; *G-banded*.
H43.14	1.6	44	TB10; BuT; 46,XY; *G-banded*.

NOTES: Emulsion NTB-2:H_2O = 2:1.

Development D19 4 min, no agitation, at 19°C.

LOCALIZATION RESULTS: See chapter 4.

AIII.5.Hybridization of *G-CSF* probe to chromosomes with APL translocation, t(15;17) (See appendix AII for karyotypes.)

Specific activity of probe = $1.7*10^7$cpm/µg.

Slide	Probe Conc. (µg/ml)	Exposure time (days)	Individual and relevant details of chromosomes (before and *after in situ* hybridization).
H49.1	0.4	14	TB11; BuT; *G-banded.*
H49.2	0.4	26	TB11; BuT; *G-banded.*
H49.3	0.4	32	TB11; BuT; *G-banded.*
H49.4	-	32	TB11; BuT;for final background level; *G-banded.*
H49.5	-	1	TB11; BuT; for initial background level; *G-banded.*
H49.6	0.4	32	SD; APL; G-banded[1] and pre-photographed.
H49.7	0.4	32	SD; APL; G-banded and pre-photographed.
H49.8	0.4	32	SD; APL; G-banded and pre-

1. slides H49.6-H49.16 G-banded before in situ hybridization with alkaline phosphate buffered Giemsa; see AII.

			photographed.
H49.9	0.4	32	SD; APL; G-banded and pre-photographed.
H49.10	0.4	32	SD; APL; G-banded and pre-photographed.
H49.11	0.4	32	SD; APL; G-banded and pre-photographed.
H49.12	0.4	32	ML; APL; G-banded and pre-photographed.
H49.13	0.4	32	ML; APL; G-banded and pre-photographed.
H49.14	0.4	32	ML; APL; G-banded and pre-photographed.
H49.15	0.4	32	ML; APL; G-banded and pre-photographed.
H49.16	0.4	32	ML; APL; G-banded and pre-photographed.
H49.19	0.4	32	SD; APL.
H49.24	0.4	32	ML; APL.
H49.25	0.4	32	ML; APL.
H49.17	0.4		TB11; BuT; *G-banded.*
H49.18	0.4		TB11; BuT; *G-banded.*
H49.20	0.4		SD; APL; *G-banded.*
H49.21	0.4		SD; APL; *G-banded.*
H49.22	0.4		SD; APL; *G-banded.*
H49.23	0.4		SD; APL; *G-banded.*

H49.26	0.4	ML; APL; *G-banded*.
H49.27	0.4	ML; APL; *G-banded*.

NOTES: Emulsion NTB-2:H_2O = 2:1.

Development D19, 4 min, no agitation, at 19^oC.

Slides H49.17-H49.27 were dipped in Ilford L4 emulsion, developed and scored in another laboratory (see Acknowledgements).

HYBRIDIZATION RESULTS:

(relative length of 17q=2.02)

Slide H49.1: S=10=r; B=81; 28 cells; λ=1.60; $P=10^{-5}$.

Slide H49.2: S=22=r; B=86; 25 cells; λ=1.70; $P<<10^{-5}$.

LOCALIZATION RESULTS: See chapter 4.

AIII.6.Hybridization of JW101 and pHPζ (alpha- and zeta-globin gene probes) to chromosomes expressing fra(16)(p12)

Specific activity of JW101 = $2.1*10^7$cpm/μg.

Specific activity of pHPζ = $2.5*10^7$cpm/μg.

Slide	Probe Conc. (µg/ml)	Exposure time (days)	Individual, probe, and relevant details of chromosomes.
H12.1	0.4	26	F15F; JW101.
H12.2	0.4	59	F15F; JW101.
H12.3	0.4	83	F15F; JW101.
H12.4	0.05	83	F2ZA; JW101; fra(16)(p12)
H12.5	0.1	83	F2ZA; JW101; fra(16)(p12).
H12.6	0.2	83	F2ZA; JW101; fra(16)(p12).
H12.7	0.4	83	F2ZA; JW101; fra(16)(p12).
H12.8	0.8	83	F2ZA; JW101; fra(16)(p12).
H12.9	1.6	83	F2ZA; JW101; fra(16)(p12).
H12.10	2.4	83	F2ZA; JW101; fra(16)(p12).
H16.1	0.6	24	F2P; pHPζ.
H16.2	0.6		F2P; pHPζ.
H16.3	0.6		F2P; pHPζ.
H16.4	0.6		F2P; pHPζ.
H16.5	-	65	F2P; pHPζ; for final background level.
H16.6	-	5	F2R; pHPζ; fra(16)(p12); for initial background level.
H16.9A	0.1	61	F2ZA; pHPζ; fra(16)(p12).
H16.9B	0.1	65	F2B; pHPζ; fra(16)(p12).
H16.10A	0.2	61	F2ZA; pHPζ; fra(16)(p12).
H16.10B	0.2	65	F2B; pHPζ; fra(16)(p12).

H16.10C	0.2	65	F2R; pHPζ; fra(16)(p12).
H16.11A	0.4	61	F2ZA; pHPζ; fra(16)(p12).
H16.11B	0.4	65	F2B; pHPζ; fra(16)(p12).
H16.11C	0.4	65	F2R; pHPζ; fra(16)(p12).
H16.12A	0.8	61	F2ZA; pHPζ; fra(16)(p12).
H16.12B	0.8	65	F2B; pHPζ; fra(16)(p12).
H16.12C	0.8	65	F2R; pHPζ; fra(16)(p12).
H16.12D	0.8	65	F2B; pHPζ; fra(16)(p12).
H16.13A	1.2	61	F2ZA; pHPζ; fra(16)(p12).
H16.13B	1.2	65	F2B; pHPζ; fra(16)(p12).
H16.13C	1.2	65	F2R; pHPζ; fra(16)(p12).
H16.14A	1.6	61	F2ZA; pHPζ; fra(16)(p12).
H16.14B	1.6	65	F2B; pHPζ; fra(16)(p12).
H16.14C	1.6	65	F2R; pHPζ; fra(16)(p12).
H16.15A	2.1	61	F2ZA; pHPζ; fra(16)(p12).
H16.15B	2.1	65	F2B; pHPζ; fra(16)(p12).
H16.15C	2.1	65	F2R; pHPζ; fra(16)(p12).

NOTES: Emulsion NTB-2:H_2O = 2:1

Development D19 3 min, with agitation.

HYBRIDIZATION RESULTS:

Slide H12.1: S=15½=r; B=244½; 50 cells; λ=3.16; $P<10^{-5}$.

Slide H12.2: S=12=r; B=148; 25 cells; λ=1.91; $P<10^{-5}$.

Slide H12.7: S=26=r; B=258; 39 cells; λ=3.33; $P<<10^{-5}$.

Slide H16.1: S=13½=r; B=295½; 100 cells; λ=3.81; $P<1.7*10^{-4}$.

Slide H16.9A: S=9=r; B=135; 51 cells; λ=1.74; $P=7*10^{-5}$.

Slide H16.15A: S=24=r; B=542; 75 cells; λ=6.99; $P<10^{-5}$.

LOCALIZATION RESULTS:

HBA:

Slide (probe conc. µg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*[1]	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H12.4 (0.05)	0	1	-	0	140	55	1
H12.5 (0.1)	0	20	20.00 *P<<0.001*	3	203	85	19
H12.6 (0.2)	5	16	5.76 *P<0.025*	3	206	88	15
H12.7 (0.4)	6	17	5.26 *P<0.025*	7	210	88	18
H12.8	3	15	8.00	1	153	73	15

1. $\chi^2_{0.05[1]} = 3.84$.

(0.8)			*P<0.005*				
H12.9	1	6	-	4	85	25	9
(1.6)							
H12.10	1	0	-	0	11	6	1
(2.4)							

HBZ:

Slide (probe conc., µg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H16.9A	1	3	-	1	147	63	5
(0.1)							
H16.10A	0	2	-	0	112	52	2
(0.2)							
H16.11A	2	7	-	2	188	74	9
(0.4)							
H16.12A	2	15	9.94	2	217	103	14
(0.8)			*P<0.005*				
H16.13A	2	12	7.14	2	205	76	12
(1.2)			*P<0.01*				
H16.14A	5	18	6.17[4]	3	213	92	20
(1.6)			*P<0.025*				
H16.15A	3	15	6.74[5]	1	179	73	17

4. Includes two tri-radial figures; H_0: prox:dist=20:22.
5. Includes two tri-radial figures; H_0: prox:dist=17:19.

(2.1)			*P<0.01*				
H16.12D	3	3	-	0	66	35	6
(0.8)							
H16.14B	3	2	-	0	66	27	5
(1.6)							
H16.15B	1	2	-	1	49	25	4
(2.1)							
H16.10C	0	1	-[6]	1	244	58	2
(0.2)							
H16.11C	0	4	-	0	268	39	3
(0.4)							
H16.12C	3	9	2.45[7]	3	508	132	10
(0.8)			*n.s.*				
H16.13C	2	6	-	0	209	65	7
(1.2)							
H16.14C	1	4	-[8]	0	321	48	4
(1.6)							
H16.15C	4	10	2.27[9]	7	240	76	19
(2.1)			*n.s.*				

6. Includes one tri-radial figure.
7. Includes one tri-radial figure; H_0: prox:dist=10:11.
8. Includes one tri-radial figure.
9. Includes one tri-radial figure; H_0: prox:dist=19:20.

RESULTS OF SUB-LOCALIZATION (grouping different concentrations):

Slide (probe conc.)	No. grains closer to f.s.	No. grains closer to telomere	X^2 (Proba-bility)	No. grains mid-way or ambiguous
H12.4	0	1	-	0
H12.5	5	11	2.25 (n.s.)	4
H12.6	9	3	3.00 (n.s.)	3
H12.7	8	9	0.06 (n.s.)	2
H12.8	3	9	3.00 (n.s.)	4
H12.9	3	5	-	0
H16.9A	1	2	-	1
H16.10A	0	1	-	0
H16.11A	3	2	-	3
H16.12A	5	9	1.14 (n.s.)	4
H16.13A	4	6	-	4
H16.14A	6	13	2.58 (n.s.)	1
H16.15A	6	10	1.00 (n.s.)	0
H16.14B	0	0	-	2
H16.15B	1	1	-	1
H16.12D	0	2	-	0
H16.10C	1	0	-	0
H16.11C	2	2	-	0
H16.12C	4	8	1.33 (n.s.)	0
H16.13C	0	4	-	2

H16.14C	0	3	-	0
H16.15C	8	7	0.07 (n.s.)	2
Combined:	69	108	8.59 (P<0.005)	33

AIII.7.Hybridization of JW101, pα3'HVR.64 and pHPζ (alpha- and zeta-globin gene probes) to translocation chromosomes (For karyotypes see appendix AII.)

Specific activity of JW101 = $4*10^7$cpm/μg.

Specific acitvity of pHPζ = $5*10^7$cpm/μg.

Specific activity of p 3'HVR.64 = $2.5*10^7$cpm/μg.

Slide	**Probe Conc. (μg/ml)**	**Exposure time (days)**	**Individual or cell line and relevant details of chromosomes.**
GM6227 (t(1;16)):			
Probe JW101:			
H24.1	0.4	16	F11C.
H24.2	0.4	28	F11C.
H24.3	0.4	41	F11C.
H24.4	0.4	103	F11C.
H24.5	0.4	42	GM6227; t(1;16); G-banded with trypsin and pre-photographed.
H24.6	0.8	42	GM6227; t(1;16); G-banded with trypsin and pre-photographed.

H24.10	0.4	42	GM6227; t(1;16).
H24.11	0.4	42	GM6227; t(1;16).
H24.B1	-	1	F11C; for initial background level.
H24.B2	-	42	F11C; for final background level.

GM2324 (t(16;22)):

Probe JW101:

H36.1	0.4	17	TB9.
H36.2	0.4	47	TB9.
H36.4	-	<1	TB9; for initial background level.
H36.14	-	50	TB9; for final background level.
H36.6	0.4	50	GM2324; t(16;22); G-banded with trypsin and photographed.
H36.7	0.4	50	GM2324; t(16;22); G-banded with trypsin and photographed.

Probe pHPζ:

H36.11	0.4	28	TB9.
H36.12	0.4	17	TB9.
H36.13	0.4	50	TB9.
H36.4	-	<1	TB9; for initial background level.
H36.14	-	50	TB9; for final background level.
H36.15	0.4	50	GM2324; t(16;22); G-banded with trypsin and pre-photographed.

Probe pα3'HVR.64:

H44.1	0.2	7	C495/86.
H44.2	0.2	14	C495/86.
H44.3	0.2	14	C495/86.
H44.4	-	1	C495/86; for initial background level.
H44.5	-	14	C495/86; for final background level.
H44.6	0.05	14	C494/86
H44.7	0.1	14	C494/86.
H44.8	0.2	14	C494/86.
H44.9	0.4	14	C494/86.
H44.10	0.8	14	C494/86.
H44.11	1.6	14	C494/86.
H44.12	0.2	14	GM2324; t(16:22); G-banded with trypsin and pre-photographed.
H44.13	0.2	14	GM2324; t(16:22); G-banded with trypsin and pre-photographed.
H44.14	0.2	14	GM2324; t(16:22); G-banded with trypsin and pre-photographed.

NOTES: Emulsion NTB-2:H2O = 2:1.

Development H24: D19 3 min, with agitation.

H36, H44: D19 4 min, no agitation, at 19°C.

HYBRIDIZATION RESULTS:

Slide H24.1: S=10=r; B=161; 50 cells; λ=2.08; P=7*10^{-5}.

Slide H24.2: S=14=r; B=214; 50 cells; λ=2.76; P<10^{-5}.

Slide H24.3: S=14=r; B=104; 25 cells; λ=1.34; P<<10^{-5}.

Slide H44.1: S=8=r; B=163; 25 cells; λ=2.10; P=0.001.

Slide H44.2: S=19½=r; B=97½; 25 cells; λ=1.26; P<<10^{-5}.

LOCALIZATION RESULTS: See chapter 5.

AIII.8.Hybridization of pULB1148 (haptoglobin gene probe) to chromosomes expressing *FRA16B* (with pBR322 control.)

Specific activity of pULB1148 = 2.6*10^7cpm/µg.

Specific activity of pBR322 = 4.0*10^7cpm/µg.

Slide	Probe Conc. (µg/ml)	Exposure time (days)	Individual and relevant details of chromosomes.
Probe pULB1148:			
H9.1	0.2	33	F22A; *FRA16B*.
H9.2	0.2	33	F22A; *FRA16B*.
H9.3	0.2		F22A; *FRA16B*.
H9.4	-	1	F22A; for initial background level; *FRA16B*.

H9.5	-	37	F22A; for final background level; *FRA16B*.
H9.6	0.05	37	F22A; *FRA16B*.
H9.8	0.1	37	F22A; *FRA16B*.
H9.10	0.2	37	F22A; *FRA16B*.
H9.12	0.4	37	F22A; *FRA16B*.
H9.14	0.8	37	F22A; *FRA16B*.
H9.16	1.6	37	F22A; *FRA16B*.
H9.7	0.05	37	F7F; *FRA16B*.
H9.9	0.1	37	F7F; *FRA16B*.
H9.11	0.2	37	F7F; *FRA16B*.
H9.13	0.4	37	F7F; *FRA16B*.
H9.15	0.8	37	F7F; *FRA16B*.
H9.17	1.6	37	F7F; *FRA16B*.

Probe pBR322:

H9.18	0.05	37	F22A; *FRA16B*.
H9.20	0.1	37	F22A; *FRA16B*.
H9.22	0.2	37	F22A; *FRA16B*.
H9.24	0.4	37	F22A; *FRA16B*.
H9.26	0.8	37	F22A; *FRA16B*.
H9.28	1.6	37	F22A; *FRA16B*.
H9.19	0.05	37	F7F; *FRA16B*.
H9.21	0.1	37	F7F; *FRA16B*.
H9.23	0.2	37	F7F; *FRA16B*.
H9.25	0.4	37	F7F; *FRA16B*.

H9.27	0.8	37	F7F; *FRA16B*.
H9.29	1.6	37	F7F; *FRA16B*.

NOTES: Hybridization at 42°C;

Emulsion NTB-2:H_2O = 1:1;

Development D19, 3 min (but H9.2 and H9.4: D19, 5 min), with agitation.

HYBRIDIZATION RESULTS:

Slide H9.1: S=36½=r; B=375½; 30 cells; λ=6.53; $P<<10^{-5}$.

Slide H9.2: S=14=r; B=229; 15 cells; λ=3.98; $P=8*10^{-5}$.

Slide H9.10: S=29½=r; B=288½; 30 cells; λ=5.02; $P<<10^{-5}$.

LOCALIZATION RESULTS:

pULB1148:

Slide (probe conc., µg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
F22A:							
H9.6	4	22	12.46	3	191	97	24
(0.05)			*P<0.001*				
H9.8	2	15	9.94	1	70	37	12
(0.1)			*P<0.005*				
H9.10	7	25	10.12	10	60	96	28
(0.2)			*P<0.005*				
H9.12	8	38	19.56	10	218	107	37
(0.4)			*P<0.001*				
H9.14	10	38	16.34	6	81	50	31
(0.8)			*P<0.001*				
H9.16	9	14	1.09	4	57	26	16
(1.6)			*n.s.*				

F7F:

Slide (probe conc., μg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H9.7 (0.05)	2	6	-	0	189	44	8
H9.9 (0.1)	2	3	-	3	208	38	7
H9.11 (0.2)	3	9	3.00 *n.s.*	1	200	26	10
H9.13 (0.4)	4	15	6.37 *P<0.025*	1	280	44	11
H9.15 (0.8)	12	15	0.33 *n.s.*	6	321	73	22
H9.17 (1.6)	14	32	7.04 *P<0.01*	6	519	95	43

pBR322:

Slide (probe conc., µg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
F22A:							
H9.18	1	1	-	0	206	100	2
H9.20	2	2	-	1	163	75	4
H9.22	2	5	-	1	179	105	8
H9.24	7	1	-	0	286	149	7
H9.26	5	3	-	1	178	94	9
H9.28	6	5	0.09 *n.s.*	0	106	52	11

AIII.9.Hybridization of *APRT* gene probe to chromosomes expressing fra(16)(q22) and fra(16)(q23) *(FRA16B* and *FRA16D)*

Specific activity of probe = (i) $1.0*10^7$cpm/µg;
(ii) $1.7*10^7$cpm/µg.

FRA16B:

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual, nick translation (n.t.) number (see above) and relevant details of slides.
H14.1	0.2	13	F22A; n.t.(ii).
H14.2	0.2	34	F22A; n.t.(ii).
H14.3	0.2	46	F22A; n.t.(ii).
H14.6	-	1	F22A; for initial background level.
H14.7	-	36	F22A; for final background level.
H14.8	0.025	36	F22A; n.t.(ii); *FRA16B*.
H14.9	0.05	36	F22A; n.t.(ii); *FRA16B*.
H14.10	0.1	36	F22A; n.t.(ii); *FRA16B*.
H14.11	0.2	36	F22A; n.t.(ii); *FRA16B*.
H14.12	0.4	36	F22A; n.t.(ii); *FRA16B*.
H14.13	0.8	36	F22A; n.t.(ii); *FRA16B*.
H14.14	1.6	36	F22A; n.t.(ii); *FRA16B*.
H14.15	0.025	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.16	0.05	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.17	0.1	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.

H14.18	0.2	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.19	0.4	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.20	0.8	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.21	1.6	36	F22A; n.t.(ii); *FRA16B*; increased stringency wash.
H14.22	0.2	36	F22A; n.t.(ii); *FRA16B*; slides spread one day before use[1].
H14.23	0.2	36	F22A; n.t.(ii); *FRA16B*; slides stored 3 months, -20°C before use.
H14.24	0.2	36	F22A; n.t.(i); *FRA16B*; slides spread one day before use.
H14.25	0.2	36	F22A; n.t.(i); *FRA16B*; slides stored 3 months, -20°C before use.

NOTES: Emulsion NTB-2:H_2O = 2:1.

Development D19 3 min, with agitation.

Hybridization at 42°C.

Increased stringency wash: Three times in 50% formamide/2*SSC, pH7.0, 39°C, 10 min each; 0.5*SSC,pH7.0, 39°C, 10 min; 0.2*SSC, pH7.0, 39°C, 10 min; 0.1*SSC, pH7.0, 39°C, 10 min; three times in 0.1*SSC, pH7.0, room temp., 10 min each.

1. Slides H14.1-H14.21 also spread one day before use.

HYBRIDIZATION RESULTS:

Slide H14.1: S=15=r; B=136; 50 cells; λ=2.36; $P<10^{-5}$.

Slide H14.2: S=26=r; B=105; 25 cells; λ=1.83; $P<<10^{-5}$.

Slide H14.11: S=6=r; B=69; 15 cells; λ=1.20; P=0.001.

Slide H14.12: S=25=r; B=233; 45 cells; λ=4.05; $P<<10^{-5}$.

Slide H14.22: S=31; B=152; 35 cells; λ=2.64; $P<<10^{-5}$.

LOCALIZATION RESULTS:

Slide (probe conc., µg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H14.8 (0.025)	0	1	-	0	53	31	1
H14.9 (0.05)	0	2	-	0	67	31	2
H14.10 (0.1)	1	3	-	0	74	37	4
H14.11 (0.2)	1	2	-	0	47	20	3
H14.12 (0.4)	7	21	7.00 *P<0.01*	0	141	74	19
H14.13 (0.8)	9	26	8.26 *P<0.005*	2	196	108	29

H14.14	1	5	-	0	65	26	3
(1.6)							
H14.15	1	2	-	0	99	53	3
(0.025)							
H14.16	0	0	-	0	74	35	0
(0.05)							
H14.17	1	5	-	0	102	56	6
(0.1)							
H14.18	2	9	4.21[1]	0	57	25	8
(0.2)			*P<0.05*				
H14.19	2	16	10.89	0	144	63	14
(0.4)			*P<0.001*				
H14.20	2	18	12.60	0	211	108	18
(0.8)			*P<0.001*				
H14.21	7	35	21.36	2	156	61	26
(1.6)			*P<<0.001*				
H14.22	3	21	13.50	2	190	104	19
(0.2)			*P<0.001*				
H14.23	4	11	3.27	2	311	158	14
(0.2)			*n.s.*				
H14.24	4	8	1.33	0	184	91	11
(0.2)			*n.s.*				
H14.25	1	9	6.4	0	174	78	7
(0.2)			*P<0.025*				

1. Includes one tri-radial figure; H_0: prox:dist=25:26.

RESULTS OF SUB-LOCALIZATION ON DISTAL ACENTRIC FRAGMENT:

Slide (probe conc.)	No. grains closer to f.s.	No. grains closer to telomere	X^2 and *Probability*	No. grains mid-way or ambiguous
H14.12 (0.4)	2	17	11.84 *P<0.001*	0
H14.13 (0.8)	10	19	2.79 *n.s.*	0
H14.18 (0.2)	1	7	-	1
H14.19 (0.4)	4	11	3.27 *n.s.*	0
H14.20 (0.8)	3	13	6.25 *P<0.025*	0
H14.21 (1.6)	12	23	3.46 *n.s.*	0
H14.22 (0.2)	5	18	7.35 *P<0.01*	0
H14.23 (0.2)	6	7	0.08 *n.s.*	0
H14.24 (0.2)	0	6	-	0
H14.25 (0.2)	3	6	-	0

fra(16)(q23):

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual and relevant details of chromosomes
H26.10	0.4	14	F52B; n.t. (ii).
H26.11	0.4	28	F52B; n.t. (ii).
H26.12	0.4	45	F52B; n.t. (ii).
H26.8	-	1	F52B; for initial background level.
H26.34	-	28	F52B; for final background level.
H26.35	0.4	35	CF3; fra(16)(q23); n.t. (ii).
H26.36	0.4	29	CF3; fra(16)(q23); n.t. (ii).
H26.37	0.4	29	CF4; fra(16)(q23); n.t. (ii).
H26.38	0.4	32	CF4; fra(16)(q23); n.t. (ii).
H26.39	0.8	32	CF3; fra(16)(q23); n.t. (ii).
H26.40	0.8	32	CF4; fra(16)(q23); n.t. (ii).

NOTES: Emulsion NTB-2:H_2O = 1:1.

Development D19 3 min, with agitation.

HYBRIDIZATION RESULTS:

Slide H26.10: S=5; B=98; 25 cells; λ=1.70; P=0.03

Slide H26.11: S=22; B=235; 50 cells; λ=4.09; $P<<10^{-5}$.

Slide H26.35: S=26; B=250; 60 cells; λ=4.41; $P<<10^{-5}$.

Slide H26.36: S=26; B=275; 60 cells; λ=4.85; $P<<10^{-5}$.

Slide H26.37: S=28; B=273; 60 cells; λ=4.75; $P<<10^{-5}$.

Slide H26.38: S=28; B=262; 60 cells; λ=4.56; $P<<10^{-5}$.

RESULTS OF LOCALIZATION:

Slide (Individual)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. chr. 16	No. f.s.	No. fs with label on GSR
H26.35 (CF3)	8	22	6.53 *P<<0.025*	4	1 064	147	32
H26.36 (CF3)	4	25	15.21 *P<0.001*	4	1 100	147	26
H26.37 (CF4)	10	22	4.50 *P<0.05*	2	1 354	263	29
H26.38 (CF4)	9	36	16.20 *P<0.001*	8	1 444	308	41
Combined	31	105	40.26 *P<<0.001*	18	4 962	865	128

AIII.10.Hybridization of metallothionein gene probes to chromosomes expressing fra(16)(q22) (i.e. *FRA16B* and *FRA16C*)

Specific activity of *MT2Ar* = $3.0*10^7$cpm/μg.

Specific activity of *MT2Au* = $2.2*10^7$cpm/μg.

Specific activity of *MT1Bu* = $3.2*10^7$cpm/μg.

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual, probe and relevant details of chromosomes.
H20.1	0.4	20	F35G; *MT2Ar*.
H20.2	0.4	26	F35G; *MT2Ar*.
H20.3	0.4	169	F35G; *MT2Ar*.
H20.4	1.6	169	F35G; *MT2Ar*.
H20.5	1.6	26	F35G; *MT2Ar*.
H20.8	-	1	F35G; no probe; for initial background level.
H20.9	-	29	F35G; no probe; for final backround level.
H20.10	0.05	29	F41A; *MT2Ar*; *FRA16B*.
H20.11	0.1	29	F41A; *MT2Ar*; *FRA16B*.
H20.12	0.2	29	F41A; *MT2Ar*; *FRA16B*.
H20.13	0.4	29	F41A; *MT2Ar*; *FRA16B*.
H20.14	0.8	29	F41A; *MT2Ar*; *FRA16B*.

H20.15	1.6	29	F41A; *MT2Ar*; *FRA16B*.
H20.16	0.1	27	F41A; *MT2Ar*; *FRA16B*; TBu.
H20.17	0.4	27	F41A; *MT2Ar*; *FRA16B*; TBu.
H20.18	1.6	27	F41A; *MT2Ar*; *FRA16B*; TBu.
H20.19	0.2	1	F38A; pHY2.1.
H35.1	0.2	18	TB8; *MT1Bu*
H35.2	0.2		TB8; *MT1Bu*
H35.3	0.2		TB8; *MT1Bu*
H35.4	0.05		TB8; *MT1Bu*
H35.5	1.2		TB8; *MT1Bu*
H35.6	-	1	TB8; no probe; for initial background level.
H35.7	-	33	TB8; no probe; for final background level.
H35.8	0.05	33	TB8; *MT1Bu*.
H35.9	0.1	33	TB8; *MT1Bu*.
H35.10	0.2	33	TB8; *MT1Bu*.
H35.11	0.4	33	TB8; *MT1Bu*.
H35.12	0.8	33	TB8; *MT1Bu*.
H35.13	1.6	33	TB8; *MT1Bu*.
H35.14	0.2	33	F4F; *MT1Bu*; *FRA16B*.
H35.15	0.2	33	F7B; *MT1Bu*; *FRA16B*.
H35.16	0.2	33	F18B; *MT1Bu*; *FRA16B*.
H35.17	0.2	23	F18B; *MT1Bu*; *FRA16B*.
H35.18	0.2	23	F22A; *MT1Bu*; *FRA16B*.
H35.19	0.2	33	F25A; *MT1Bu*; *FRA16B*.

H35.20	0.2	33	F36A; *MT1Bu*; *FRA16B*.
H35.21	0.2	33	F36A; *MT1Bu*; *FRA16B*.
H35.22	0.2	33	F38A; *MT1Bu*; *FRA16B*.
H35.23	0.2	33	F38A; *MT1Bu*; *FRA16B*.
H35.24	0.2	23	F41A; *MT1Bu*; *FRA16B*.
H35.25	0.2	33	F42E; *MT1Bu*; *FRA16B*.
H35.26	0.2	33	F42E; *MT1Bu*; *FRA16B*.
H35.27	0.2	33	F42G; *MT1Bu*; *FRA16B*.
H35.28	0.2	33	F42G; *MT1Bu*; *FRA16B*.
H35.29	0.2	23	F50C; *MT1Bu*; *FRA16B*.
H35.30	0.2	23	F52A; *MT1Bu*; *FRA16B*.
H35.31	0.2	23	F53C; *MT1Bu*; *FRA16B*.
H35.32	0.2	23	F56B; *MT1Bu*; *FRA16B*.
H35.33	0.2	33	F63A; *MT1Bu*; *FRA16B*.
H40.1	0.4	19	F50I; *MT2Au*.
H40.2	0.4	28	F50I; *MT2Au*.
H40.3	0.4		F50I; *MT2Au*.
H40.4	-	5	F50I; no probe; for initial background level.
H40.5	-	33	F50I; no probe; for final background level.
H40.6	0.05	33	F50I; *MT2Au*.
H40.7	0.1	33	F50I; *MT2Au*.
H40.8	0.2	33	F50I; *MT2Au*.
H40.9	0.4	33	F50I; *MT2Au*.
H40.10	0.8	33	F50I; *MT2Au*.

H40.11	1.6	33	F50I; *MT2Au*.
H40.12	0.4	33	F4F; *MT2Au*; *FRA16B*.
H40.13	0.4	33	F4F; *MT2Au*; *FRA16B*.
H40.14	0.4	33	F7B; *MT2Au*; *FRA16B*.
H40.15	0.4	33	F7B; *MT2Au*; *FRA16B*.
H40.16	0.4	33	F18B; *MT2Au*; *FRA16B*.
H40.17	0.4	33	F18B; *MT2Au*; *FRA16B*.
H40.18	0.4	33	F36A; *MT2Au*; *FRA16B*.
H40.19	0.4	33	F36A; *MT2Au*; *FRA16B*.
H40.20	0.4	33	F38A; *MT2Au*; *FRA16B*.
H40.21	0.4	32	F41A; *MT2Au*; *FRA16B*.
H40.22	0.4	33	F41A; *MT2Au*; *FRA16B*.
H40.23	0.4	32	F42E; *MT2Au*; *FRA16B*.
H40.24	0.4	33	F42E; *MT2Au*; *FRA16B*.
H40.25	0.4	33	F42G; *MT2Au*; *FRA16B*.
H40.26	0.4	33	F42G; *MT2Au*; *FRA16B*.
H40.27	0.4	33	F50C; *MT2Au*; *FRA16B*.
H40.28	0.4	33	F50C; *MT2Au*; *FRA16B*.
H40.29	0.4	33	F52A; *MT2Au*; *FRA16B*.
H40.30	0.4	33	F53C; *MT2Au*; *FRA16B*.
H40.31	0.4	33	F56B; *MT2Au*; *FRA16B*.
H40.32	0.4	33	F56B; *MT2Au*; *FRA16B*.
H40.33	0.4	33	F63A; *MT2Au*; *FRA16B*.
H40.34	0.4	33	F63A; *MT2Au*; *FRA16B*.
H40.35	0.4	33	F22A; *MT2Au*; *FRA16B*.
H40.36	0.4	33	F25A; *MT2Au*; *FRA16B*.
H40.37	0.4	33	F25A; *MT2Au*; *FRA16B*.

H42.1	0.4	22	F50I; *MT1Bu*.
H42.2	0.4	29	F50I; *MT1Bu*.
H42.3	0.4	38	F50I; *MT1Bu*.
H42.4	-	38	F50I; no probe; for final background level.
H42.5	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.6	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.7	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.8	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.9	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.10	0.4	38	CF6; *MT1Bu*; *FRA16C*, pre-located.
H42.11	0.4	38	CF6; *MT1Bu*; *FRA16C*.
H42.12	0.4	38	CF6; *MT1Bu*; *FRA16C*.
H42.13	0.4	38	CF6; *MT1Bu*; *FRA16C*.
H42.14	0.4	38	CF6; *MT1Bu*; *FRA16C*.
H42.15	0.4	22	F50I; *MT2Au*.
H42.16	0.4	29	F50I; *MT2Au*.
H42.17	0.4	38	F50I; *MT2Au*.
H42.18	-	38	F50I; no probe; for final background level.
H42.19	0.4	38	CF6; *MT2Au*; *FRA16C*, pre-located.
H42.20	0.4	38	CF6; *MT2Au*; *FRA16C*, pre-located.
H42.21	0.4	38	CF6; *MT2Au*; *FRA16C*, pre-located.
H42.22	0.4	38	CF6; *MT2Au*; *FRA16C*, pre-located.
H42.23	0.4	38	CF6; *MT2Au*; *FRA16C*, pre-located.
H42.24	0.4	38	CF6; *MT2Au*; *FRA16C*.

H42.25	0.4	38	CF6; *MT2Au*; *FRA16C*.
H42.26	0.4	38	CF6; *MT2Au*; *FRA16C*.
H42.27	0.4	38	CF6; *MT2Au*; *FRA16C*.
H42.28	0.4	38	CF6; *MT2Au*; *FRA16C*.
H41.5	-	1	F50I; no probe; for initial background level.

NOTES: H20.1-H20.19: Emulsion NTB-2:H_2O = 1:1.
Development D19, 3 min, with agitation.

H35.1-H35.33: Emulsion NTB-2:H_2O = 2:1.
Development D19, 3 min, with agitation.

H40.1-H40.37: Emulsion NTB-2:H_2O = 2:1
Development D19, 4 min; no agitation, at 19°C.

H42.1=H42.28: Emulsion NTB-2:H_2O = 2:1.
Development D19, 4 min; no agitation at 19°C.

HYBRIDIZATION RESULTS:

Slide H20.1: S=29=r; B=191; 25 cells; λ =3.37; $P<<10^{-5}$.

Slide H20.10: S¼57=r; B¼484; 50 cells; λ =8.42; $P<<10^{-5}$.[1]

Slide H20.11: S¼9=r; B¼161; 8 cells; λ =2.80; P=0.002.[1]

Slide H35.1: S=12=r; B=56; 25 cells; λ =0.99; $P<10^{-5}$.

Slide H35.10: S=48=r; B=143; 49 cells; λ =2.52; $P<<10^{-5}$.

1. Many clusters of three or more silver grains were present, hence the figure is only an estimate. Few of the clusters were seen over 16q on these slides, 16q being a small region; however, slides with higher concentrations of probe were unanalysable for signal and background.

Slide H35.11: S=44=r; B=169; 49 cells; λ=2.98; $P<<10^{-5}$.

Slide H40.1: S=27=r; B=157; 50 cells; λ=2.77; $P<<10^{-5}$.

Slide H40.2: S=37=r; B=168; 75 cells; λ=2.96; $P<<10^{-5}$.

Slide H42.1: S=21=r; B=155; 25 cells; λ=2.73; $P<<10^{-5}$.

Slide H42.2: S=16=r; B=206; 25 cells; λ=3.63; $P<10^{-5}$.

Slide H42.15: S=10=r; B=58; 25 cells; λ=1.02; $P<10^{-5}$.

Slide H42.16: S=21=r; B=131; 50 cells; λ=2.31; $P<<10^{-5}$.

LOCALIZATION RESULTS:

FRA16B: **(individual F41A)**

MT2Ar:

Slide (probe conc., u g/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H20.10 (0.05)	70	16	33.9 *P<<0.001*	10	597	273	44
H20.11 (0.1)	45	9	24.00 *P<<0.001*	4	414	135	32

H20.16	11	9	0.20	1	303	77	12
(0.1)			*n.s.*				
H20.17	21	5	9.85	2	382	101	21
(0.4)			*P<0.005*				
H20.18	23	6	9.97	1	223	54	15
(1.6)			*P<0.005*				

FRA16B:

*MT1B*u:

Slide	No. grains prox.	No. grains dist.	X^2 and *Proba-bility*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H35.14	4	0	-	1	70	51	4
(F4F)							
H35.15	0	0	-	0	58	10	0
(F7B)							
H35.16	8	2		0	79	31	4
H35.17	4	0		0	85	36	3
Combined	12	2	7.14	0	164	67	7
(F18B)			*P<0.01*				
H35.18	6	0	-	2	31	17	5
(F22A)							

H35.19	36	5	23.44	2	338	158	36
(F25A)			*P<<0.001*				
H35.20	11	0		0	281	87	8
H35.21	15	0		1	156	57	12
Combined	26	0	26.00	1	437	144	20
(F36A)			*P<<0.001*				
H35.22	0	0		0	10	1	0
H35.23	0	0		0	30	4	0
Combined	0	0	-	0	40	5	0
(F38A)[2]							
H35.24	6	2	-	0	91	45	6
(F41A)							
H35.25	6	0		1	61	15	4
H35.26	7	1		0	187	55	6
Combined	13	1	10.29	1	248	70	10
(F42E)			*P<0.005*				
H35.27	9	1		1	79	35	7
H35.28	3	1		0	64	27	3
Combined	12	2	7.14	1	143	62	10
(F42G)			*P<0.01*				
H35.29	7	1	-	1	88	54	8
(F50C)							
H35.30	5	2	-	0	171	70	5
(F52A)							
H35.31	4	0	-	0	62	31	4
(F53C)							

2. Data not included for this individual in Simmers et al., 1987A.

H35.32	2	0	-	0	29	6	1
(F56B)							
H35.33	4	0	-	0	15	7	3
(F63A)							

FRA16B:

MT2Au:

Slide	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H40.12	1	2		5	94	38	4
H40.13	4	0		1	89	21	4
Combined	5	2	-	6	183	59	8
(F4F)							
H40.14	3	0		1	168	21	3
H40.15	3	0		2	171	14	2
Combined	6	0	-	3	339	35	5
(F7B)[2]							
H40.16	15	5		5	218	69	19
H40.17	11	1		4	178	49	13
Combined	26	6	12.5	9	396	118	32
(F18B)			*P<0.001*				
H40.18	35	18		8	1 414	245	42

2. Data not included for this individual in Simmers et al., 1987A.

H40.19	13	6		0	492	54	14
Combined	48	24	8.00	8	1 906	299	56
(F36A)			*P<0.005*				
H40.20	2	1	-	1	128	16	3
(F38A)[2]							
H40.21	47	13		6	645	279	44
H40.22	26	6		4	410	169	25
Combined	73	19	31.70	10	1 055	448	69
(F41A)			*P<<0.001*				
H40.23	21	3		3	356	79	18
H40.24	3	1		0	322	33	3
Combined	24	4	14.29	3	678	112	21
(F42E)			*P<0.001*				
H40.25	15	3		5	194	47	15
H40.26	8	1		1	95	39	9
Combined	23	4	13.37	6	289	86	24
(F42G)			*P<0.001*				
H40.27	51	8		3	864	191	47
H40.28	43	5		8	767	179	39
Combined	94	13	61.32	11	1 631	370	86
(F50C)			*P<<0.001*				
H40.29	57	6	41.29	3	1046	248	45
(F52A)			*P<<0.001*				
H40.30	110	24	55.19	17	1 465	528	95
(F53C)			*P<<0.001*				
H40.31[3]							

3. Not scored, as results from previous slide sufficient.

(F53C)							
H40.32	19	2	13.76	2	872	129	18
(F56B)			*P<0.001*				
H40.33[3]							
(F56B)							
H40.34	14	3	7.12	3	537	136	16
(F63A)			*P<0.01*				
H40.35	4	0	-	3	164	47	6
(F22A)							
H40.36	59	12	31.11	8	4 011	371	58
(F25A)			*P<<0.001*				

FRA16C:

*MT1B*u:

Slide	No. grains prox.	No. grains dist.	X^2 and *Proba-bility*	No. grains cent.	No. f.s.	No. fs with label on GSR
H42.5	0	0		4	9	2
H42.6	5	1		2	15	6
H42.7	1	0		0	4	1
H42.8	2	0		1	8	3
H42.9	7	2		1	31	6
H42.10	2	1		0	17	3

Combined (CF6)	17	4	8.05 *P<0.005*	8		84	21

FRA16C:

*MT2A*u:

Slide	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H42.19	5	0		0		22	3
H42.20	0	0		0		7	0
H42.21	1	2		0		17	1
H42.22	5	0		0		23	4
H42.23	2	1		4		23	6
Combined (CF6)	13	3	6.25 *P<0.025*	4		92	14
H42.24	1	0		0	182	4	1
H42.25	1	0		0	212	5	1
H42.26	0	0		0	156	4	0

AIII.11.Hybridization of two anonymous DNA fragments from chromosome 16 to chromosomes expressing fra(16)(q22) (*FRA16B*)

Specific activity of ACH207 = $2.7*10^7$cpm/μg.

Specific activity of ACH224 = $2.3*10^7$cpm/μg.

Specific activity of λSL = $4.0*10^7$cpm/μg.

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual and relevant details of chromosomes (before and *after in situ* hybridization).
ACH207:			
H51.1	0.4	11	F42B.
H51.2	0.4	21	F42B.
H51.3	0.4	28	F42B.
H51.4	0.1	34	F42B.
H51.5	0.1	34	F42B.
H51.6	-	3	F42B; for initial background level.
H51.7	-	34	F42B; for final background level.
H51.8	0.05	34	F53C; fra(16)(q22).
H51.9	0.1	34	F53C; fra(16)(q22).
H51.10	0.2	34	F53C; fra(16)(q22).
H51.11	0.4	34	F53C; fra(16)(q22).
H51.12	0.8	34	F53C; fra(16)(q22).
H51.13	1.6	34	F53C; fra(16)(q22).

ACH224:

H51.14	0.4	11	F42B.
H51.15	0.4	21	F42B.
H51.16	0.4	28	F42B.
H51.17	0.1	34	F42B.
H51.18	0.1	34	F42B.
H51.19	0.05	34	F53C; fra(16)(q22).
H51.20	0.1	34	F53C; fra(16)(q22).
H51.21	0.2	34	F53C; fra(16)(q22).
H51.22	0.4	34	F53C; fra(16)(q22).
H51.23	0.8	34	F53C; fra(16)(q22).
H51.24	1.6	34	F53C; fra(16)(q22).
H51.25	-	34	F42B; for final background level.

λ**SL:** (control)

H51.26	0.05	34	TB10.
H51.27	0.1	34	TB10.
H51.28	0.2	34	TB10.
H51.29	0.4	34	TB10.
H51.30	0.8	34	TB10.
H51.31	1.6	34	TB10.

NOTES: Emulsion NTB-2:H_2O = 2:1.

Development D19, 4 min, no agitation, at 19°C.

HYBRIDIZATION RESULTS:

Slide H51.1: S=15=r; B=174; 50 cells; λ=3.03; $P<10^{-5}$.

Slide H51.2: S=43=r; B=200; 50 cells; λ=3.48; $P<<10^{-5}$.

Slide H51.3: S=3=r; B=43; 25 cells; λ=0.75; P=0.04.[1]

Slide H51.14: S=8=r; B=74; 25 cells; λ=1.29; $P=6*10^{-5}$.

Slide H51.15: S=15=r; B=93; 25 cells; λ=1.62; $P<10^{-5}$.

Slide H51.16: S=2=r; B=35; 25 cells; λ=0.61; P=0.12.

LOCALIZATION RESULTS:

Slide (probe conc., μg/ml)	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
ACH207:							
H51.11 (0.4)	28	82	26.51 *P<<0.001*	27	824	250	84
ACH224:							
H52.21 (0.2)	1	14	12.27 *P<0.001*	3	128	84	15

1. This and slide H51.16 developed together: apparently development faulty.

AIII.12.Hybridization of 5.4*ETS1* to chromosomes expressing fra(11)(q23) and the LCL-specific gap at 11q23.1

Specific activity of probe: $4.1*10^7$cpm/μg.

Slide	Probe Conc. (μg/ml)	Exposure time (days)	Individual and relevant details of chromosomes.
H41.1	0.2	29	C379/86; TBu; G-banded with trypsin and pre-photographed.
H41.2	0.2	35	C379/86; TBu; G-banded with trypsin and pre-photographed.
H41.3	0.2	118	F59B2; fra(11)(q13) (for identification of chromosome 11).
H41.5	-	1	F50I; for initial background level.
H41.6	-	35	F50I; for final background level.
H41.13	0.2	35	C208/79; LCL (with 11q23.1 gap); BuBu; solid stained and pre-photographed.
H41.14	0.2	35	C208/79; LCL (with 11q23.1 gap); BuBu; solid stained and pre-photographed.

H52.1	0.2	11	TB10; BuT; *G-banded.*
H52.2	0.2	21	TB10; BuT; *G-banded.*
H52.3	0.2	28	TB10; BuT; *G-banded.*
H52.4	-	3	TB10; for initial background level.
H52.5	0.2	35	F59B2; fra(11)(q13)
H52.6	0.2	35	F59B2; fra(11)(q13).
H52.7	0.2	35	F59B2; fra(11)(q13).
H52.8	0.2	35	F65A; fra(11)(q23).
H52.9	0.2	35	F65A; fra(11)(q23).
H52.10	0.2	35	F65A; fra(11)(q23).
H52.11	0.2	35	F65A; fra(11)(q23).
H52.12	0.2	35	F65A; fra(11)(q23).
H52.13	0.2	35	TB10; for final background level.

NOTES: H41.1-H41.14: Emulsion NTB-2:H_2O = 2:1.
Development D19, 4 min; no agitation,
at 19°C.

H52.1-H52.13: Emulsion NTB-2:H_2O = 2:1.
Development D19, 4 min, no agitation,
at 19°C.

HYBRIDIZATION RESULTS:

Slide H41.1: S=36=r; B=96; 19 cells; λ=2.67; $P<<10^{-5}$.

Slide H52.1: S=25=r; B=98; 25 cells; λ=2.77; $P<<10^{-5}$.

Slide H52.2: S=28=r; B=140; 25 cells; λ=3.96; $P<<10^{-5}$.

LOCALIZATION RESULTS:

fra(11)(q23) (all 0.2µg/ml probe):

Slide	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells	No. f.s.	No. fs with label on GSR
H52.8	6	22	9.14 *P<0.005*	3	439	32	20
H52.9	3	17	9.80 *P<0.005*	0	389	34	14
H52.10	0	23	23.00 *P<<0.001*	1	328	25	15
H52.11	1	27	24.14 *P<<0.001*	2	489	40	16
H52.12	1	13	10.29 *P<0.005*	2	429	32	11
Combined H52.8-H52.12	11	102	73.28 *P<<0.001*	8	2 074	163	76

LCL-specific gap at 11q23.1 (all 0.2μg/ml probe):

Slide	No. grains prox.	No. grains dist.	X^2 and *Probability*	No. grains cent.	No. cells with gap	No. gaps	No. fs with label on GSR
H41.13	6	18	6.00 *P<0.025*	9	52	60	24
H41.14	0	14	14.00 *P<0.001*	4	32	39	12
Combined H41.13-H41.14	6	34	19.60 *P<<0.001*	13	84	99	36

RESULTS OF SUBLOCALIZATION ON DISTAL ACENTRIC FRAGMENT:

fra(11)(q23) (all 0.2μg/ml):

Slide	No. grains closer to f.s.	No. grains closer to telomere	X^2 and *Probability*	No. grains mid-way or ambiguous
H52.8	7	8	0.07 *n.s.*	7

H52.9	6	7	0.08 *n.s.*	5
H52.10	4	6	0.40 *n.s.*	4
H52.11	13	11	0.17 *n.s.*	2
H52.12	5	9	1.14 *n.s.*	1
Combined H52.8-H52.12	35	41	0.47 *n.s.*	19

LCL-specific gap at 11q23.1 (all 0.2μg/ml):

Slide	No. grains closer to gap	No. grains closer to telomere	X^2 and *Probability*	No. grains mid-way or ambiguous
H41.13	7	11	0.89	10
H41.14	4	12	4.00 *P<0.05*	3

Combined H41.13-H41.14	11	23	4.24	13
			P<0.05	

APPENDIX AIV

SIGNAL AND BACKGROUND LEVELS FOR CERTAIN PROBES

pULB1148:

Slide	probe conc. (µg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H9.6	0.05	53	39	*0.74*	273	*5.15*	0.14
H9.8	0.1	14	13	*0.93*	147	*10.50*	0.088
H9.10	0.2	49	44	*0.90*	495	*10.10*	0.089
H9.12	0.4	70	133	*1.90*	1114	*15.91*	0.12
H9.14	0.8	29	99	*3.41*	530	*18.28*	0.19
H9.16	1.6	34	91	*2.68*	1591	*46.79*	0.057

APRT probe:

Slide	probe conc. (µg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H14.8	0.025	21	3	*0.14*	39	*1.86*	0.077
H14.9[1]	0.05	13	0	*0.00*	29	*2.23*	0
H14.10	0.1	24	8	*0.33*	60	*2.50*	0.13
H14.11	0.2	15	6	*0.40*	69	*4.60*	0.087
H14.12	0.4	45	25	*0.56*	233	*5.18*	0.11
H14.13	0.8	52	47	*0.90*	490	*9.42*	0.096
H14.14	1.6	28	27	*0.96*	384	*13.71*	0.070
(increased stringency wash, see AIII:)							
H14.15[2]	0.025	23	2	*0.09*	39	*1.70*	0.051
H14.16	0.05	24	7	*0.29*	59	*2.46*	0.12
H14.17[3]	0.1	27	2	*0.07*	70	*2.59*	0.029
H14.18	0.2	17	8	*0.47*	124	*7.29*	0.065
H14.19	0.4	41	33	*0.80*	207	*5.05*	0.16
H14.20	0.8	41	28	*0.68*	321	*7.83*	0.087
H14.21	1.6	53	59	*1.11*	849	*16.02*	0.069

1. n.s.
2. P=0.16; n.s.
3. P=0.34; n.s.

(other conditions varied, see AIII:)

H14.22	0.2	35	31	*0.89*	152	*4.34*	0.20
H14.23	0.2	72	23	*0.32*	345	*4.79*	0.067
H14.24	0.2	25	9	*0.36*	170	*6.80*	0.053
H14.25	0.2	25	13	*0.52*	206	*8.24*	0.063

JW101:

Slide	probe conc. (μg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H12.4[4]	0.05	64	4	*0.06*	160	*2.50*	0.025
H12.5	0.1	42	25	*0.60*	250	*5.95*	0.10
H12.6	0.2	45	10	*0.22*	274	*6.09*	0.036
H12.7	0.4	39	26	*0.70*	258	*6.97*	0.10
H12.8	0.8	20	8	*0.40*	185	*9.25*	0.043

4. P=0.16; n.s.

pHPζ:

Slide	probe conc. (µg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H25.9[5]	0.05	25	4	*0.16*	104	*4.16*	0.038
H25.10	0.1	25	5	*0.20*	102	*4.08*	0.049
H25.11	0.2	25	8	*0.32*	138	*5.52*	0.058
H25.12	0.4	25	7	*0.28*	199	*7.96*	0.035
H25.13	0.8	25	9½	*0.38*	347½	*13.90*	0.027

pα3'HVR.64:

Slide	probe conc. (µg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H44.6	0.05	25	11½	*0.46*	112½	*4.50*	0.10
H44.7	0.1	25	22	*0.88*	131	*5.24*	0.17
H44.8	0.2	25	24	*0.96*	143	*5.72*	0.17
H44.9	0.4	25	22	*0.88*	129	*5.16*	0.17
H44.10	0.8	25	21	*0.84*	170	*6.80*	0.12
H44.11	1.6	25	31	*1.24*	191	*7.64*	0.16

5. Not given in appendix AIII; chromosomes F11C used (see appendix AII), exposure 28 days.

G-CSF probe:

Slide	probe conc. (μg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H43.9[6]	0.05	25	2	*0.08*	64	*2.56*	0.031
H43.10	0.1	25	4	*0.16*	60	*2.40*	0.067
H43.11[7]	0.2	25	3	*0.12*	84	*3.36*	0.036
H43.12	0.4	25	4	*0.16*	58	*2.32*	0.069
H43.13[8]	0.8	25	4	*0.16*	72	*2.88*	0.056
H43.14[9]	1.6	25	5	*0.20*	111	*4.44*	0.045

6. P=0.37; n.s.
7. P=0.24; n.s.
8. P=0.054; n.s.
9. P=0.073; n.s.

ACH207:

Slide	probe conc. (μg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H51.8	0.05	25	13	*0.52*	106	*4.24*	0.12
H51.9	0.1	25	24	*0.96*	168	*6.72*	0.14
H51.10	0.2	25	9	*0.36*	178	*7.12*	0.051
H51.11	0.4	25	30	*1.20*	189	*7.56*	0.15
H51.12	0.8	25	58	*2.32*	323	*12.92*	0.18
H51.13	1.6	25	79	*3.16*	808	*32.32*	0.098

ACH224

Slide	probe conc. (μg/ml)	Cell no. (c)	Signal (S)	*S/c*	Back-ground (B)	*B/c*	S/B
H51.19	0.05	25	9	*0.36*	99	*3.96*	0.091
H51.20	0.1	25	12	*0.48*	123	*4.92*	0.098
H51.21	0.2	25	26	*1.04*	212	*8.48*	0.12
H51.22	0.4	25	23	*0.92*	223	*8.92*	0.10
H51.23	0.8	25	34	*1.36*	336	*13.44*	0.10

APPENDIX AV

PAPERS PUBLISHED AND IN PRESS.

This appendix contains papers published, or accepted for publication, on work directly resulting from this thesis.

Listed below are the roles I played in the work described in these papers.

1. (Chapter 5). Simmers, R.N., Stupans, I. and Sutherland, G.R. 1986A. Localization of the human haptoglobin genes distal to the fragile site at 16q22 using in situ hybridization. Cyogenet. Cell Genet. 41:38-41.
I carried out all the work, analysed the results and wrote the paper. Drs Stupans and Sutherland provided supervision of this work.

2. (Chapter 5). Fratini, A., Simmers, R.N., Callen, D.F., Hyland, V.J., Tischfield, J.A., Stambrook, P.J. and Sutherland, G.R. 1986. A new location for the human adenine phosphoribosyltransferase gene (*APRT*) distal to the haptoglobin (*HP*) and fra(16)(q23) (*FRA16D*) loci. Cytogenet. Cell Genet. 43:10-13.
I carried out and analysed the results of the *in situ* hybridization, and wrote those parts of the paper pertaining to this.

3. (Chapter 6). Simmers, R.N. and Sutherland, G.R. 1987. Further localization of *ETS1* indicates that the chromosomal rearrangement in Ewing sarcoma does not occur at fra(11)(q23). Hum. Genet. (In Press)

I carried out the work, analysed the results, and wrote the paper. Dr Sutherland provided supervision.

4. (Chapters 4, 5). Simmers, R.N., Sutherland, G.R., West, A. and Richards, R.I. 1987A. Fragile sites at 16q22 are not at the breakpoint of the chromosomal rearrangement in AMMoL. Science, 236:92-94.

I carried out the work, analysed the results and wrote the paper. Dr Sutherland provided supervision and Drs West and Richards provided the DNA clones.

5. (Chapter 4). Simmers, R.N., Webber, L.M., Shannon, M.F., Garson, O.M., Wong, G., Vadas, M.A. and Sutherland, G.R. 1987B. Localization of the *G-CSF* gene on chromosome 17 proximal to the breakpoint in the t(15;17) in APL. Blood 70:330-332.

I carried out the work, with assistance from Dr Webber, analysed the results, and wrote the paper. Chromosome preparations were provided by Drs Webber and Garson. DNA preparations were provided by Professor Vadas and Dr Wong, and DNA analysis was performed by Dr Shannon.

6. (Chapter 5). Simmers, R.N., Mulley, J.C., Hyland, V.J., Callen, D.F. and Sutherland, G.R. 1987C. Mapping the human α-globin gene complex to 16p13.2->pter. J. Med. Genet. (In press).
I carried out and analysed the results of the *in situ* hybridization, and wrote those sections of the paper pertaining to this.

7. (Chapter 4). Simmers, R.N., Smith, J., Shannon, M.F., Wong, G., Lopez, A.F., Baker, E., Sutherland, G.R. and Vadas, M.A. 1987D. Localization of the human G-CSF gene to the region of a breakpoint in the translocation typical of acute promyelocytic leukaemia. Hum. Genet. (In Press).
I performed the *in situ* hybridizations and analysed the results, with assistance from Mrs Baker, and helped write the paper.

8. (Chapter 6). Sutherland, G.R. and Simmers, R.N. 1987. No statistical association between common fragile sites and non-random chromosome breakpoints in cancer cells. Cytogenet. Cell Genet. (In Press).
I provided the experimental results (papers 3 and 4) discussed in this paper, and discussed the content of the paper with Dr Sutherland.

Simmers, R.N., Stupans, I., and Sutherland, G.R., (1986) Localization of the human haptoglobin genes distal to the fragile site at 16q22 using in situ hybridization. *Cytogenetics and Cell Genetics, v. 41 (1), pp. 38-41.*

Fratini, A., Simmers, R.N., Callen, D.F., Hyland, V.J., Tischfield, T.A., Stambrook, P.J., and Sutherland, G.R., (1986) A new location for the human adenine phosphoribosyltransferase gene (APRT) distal to the haptoglobin (HP) and fra(16)(q23)(FRA16D) loci.
Cytogenetics and Cell Genetics, v. 43 (1-2), pp. 10-13.

CGCSFGE3

FURTHER LOCALIZATION OF *ETS1* INDICATES THAT THE CHROMOSOMAL REARRANGEMENT IN EWING SARCOMA DOES NOT OCCUR AT fra(11)(q23)

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SUMMARY

A genomic probe homologous to 5.4kb of the c-*ets*-1 gene was hybridized *in situ* to chromosomes expressing fra(11)(q23). This probe hybridized distal to the fragile site, which is just distal to the mid-point of band 11q23.3. This result localizes *ETS1* from the *FRA11B* locus to 11q24. The result also distinguishes the *FRA11B* locus from the site of translocation at 11q23-q24 in the Ewing sarcoma- and peripheral neuroepithelioma-specific t(11;22), indicating that the chromosomes of a previously reported patient heterozygous for fra(11)(q23) did not rearrange at this fragile site to give rise to Ewing sarcoma. This adds to the mounting evidence against individuals with fragile sites being predisposed to developing cancer.

INTRODUCTION

The retroviral oncogene v-*ets* has its human cellular counterparts, c-*ets*-1 and c-*ets*-2, at two distinct loci: *ETS1* on chromosome 11 and *ETS2* on chromosome 21 (Watson et al. 1986). The *ETS1* locus has been mapped to bands 11q23->q24 using *in situ* hybridization (de Taisne et al. 1984). The region 11q23-24 is also involved in many consistent chromosome rearrangements in cancer, some of which have been found to involve the proto-oncogene c-*ets*-1.

Rearrangements involving this region have been reported for lymphocytic small cell lymphoma (Yunis et al. 1982; Yunis et al. 1984); myelodysplastic syndrome (Ohyashiki et al. 1986; Feder et al. 1985); Ewing sarcoma (Aurias et al. 1983; Turc-Carel et al. 1983), and the peripheral neuroectodermal tumours, peripheral neuroepithelioma (Whang-Peng et al. 1984; Whang-Peng et al. 1986) and Askin tumour (Whang-Peng et al. 1986); and an acute leukemia with a characteristic (4;11) translocation (Van den Berghe et al. 1979; Prigogina et al. 1979). Rearrangements involving 11q in all forms of acute non-lymphocytic leukemia (ANLL) are most strongly associated with M4 and M5, especially M5a with poorly differentiated blast cells, and most of these rearrangements occur at band q23 (Fourth International Workshop 1984).

Fragile sites are non-staining gaps or breaks at specific points on chromosomes (Sutherland 1979). The common, or constitutive, fragile sites occur at frequencies approaching homozygosity (Sutherland and Hecht 1985; Yunis and Soreng 1984), whereas rare fragile sites occur only in some members of the population. The folate-sensitive fragile (X) is the only fragile site associated with a known phenotypic abnormality - fragile (X)-linked mental retardation - which is the most common cause of inherited mental retardation (Sutherland and Hecht 1985). Fra(11)(q23) is a rare,

folate-sensitive fragile site localized near the distal end of band 11q23.3 (Sutherland et al. 1983). Fragile sites have recently become the subject of much discussion regarding their possible role as factors predisposing to cancer (Yunis 1984; Yunis 1983; Le Beau and Rowley 1984; Le Beau 1986). One line of evidence for this relationship is a number of patients reported to carry a particular rare fragile site, and to have a chromosome rearrangement in their tumor cells with a breakpoint in the same band as the fragile site (Le Beau 1986; Sessarego et al. 1983; Gollin et al. 1986). One patient has been reported to be heterozygous for fra(11)(q23) and to have Ewing sarcoma with the t(11;22)(q23;q11) in the malignant cells (Gollin et al. 1986).

We have used fra(11)(q23) as a precise point to obtain more information about the location of *ETS1* and to elucidate any possible role of this fragile site in the development of cancer.

MATERIALS AND METHODS

Chromosomes expressing fra(11)(q23) (Sutherland et al. 1983) were prepared by culturing and harvesting lymphocytes from an individual heterozygous for this fragile site in MEM-FA as described (Sutherland 1979). These were stored in fixative (methanol:acetic acid, 3:1) until required and then spread onto microscope slides.

A c-*ets*-1 probe consisting of a 5.4kb genomic DNA fragment, containing no repeats, in the plasmid pKH47 (de Taisne et al. 1984) was used. This was labeled to a specific activity of $4X10^7$cpm/ μg with three tritiated nucleotides and hybridized *in situ* to chromosomes expressing fra(11)(q23), as described (Simmers et al. 1986), with denaturation of the chromosomes in 70% deionized formamide, 2X SSC, at 70°C, pH 7.0 for two minutes and hybridization of 0.2μg/ml probe at 37°C. The slides were dipped in Kodak NTB-2 nuclear research emulsion diluted 2:1 with water, exposed for 35

days, developed and stained. The probe was also hybridized to normal male metaphase and prometaphase chromosomes prepared by the method of Zabel et al. (1983). These slides were developed sequentially and banded by the method of Zabel et al. (1983) to assess the level of specific labelling.

RESULTS

Twenty-five normal male metaphases developed after 21 days of exposure showed 28 silver grains over 11q and 140 grains over other chromosomal regions ($p << 10^{-5}$ using cumulative Poisson frequencies). Thus the c-*ets*-1 probe had hybridized specifically at the *ETS1* locus.

2074 metaphases from a culture with fra(11)(q23) expression induced were scored after 35 days of exposure. The fragile site was expressed in 163 of these metaphases and in 76 of these a silver grain or grains was touching part of the region from 11qter to a point equidistant from the fragile site on the proximal side. Of these, there were 102 silver grains distal to the fragile site; 11 silver grains proximal; and 8 spanning the fragile site and touching both or neither of the distal and proximal regions. Compared to the random expected distribution of the 113 informative proximal and distal silver grains (i.e. $56\,1/2$ proximal and 56 $1/2$ distal), the distribution of proximal and distal grains, with an excess of grains distal to the fragile site, is highly significant ($\chi^2 = 73.28$; $p << 10^{-3}$). This indicates that the region of the *ETS1* locus homologous to the probe is distal to the fragile site.

DISCUSSION

The results show that the region of the *ETS1* locus homologous to the probe used for *in situ* hybridization is distal to the fra(11)(q23) locus (*FRA11B*). There is a small number of grains proximal to the fragile site, but these may be accounted for by random background and scatter from the site of hybridization (see Simmers et al. 1987 for discussion). However, sequences homologous to only part of the gene were used for hybridization, and the possibility of the fragile site occurring within the c-*ets*-1 gene has not been excluded. Griffin et al. (1986) have sub-localized *ETS1* to the region 11q23.3->q24 using a constitutional chromosome rearrangement at 11q23.3. Our results further localize *ETS1* within this region, from *FRA11B* to 11q24. *FRA11B* is just distal to the mid-point of band 11q23.3 (Fig.2).

Gollin et al. (1987) reported the t(11;22) in the malignant cells of a patient with Ewing sarcoma, who was a heterozygote for fra(11)(q23). This case can be added to a growing body of anecdotal reports of individuals with a rare fragile site, expressed in chromosomes from normal cells, in the same band as the site of a rearrangement in their malignant cells. These cases involve band 11q13 in two patients with lymphocytic small cell lymphoma (Yunis 1983; Yunis et al. 1984) and a patient with myelofibrosis (Sessarego et al. 1983), band 12q13 in a patient with malignant lymphoma (Yunis 1983), and band 16q22 in 17 of 24 patients with the M4 form of ANLL (Le Beau 1986; Glover et al. 1986). We recently showed that the inversion of chromosome 16 with a breakpoint at band 16q22 in AMMoL does not occur at the rare fra(16)(q22) locus (*FRA16B*) (Simmers et al. 1987), nor at the common fragile site locus in the same band (*FRA16C*).

Griffin et al. (1986) found the region of DNA homologous to the same c-*ets*-1 genomic DNA probe used in our study to be proximal to the breakpoint at 11q23-24 in the specific chromosome rearrangements of two

Ewing sarcoma and two peripheral neuroepithelioma cell lines. Using Southern analysis they could not detect rearrangement of c-*ets*-1 by these translocations for 25kb around the probe in thirteen of such tumor cell lines. Although they could not exclude the possibility that these specific rearrangements involved another part of the *ETS1* locus, their results and ours clearly separate the breakpoints from the fragile site locus by at least the region homologous to the probe (Fig.3). Therefore, the translocation observed in the patient with the t(11;22) in Ewing sarcoma (Gollin et al. 1986) did not result from breakage and rearrangement at fra(11)(q23). Our results also exclude the possibility that fra(11)(q23) occurs at the 11q23-q24 breakpoint in peripheral neuroepithelioma, and suggests that fra(11)(q23) does not occur at this breakpoint in Askin tumour. Askin tumour cells have a t(11;22) cytogenetically identical to that in Ewing sarcoma and peripheral neuroepithelioma.

Whilst mechanisms for fragile sites predisposing to oncogenic translocations other than by breakage directly at the fragile site might be invoked, they would infer that the fragile site is acting from a distance and is not involved solely because of its fragility. The results of *in situ* hybridization using the *ETS1* locus and fra(11)(q23) (this study), and the metallothionein locus and fra(16)(q22) (Simmers et al. 1987), show that the chromosomal translocations in question in Ewing sarcoma and AMMoL did not result from breakage at either of these two rare fragile sites. There are two lines of circumstantial evidence linking fragile sites and cancer breakpoints: the occurrence of these two phenomena in the same chromosome band, and anecdotal reports of individuals who are heterozygous for a rare fragile site and have a chromosomal rearrangement in malignant cells with one breakpoint in the rearrangement being in the same band as the fragile site. When direct evidence of a true coincidence between a fragile site and a chromosomal breakpoint has been sought, it has pointed to these two

phenomena not being causally related. Equally, there is no evidence of any increase in malignant disease in families in which fragile sites segregate and cancer families have not been found to be segregating for fragile sites.

There also exists a common fragile site in subband 11q23.3 (Yunis and Soreng 1984). It may be present on both chromosomes 11 in all individuals; no cases of possible involvement of this common fragile site in the evolution of cancer have been reported. This fragile site has not been included in this study, for these reasons and because of the difficulties involved in its induction and detection in large numbers of metaphases.

There are a number of other specific chromosome rearrangements with a breakpoint at band 11q23. The current study provides no further evidence for the concurrence of fra(11)(q23) and these breakpoints, although all of those studied are excluded from the region distal to *ETS1*. Probes homologous to parts of c-*ets*-1 have been found to hybridize distal to the breakpoints at 11q23 in the t(9;11)(p22;q23) in ANLL-M5 (Diaz et al. 1986) and the t(4;11)(q21;q23) in acute leukemia (Sacchi et al. 1984) (see Fig.3), and no rearrangement of the c-*ets*-1 gene has been detected using Southern analysis in either case. Sacchi et al (1986) found a lower level of expression of the c-*ets*-1 gene in cells with the t(4;11) however, indicating that the breakpoint at 11q23 may be through an adjacent regulatory region of this gene. Rearrangement and amplification of c-*ets*-1 has been detected in two other cancers where band 11q23 was involved in the malignant karyotype (Rovigatti et al. 1986).

A tenuous relationship between the locations of fragile sites and oncogenes has been observed (Hecht and Sutherland 1984; Yunis and Soreng 1984; Sutherland and Hecht 1985), although this may only reflect the closer

correspondence of locations of fragile sites and cancer breakpoints (Daniel 1986). We have refined the map position of *ETS1* and provided further evidence against any causal relationship between fragile sites and cancer. The only caveat which needs to be placed on our results is that the probe used was 5.4kb of genomic DNA from within a locus which is probably in excess of 25kb in length (Griffin et al. 1986). It remains possible that the fragile site could be within the oncogene but there is no doubt that the fragile site and the chromosome breakpoints in Ewing sarcoma, peripheral neuroepithilioma and probably Askin tumor are not coincidental.

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LEGEND TO FIGURES

Fig.1 Metaphase after *in situ* hybridization showing a silver grain (large arrow) distal to fra(11)(q23) (small arrow).

Fig.2 Idiogram indicating *FRA11B* (arrow) and the regional localization of *ETS1* (bar) determined in this study.

Fig.3 Diagrams of 11q showing the linear order of markers (not drawn to scale). (a) *FRA11B* and the t(11;22) breakpoint(s) are separated by at least 5.4kb of DNA homologous to the c-*ets*-1 probe. (b) The t(9;11) in ANLL and the t(4;11) in ALL showed breakpoints proximal to the regions of c-*ets*-1 homologous to the v-*ets* sequences in *pets*BB and a 5.7kb genomic sequence homologous to c-*ets*-1 in pRD6K.

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Figure 1. See figure 6.3(c).

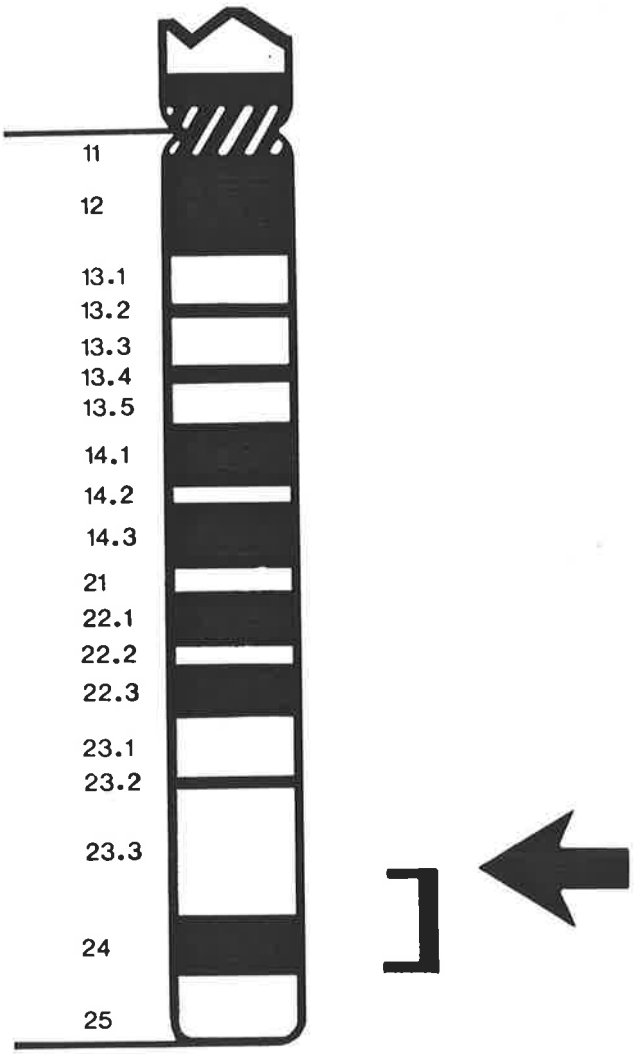
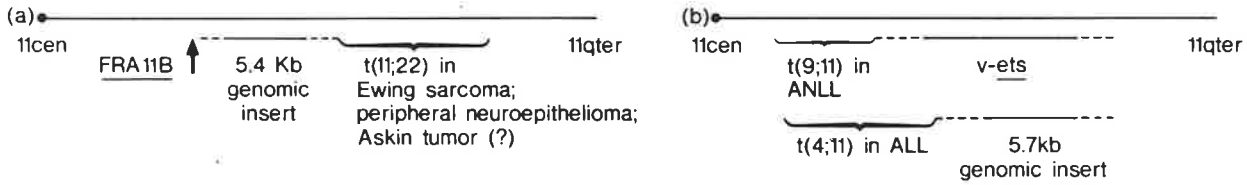


Figure 2.


(a)
11cen
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5.4 Kb
genomic
insert
t(11;22) in
Ewing sarcoma;
peripheral neuroepithelioma;
Askin tumor (?)
11qter
(b)
11cen
t(9;11) in
ANLL
v-ets
11qter
t(4;11) in ALL
5.7kb
genomic insert

Figure 3.

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LOCALIZATION OF THE HUMAN G-CSF GENE TO THE REGION OF A BREAKPOINT IN THE TRANSLOCATION TYPICAL OF ACUTE PROMYELOCYTIC LEUKAEMIA

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SUMMARY

The colony stimulating factors regulate growth, differentiation and function of blood cells. The effect of granulocyte-colony stimulating factor (G-CSF) on myeloid leukaemias is unique among colony stimulating factors in driving the leukaemic cells from a self-renewing malignant state to a mature differentiated phenotype with the concomitant loss of tumourigenicity. This property of G-CSF has led to suggestions that its absence is responsible for lack of differentiation of leukaemic cells and that the therapeutic administration of G-CSF could reverse this defect resulting in a cure for leukaemia. Here we show that the gene coding for human G-CSF is localized to chromosome 17, bands q11.2-21. The translocation of the long arm of chromosome 17 at q12-21 to chromosome 15 is a specific abnormality occurring in a high proportion of, if not all, patients with acute promyelocytic leukaemia, a disease characterised by undifferentiated myeloid cells and dismal prognosis. Abnormalities of the regulation of a specific differentiation factor gene mediated by a specific chromosomal rearrangement may be directly implicated in the pathogenesis of human leukaemia.

INTRODUCTION

Colony stimulating factors (CSF) are a group of glycoproteins that regulate the growth, differentiation and function of blood cells (Metcalf 1977; Nicola and Vadas 1984; Burgess and Metcalf 1980a). Amongst the human CSFs, the recently cloned granulocyte-colony stimulating factor (G-CSF) (Nagata et al. 1986), the active molecule in a species previously referred to as CSF-ß (Nicola et al. 1985), is characterized by its ability to stimulate predominantly neutrophil colonies. In contrst to GM-CSF, G-CSF is fully cross-reactive between mouse and human species in terms of receptor binding and its ability to stimulate the differentiation of neutrophils and to augment their mature cell function (Nicola et al. 1985; Lopez et al. 1983). G-CSF is unique in that it not only promotes the proliferation and differentiation of progenitor cells into neutrophils (Nicola et al. 1983; Metcalf and Nicola 1983) but also stimulates the differentiation of leukaemic cells or leukaemic cell lines (Lotem et al. 1980; Burgess and Metcalf 1980b; Metcalf 1980; Nicola and Metcalf 1984) to form mature neutrophils. This effect can be measured by the suppression of self-renewal of myeloid leukaemia stem cells *in vitro* (Metcalf 1980) and by the suppression of leukaemogenicity of myeloid leukaemic cells *in vivo* (Ichikawa 1970; Fibach and Sachs 1975; Honma et al. 1979; Metcalf 1982; Lotem and Sachs 1981). These properties have led to the name 'differentiation factor' being used for this activity of G-CSF denoting its unusual capacity of stimulating differentiation.

The recent cloning of human G-CSF cDNA has revealed a single gene coding for a 177 (Nagata et al. 1986) or 180 (Souza et al. 1986) amino acid mature protein of molecular weight 19,600. Southern hybridization analysis revealed a single fragment encoding the G-CSF gene suggesting a single copy

in the genome. We have mapped this gene to human chromosome 17 using *in situ* hybridization.

MATERIALS AND METHODS

Hybridization probe

The probe for the G-CSF gene was cloned from the TPA 30-1 cell line using an oligonucleotide probe designed from published sequence data. The full length molecule identical in nucleotide sequence to that published by Souza et al. (1986) was filled at both ends with EcoRI adaptors and was inserted in the 5′ to 3′ orientation into a pXMT2 plasmid at the XhoI restriction site. The cDNA probe was nick translated with three tritiated nucleotides to a specific activity of 1.7×10^7 cpm/μg.

In situ hybridization

Prometaphase and metaphase chromosomes from normal human lymphcoytes with a karyotype 46,XY were G-banded with trypsin and photographed. The labelled G-CSF gene probe was hybridized *in situ* as described (Simmers et al. 1986) with denaturation of the chromosomes in 70% deionized formamide, 2XSSC, at 70°C, pH7.0 for two minutes and hybridization of 0.4 μg/ml probe at 37°C. The slides were dipped in Kodak NTB-2 nuclear research emulsion diluted 2:1 with water, exposed for 39 days, developed, stained, and the metaphases relocated. Additional preparations of prometaphase and metaphase chromosomes were obtained and G-banded after *in situ* hybridization using the previously described methods (Zabel et al. 1983).

RESULTS

In situ hybridization of the G-CSF probe to human chromosomes (Figure 1) resulted in 29 (13%) of 226 silver grains over the long arm of chromosome 17 in 58 metaphases and prometaphases. This chromosome arm comprises 2% of the total chromosome length (ISCN 1985). 17q is significantly labelled

($p<<10^{-5}$, using cumulative Poisson probabilities). The majority of grains were seen over 17q11.2-21, with a peak at 17q12.

DISCUSSION

The functional effects of G-CSF on leukaemic cells has led to the hypothesis that an abnormality in the production of this factor, or of its receptor, is involved in the lack of differentiation of some leukaemia cells, and the capacity of murine and more recently, human leukaemia cells to differentiate under the influence of G-CSF has suggested considerable therapeutic potential. Cells from patients with acute promyelocytic leukaemia have been shown to possess a receptor for G-CSF (Nicola et al. 1985) and to respond *in vitro* to cloned G-CSF by differentiating into mature cells (Souza et al. 1986).

The human acute promyelocytic leukaemia, or acute non-lymphoblastic leukaemia-M3, is characteristically associated with a cytogenetic abnormality, a reciprocal translocation with breakpoints on the long arm of chromosome 17 at band q12-21 and the long arm of chromosome 15 at band q22. This abnormality is present in possibly all of these patients and is virtually diagnostic of this condition (Rowley 1984). Interestingly, genetic analysis of the 15;17 translocation in leukocytes from a patient with acute promyelocytic leukaemia (Sheer et al. 1983) has strongly suggested that the c-*fes* oncogene is on the part of chromosome 15 translocated to chromosome 17 in this leukaemia.

Our results clearly demonstrate a unique chromosomal location for the G-CSF gene at 17q11.2-21, the region of one of the breakpoints in the 15;17 translocation in acute promyelocytic leukaemia. Should the translocation breakpoint coincide with, or be adjacent to the G-CSF gene, a disturbance in its regulation could explain the lack of cellular differentiation seen

in this disorder and would provide a rationale for the therapeutic administration of G-CSF.

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LEGEND TO FIGURES

Fig. 1 A diagrammatic representation of silver grains scored over chromosomes 1-22, X and Y in 58 metaphases after *in situ* hybridization. The combined distribution on the long arm of chromosome 17 from these 58 and a further 26 metaphases with label on 17q is also shown.

Fig. 2 Four partial metaphases showing a silver grain (arrow) over the region 17q12-q21.1.

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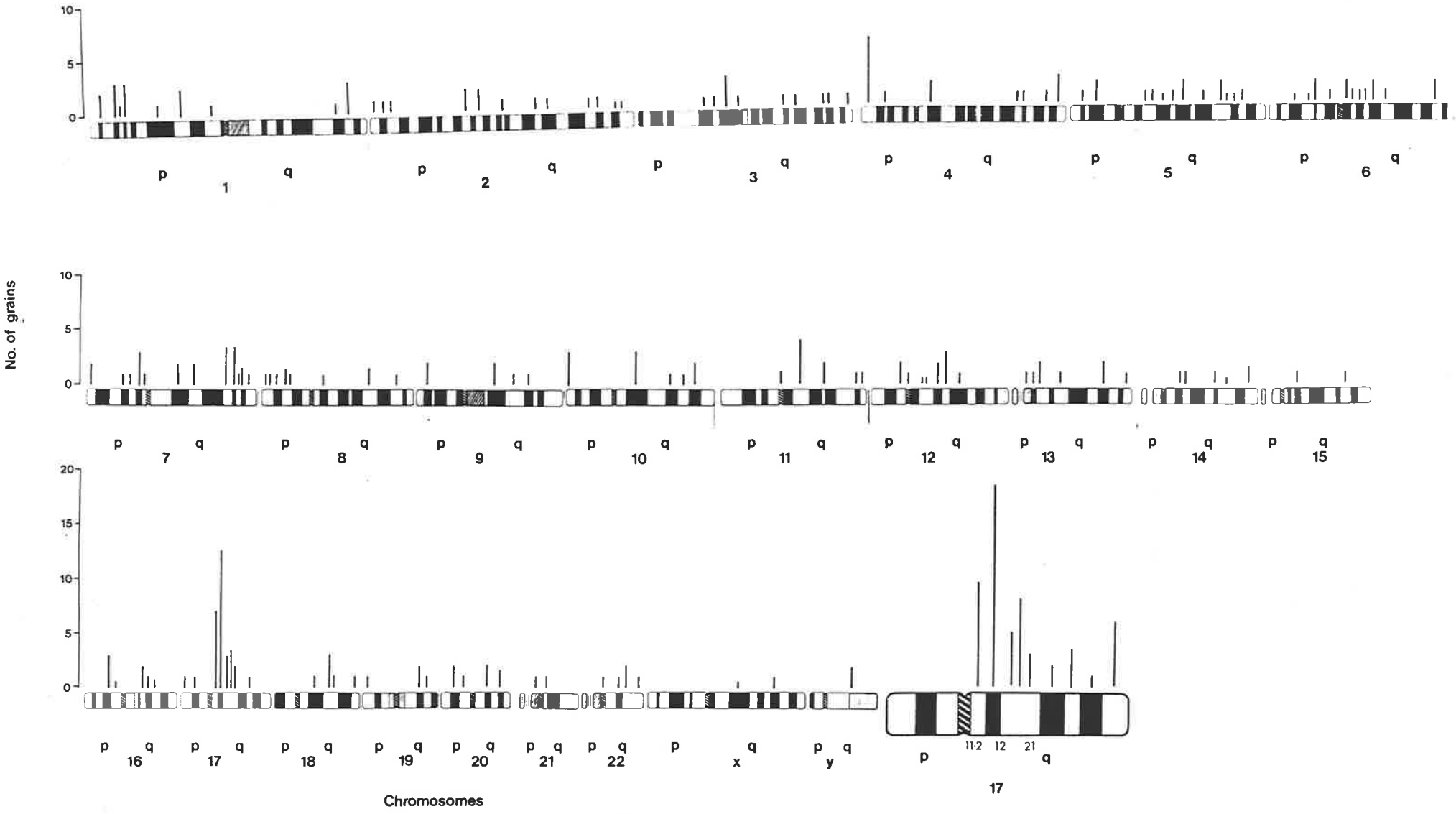
No. of grains
Chromosomes
1
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Figure 1

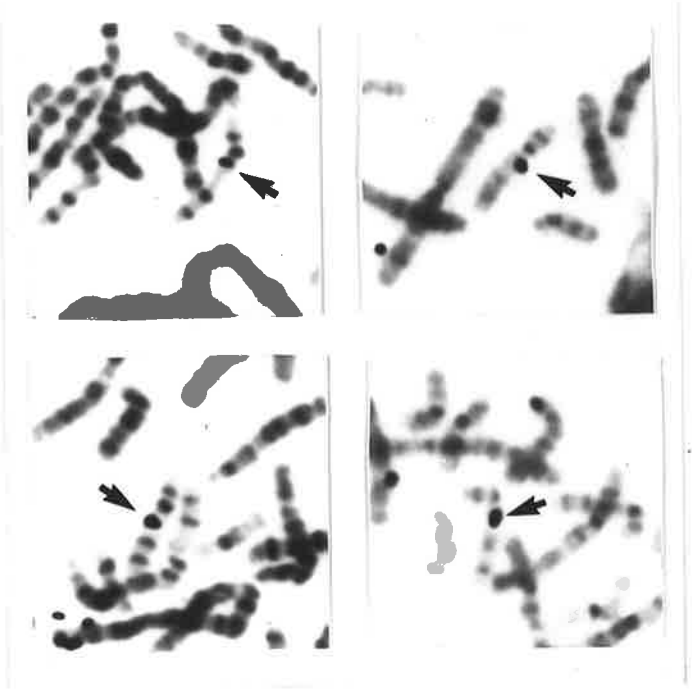

Figure 2

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