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THE MOLECULAR GENETICS OF HEREDITARY HAEMOCHROMATOSIS AND CHARACTERIZATION OF THE IRON-BINDING PURPLE PHOSPHATES OF THE HUMAN MACROPHAGE.

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ABSTRACT

Hereditary haemochromatosis is a disorder of iron metabolism in man that is characterized by the pathological accumulation of iron in the tissues with associated functional impairment. HLA studies have shown haemochromatosis to be transmitted by an autosomal recessive gene and indicate that this gene is in tight physical linkage with HLA-A alleles in the class I region of the Major Histocompatability Complex (MHC) on chromosome 6.

To investigate more precisely the position of the gene for haemochromatosis, a detailed study of the class I region was carried out using pulsed-field gel electrophoresis in conjunction with a panel of MHC probes. There was no difference in hybridization patterns between affected and non-affected individuals for most probe and enzyme combinations. Where heterozygosity did occur, no evidence for direct linkage with the disease phenotype was found. The overall results of these studies suggest that a major alteration of DNA structure in the class I MHC region is not responsible for haemochromatosis in the patients investigated.

In an additional study of haemochromatosis, the cellular expression and chromosomal localization of the human type 5 (tartrate-resistant) acid phosphatase, an iron-binding protein present within macrophages, was investigated. Utilizing the polymerase chain reaction, the cDNA encoding the type 5 acid phosphatase was cloned and sequenced and found to comprise 1359 base pairs encoding 325 amino acids. Putative metal-binding domains were identified by examination of the primary structure of this protein. Northern analysis revealed a single RNA species of approximately 1.5 kb present within cultured human macrophages, but only trace amounts of this transcript were identified in resting monocytes - findings consistent with macrophage specific expression of this protein. These observations were confirmed in similar studies of RNA prepared from osteoclasts and other cells of the monohistiocytic phenotype. In collaboration with colleagues from the Imperial Cancer Research Fund laboratories, the human acid phosphatase gene was localized to chromosome 19p.

The mapping of the acid phosphatase gene to chromosome 19 and not to human chromosome 6 suggests that this protein does not have a primary causal role in the aetiology of haemochromatosis. The findings, however, open the way for further examination of the regulation and expression of this enzyme in cells of the mononuclear phagocyte series and its possible role in human iron metabolism.

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CHAPTER 1: INTRODUCTION

Chapter 1: Introduction

1.1 Definition of Hereditary Haemochromatosis

The pathological accumulation of iron in the tissues with parenchymal injury and associated functional impairment is known as haemochromatosis or iron-storage disease.

Owing to the limited capacity for excretion of iron, iron homeostasis is maintained by controlled absorption of iron from dietary sources (McCance and Widdowson 1937). Absorption of excess iron occurs as a result of a disturbance of the regulatory mechanism in the intestine and is especially prominent in blood disorders in which ineffective erythropoeisis occurs e.g., thalassaemia major (Erlandsen et al. 1962). In this case the tendency to absorb excessive iron probably results from an inability of the iron stores to supply the markedly increased needs of the expanded erythroid marrow. Haemochromatosis may also arise because of administration of excess iron, either by prolonged ingestion (as in the case of Bantu-siderosis which results from the use of iron pots for the brewing of beer (Charlton et al. 1973)) or more usually because of regular blood transfusions (Ley et al. 1982), in which iron from haemoglobin by-passes the mechanism that controls iron absorption.

Haemochromatosis in the absence of an identifiable predisposing disorder is termed hereditary haemochromatosis and is the result of an inherited metabolic defect, the nature of which has still to be established.

The clinical expression of hereditary haemochromatosis depends on a number of factors. Sex is a major determinant, with up to ten-fold greater incidence of the disease in men compared with women. This probably reflects the iron lost through menstruation, pregnancy and lactation in women. In most clinical cases, symptoms of

iron-storage disease do not become apparent until middle age and after the menopause in women. Dietary factors also influence the amount of iron that is bioavailable in the diet. In this context there appears to be an association between alcohol consumption and the development of clinical symptoms of hereditary haemochromatosis (Bothwell et al. 1979).

1.2 Mechanism of Tissue Damage

Excess iron is deposited in the liver, heart, pancreas, endocrine organs, skin and joints but the mechanism of tissue damage in hereditary haemochromatosis is still a cause for debate. In the fully established disease state, up to 30% of the total serum iron is non-transferrin bound, as contrasted with more than 95% that is transferrin-bound in the normal individual (Batey et al. 1980). The non-transferrin bound iron (NTBI) fraction may play an important rôle in the pathogenesis of haemochromatosis: it is taken up more efficiently than transferrin-bound iron (TBI) by liver cells and is localized in lysosomes (predominantly in zone 1 hepatocytes) (Batey et al. 1981; Brissot et al. 1985), a feature also observed in hereditary haemochromatosis (Seymour and Peters 1978). In contrast to TBI, NTBI is capable of stimulating lipid peroxidation of membranes (Gutteridge et al. 1985). Disruption of lysosomal membranes has been described in hereditary haemochromatosis and has been suggested as a potential source of tissue damage (Seymour and Peters 1978). Increased lysosomal fragility was apparently related to the amount of haemosiderin in the tissues, however, peroxidative damage of lysosomal membranes induced by NTBI may also contribute to this effect. It is noteworthy that in thalassaemia major, a fraction of the serum iron is also non-transferrin bound (Hershko et al. 1978) and iron overload in this disease may progress to a clinicopathological picture that is identical to that seen in hereditary haemochromatosis. Taken as a whole, these observations suggest that the toxic NTBI fraction may be the predominant source of tissue iron in hereditary haemochromatosis (and other iron-storage diseases) and may be responsible for the tissue damage.

1.3 Diagnosis of Haemochromatosis

By the time symptoms have developed in hereditary haemochromatosis, there is irreversible parenchymal injury associated with widespread deposition of iron in the tissue. There are signs of liver disease, endocrine failure, cardiomyopathy, joint involvement and dermal pigmentation. The diagnosis of haemochromatosis is usually based on the demonstration of abnormal parameters of iron status in the serum. In the untreated disease, the serum iron is usually elevated and in more that 90% of individuals the saturation of plasma transferrin has been found to exceed 62%. The serum ferritin, although a less sensitive indicator of iron overload, is generally elevated at the time of diagnosis. Confirmation of iron-storage disease normally requires histological quantification or chemical determination of the iron content of a liver biopsy sample. Further information regarding the amount of mobilizable storage iron can be obtained by measuring the quantity of iron excreted in the urine after injecting a chelator. Several groups have reported non-invasive tests for *in vivo* detection of iron storage disease such as nuclear magnetic resonance spectroscopy (Bydder and Steiner 1984) but as yet these procedures are mainly restricted to specialised centres.

1.4 Treatment and Prognosis

Hereditary haemochromatosis is best treated by repeated venesection. The erythroid marrow responds by stepping up the rate of erythropoiesis, and iron is mobilized rapidly from the tissue stores to replace that which has been removed. Treatment is continued until iron depletion is shown by development of mild anaemia. Iron chelation therapy is not generally used in the management of iron overload because the efficiency of iron removal does not match that which can be achieved by repeated bleeding.

Removal of iron and prevention of its re-accumulation in the tissue is of proven benefit in patients with haemochromatosis, improving life expectancy as well as hepatic and

cardiac function (Bomford and Williams 1976). In a long term study it was shown that patients with haemochromatosis but without cirrhosis have a normal life expectancy after treatment (Niederau et al. 1985). In patients with cirrhosis, life expectancy was also improved but removal of iron did not prevent the development of cancer in the cirrhotic liver. Liver failure and liver cancer are frequent causes of death in patients with established cirrhosis and haemochromatosis - primary hepatocellular carcinoma causing death in about 30% of individuals with the disease. Thus, it is of critical importance to identify asymptomatic individuals with haemochromatosis in the pre-cirrhotic stage.

1.5 Genetics of Haemochromatosis

Hereditary haemochromatosis has for a long time been known to show a familial incidence and for many years autosomal dominant transmission of the disease was suspected. However, clarification of a recessive mode of inheritance was accomplished by the demonstration of an association between haemochromatosis and the Major Histocompatability Complex (MHC).

The association between haemochromatosis and HLA-A3 (Simon et al. 1975), B7 (Walters et al. 1975; Simon et al. 1976) and B14 (Simon et al. 1976) was described over a decade ago and has been confirmed independently in a large number of studies. It is now clear that the primary association of haemochromatosis is with HLA-A3, with HLA-B7 and B14 antigens in linkage disequilibrium (Simon et al. 1987). The frequency of A3 in most of the patients is 70-80% compared with about 25% in population controls without haemochromatosis (Simon and Brissot 1988).

HLA typing of affected families confirmed the recessive mode of transmission of haemochromatosis (Simon et al. 1977). The haemochromatosis gene was transmitted together with a specific haplotype within a family: siblings of propositi who shared

both parental haplotypes frequently developed iron-storage disease, whereas siblings with only one HLA haplotype in common with the propositus had only minor abnormalities of iron metabolism and did not develop clinical disease.

Recombination between the putative haemochromatosis gene and HLA genes is rare (~ 1%) indicating tight physical linkage of the disease allele to the MHC complex on the short arm of chromosome 6. Recently a combined lod score of ~ 60 has been calculated (Simon et al. 1987) indicating that the probability of linkage is 10^{60} times greater than of there being no linkage.

The primary association between hereditary haemochromatosis and HLA-A3, as well as information gained from an informative recombination in the class I region of a family affected by the disease (Edwards et al. 1980) indicates that the predisposing allele for haemochromatosis may lie somewhat closer to the A locus than the B locus. Moreover, in another documented family, in which an informative recombination occurred, the haemochromatosis gene appeared to segregate with the HLA-B gene suggesting that the disease locus may lie between the HLA-A and B genes (Edwards et al. 1986) (see Figure 1.1).

The availability of DNA probes for this chromosomal region on human chromosome 6 opens up the possibility for investigation of the putative haemochromatosis locus at the molecular level. Hybridization of MHC class I probes to Southern blots of human genomic DNA has revealed 15 to 25 restriction fragments, depending upon the enzyme used. This is consistent with 20-30 related class I sequences which are thought to represent pseudogenes and related functional genes in this region (Orr et al. 1982). The possibility that these additional class I sequences may be candidates for the abnormal gene in haemochromatosis has been investigated by Cragg et al. (1988). An MHC class I probe was hybridized to digests of DNA from haemochromatosis patients and control subjects. Restriction fragments that correlated with particular HLA antigens

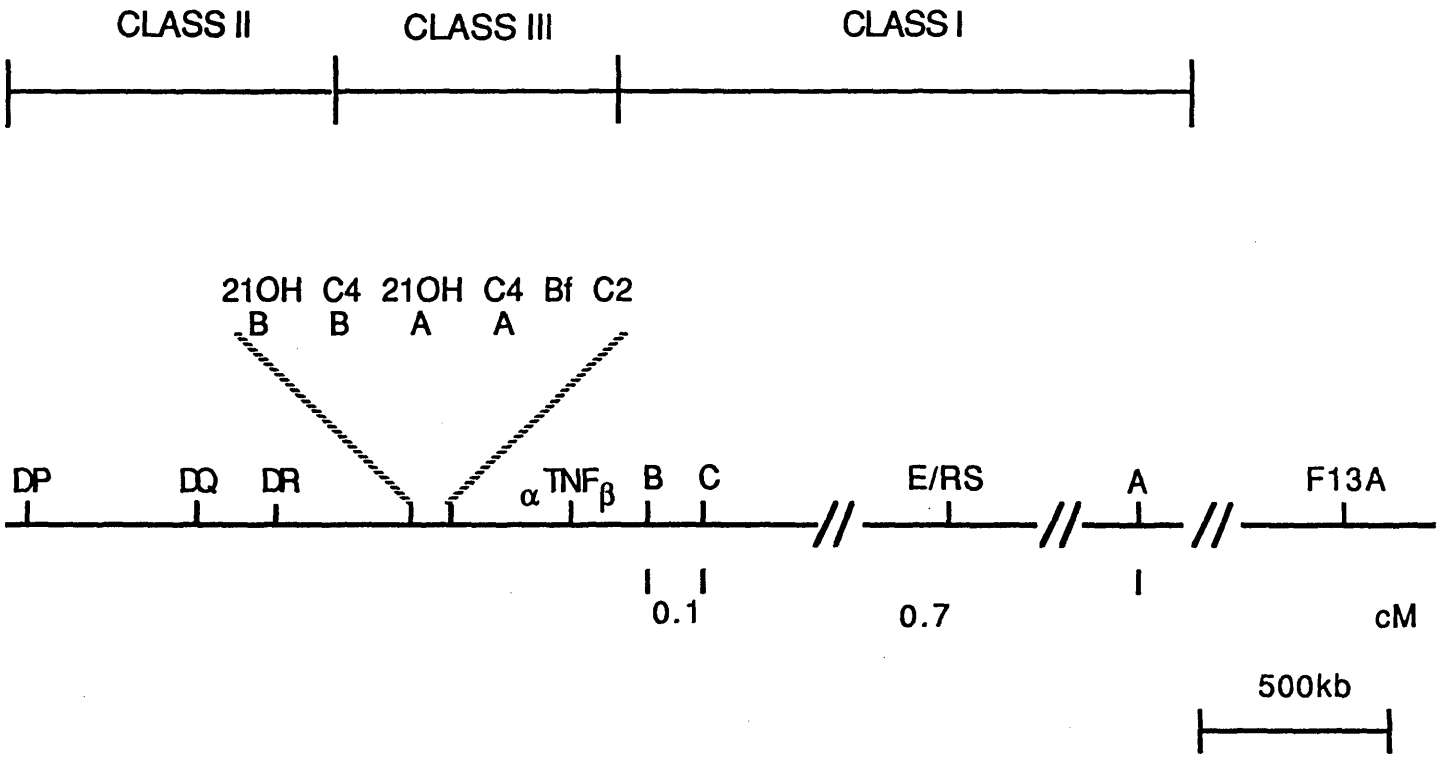


Figure 1.1. Schematic diagram showing the localization of genes on the short arm of human chromosome 6. HLA - DP, DQ, DR = Major Histocompatability Complex class II antigens. HLA - A, B, C, E/RS = Major Histocompatability Complex class I antigens. BF = Properdin factor B. C4A, C4B, C2 = Complement components 4A, 4B, and 2. 21OH A, 21OH B = 21-hydroxylase. TNF α, β=Tumour necrosis factor. F13A = Coagulation factor 13 A.

were observed but no fragments specific for haemochromatosis were detected. In a similar study, Lucotte and colleagues (1986) reported an association between a 10 kb *Pvu* II class I-specific restriction fragment and the haemochromatosis susceptibility gene. However, this fragment was found to correlate with the A3 serological allele and was not specific for haemochromatosis. In an alternative approach, David et al. (1986) have investigated a pedigree in which only one of three HLA identical siblings were affected by haemochromatosis. RFLP analysis suggested that a cross-over had occurred in the class I region of the MHC in this family. This rearrangement was accompanied by the absence of a 7.4 kb *Hin*d III class I fragment and it was postulated that the haemochromatosis gene mapped to this restriction fragment. However, at the time of writing no further information about this fragment has been published.

The demonstration that the haemochromatosis gene is in tight linkage with the HLA-A locus has important implications for clinical genetics. In families with an affected homozygote, further homozygotes and heterozygote carriers can be identified by HLA typing (Muir et al. 1984). Thus, it is now possible to identify asymptomatic individuals within an affected pedigree who are homozygous for the haemochromatosis gene, the importance of which has already been discussed.

1.6 Prevalence of the Haemochromatosis Gene

Epidemiological studies based on indices of iron metabolism (degree of transferrin saturation, serum ferritin) carried out in the United Kingdom, Canada, Sweden and South Africa have indicated that the frequency of haemochromatosis lies between 0.2% and 0.5% of the population (Bothwell et al. 1989). These findings have been substantiated by a recent large-scale study in Utah in which 11,065 presumably healthy blood donors were screened for elevated transferrin saturation (Edwards et al. 1988). Thirty eight donors with transferrin saturation of 62% or more were considered as potential homozygotes for the haemochromatosis gene and underwent liver biopsies.

Seven of these individuals had liver biopsies diagnostic of haemochromatosis and a further nine had clearly increased hepatocellular iron deposition. According to likelihood analysis of the affected pedigrees, twenty six of the thirty eight probands were homozygous and twelve were heterozygous. The estimated frequency of homozygosity was based on the data in men, due to the low phenotypic expression of the haemochromatosis genotype in women. The frequency of homozygotes was 0.5% corresponding to a gene frequency of 0.07. The data suggest that the haemochromatosis gene is one of the most common abnormal genes in populations of European origin. This remarkable prevalence allows matings between homozygotes and heterozygotes to occur. Since one half of the offspring from such matings will be homozygous for the mutant allele, the explanation for the occasional occurence of the disease in successive generations of the same family is apparent (Edwards et al. 1981; Basett et al. 1982). It is likely that such pseudodominant transmission obscured, for some time, an appreciation of the recessive mode of transmission of haemochromatosis.

The reason for the apparently high frequency of the haemochromatosis gene in the European population is unknown but it is possible that in the past, possession of the haemochromatosis gene would have conferred a selective advantage. The homozygote or heterozygote with an abnormality of iron metabolism leading to an enhanced absorption of dietary iron would be relatively protected against the development of iron deficiency with its accompanying reduction of growth and vigour (Cox 1980). It is also possible that the accumulation of large body reserves improved survival during infancy, childhood and pregnancy in comparison with the rest of the population. Evolutionary selection against subjects homozygous for hereditary haemochromatosis would be minimal since the disease does not usually become evident until after the fifth decade, when reproductive activity has declined.

1.7 Origin of the Haemochromatosis Gene

There are many reports of HLA typing of patients with haemochromatosis and in all cases the association of haemochromatosis with the HLA-A3 allele is significant. In particular, the association of the A3 gene with haemochromatosis in populations of European origin has been used to propose a common origin of the gene from a discrete founder group that was subsequently modified by recombinations and geographical scattering due to migration and, possibly preferential survival (Simon et al. 1987).

1.8 Nature of the Genetic Defect

Although much is known about the proteins and tissues of iron metabolism, the means by which levels of iron in the body are regulated is not understood. In hereditary haemochromatosis the mechanism of iron homeostasis appears to be disturbed, thus leading to hyperabsorption of dietary iron. The identification of the genetic defect involved would not only be important with regard to the disease itself, perhaps enabling earlier detection of any asymptomatic individuals, but would also lead to a better understanding of iron metabolism.

1.8.1 Small intestine

The capacity of the body to excrete iron is extremely limited and the net iron balance is regulated at the level of absorption by the mucosa of the small intestine. As inferred above, the signal by which the mucosa is 'informed' of the body requirements is unknown. In hereditary haemochromatosis, this regulatory mechanism in someway appears to break down so that there is excessive retention of iron from the diet. In established haemochromatosis, clinical measurements have generally shown iron absorption to be normal; although in young patients with rapidly-progressive disease, markedly increased absorption of iron has been found (Alper et al. 1951). After

depletion therapy, the rate of recovery of iron stores is enhanced for five to ten years after treatment (Smith et al. 1969). Thus,it can be said that in haemochromatosis the net absorption of dietary iron by the small intestine is, if not actually increased, inappropriate in relation to body iron stores (Walters et al. 1975).

Ultra-structural studies of the small intestinal cells from patients with haemochromatosis have shown a markedly reduced ferritin content (Crosby 1963). However, biochemical studies have shown no defect in the biosynthesis of ferritin by the duodenal mucosa in haemochromatosis (Halliday et al. 1978). Experiments carried out using radiolabelled iron chelates that were incubated with samples of duodenal mucosa suggested that in haemochromatosis, the mucosal epithelium had an abnormal avidity for iron in relation to body iron stores (Cox and Peters 1978) although the basis of this phenomenon is unknown. Thus, although there is evidence that the intestinal mucosa is implicated in the development of iron overload in haemochromatosis, it is not known whether it is the site of the primary genetic defect. Indeed,an abnormality of iron metabolism in another tissue might affect iron absorption in the intestine. Such a mechanism would appear to be the case in certain refractory anaemias where ineffective erythropoiesis leads to increased absorption of dietary iron.

1.8.2 The liver

The liver plays an important rôle in iron exchange within the body. Not only is the liver the principal site of transferrin synthesis - a specialized protein responsible for iron transport, but it also interacts with newly absorbed iron delivered via the plasma. Liver iron in the form of ferritin and haemosiderin constitutes the main storage pool within the body and the liver is also able to take up plasma ferritin probably via a receptor-mediated reaction (Mack et al. 1983).

The deposition of iron in the liver is an early and consistent finding in haemochromatosis patients and suggests that the hepatocyte may be primarily involved in the expression of the genetic defect. Indeed, studies by Batey and colleagues (1978) have demonstrated increased uptake of [^{52}Fe]-labelled transferrin by the liver in patients with treated haemochromatosis compared with controls with similar parameters of iron status. Thus, these studies would suggest an abnormality of iron uptake by the hepatocyte in hereditary haemochromatosis.

1.8.3 Abnormalities in candidate proteins of iron storage and transport

Plasma transferrin is responsible for the transport of iron between the different compartments of the body. Most of the iron entering the plasma is contributed by mononuclear phagocytes that constitute the so-called reticulo-endothelial system and is derived from the breakdown of red blood cells, with smaller amounts originating from storage compounds and the diet. There is active transport and exchange between the erythron where the iron is incorporated into haemoglobin, parenchymal cells, particularly those of the liver and the extravascular fluids. Cellular uptake of transferrin iron is brought about by the membrane receptors for transferrin which participate in a unique cycle of endocytosis, iron removal, and exocytosis, known as the transferrin cycle (Morgan 1983).

Iron is stored within cells as ferritin or aggregates of haemosiderin, the latter are most probably derived from the proteolytic degradation of ferritin in secondary lysosomes (Wixom et al. 1980). Ferritin consists of a protein shell made up of twenty four subunits surrounding an iron core, the iron being readily available for incorporation into functional compounds when required. Ferritins from different tissues display a great deal of heterogeneity which is related to differences in composition of heavy (H) and light (L) subunits. The more basic isoferritins are rich in L subunits and are abundant in iron-storage tissues such as liver and spleen, whereas the more acidic

isoferritins are rich in H subunits and are predominant in organs with a low non-haem iron content such as heart and pancreas (Arosio et al. 1978). In a study of various tissues obtained from patients with haemochromatosis, Powell et al. (1974) found an absence of the more acidic isoferritins. The haemochromatotic tissue ferritins closely resembled normal liver in their isoferritin profile. However, later studies showed that this anomalous isoferritin tissue pattern could also be found in patients with other types of iron-storage disease (Powell et al. 1975). Indeed, the isoferritin pattern reverted to normal after depletion therapy. Thus, the abnormal distribution of tissue isoferritins in haemochromatosis appears to be a response to iron loading and is unlikely to be related to an underlying genetic defect in ferritin metabolism. Thus far, no primary defect in ferritin has been detected in hereditary haemochromatosis. Small amounts of ferritin also circulate in the plasma. There is a close correlation between this plasma ferritin and body iron stores, and in iron-overload plasma ferritin levels are greatly elevated. However, plasma ferritin contains very little iron and it seems unlikely that changes in ferritin turnover could account for the development of iron-overload.

Physico-chemical analysis of transferrin from subjects with haemochromatosis has failed to show any difference from normal human transferrin (Bothwell et al. 1962). A cross-over study has also shown that transferrin from subjects with haemochromatosis takes up and releases iron normally *in vivo* (Wheby et al. 1964). Since the genes for transferrin and transferrin receptor are located on chromosome 3 (Yang et al. 1984) and the haemochromatosis gene has been localized to the short arm of chromosome 6, it is perhaps not surprising that abnormalities of these proteins have not been found in haemochromatosis. Similarly, the functional ferritin H and L subunit genes are coded for on chromosome 11 and 19, respectively (Caskey et al. 1983; Worwood et al. 1985). However, it is noteworthy that the ferritins are a multigene family which have been mapped to ten different chromosomes including chromosome 6 (McGill et al. 1987). This latter observation still awaits confirmation and at present it is not clear

whether this H subunit gene sequence constitutes an expressed gene or represents a pseudogene.

It remains possible that the molecular defect in hereditary haemochromatosis affects the expression of a major protein of iron metabolism. A number of studies have been carried out on the regulation of expression of ferritin, transferrin and the transferrin receptor which may shed light on the factors involved in the fine control of cellular iron metabolism. The rate of biosynthesis of ferritin is dependent on iron: it increases when cells are exposed to iron donors and decreases when cells are exposed to iron limiters, which reduce intracellular iron availability (Mattia et al. 1986). There is evidence that this iron-mediated regulation of ferritin biosynthesis occurs by stabilisation of ferritin mRNA's, possibly by a trans-activating factor induced or activated by the presence of iron (Mattia et al. 1989). It has also been shown that in a 'short term response' after a few hours of exposure to an iron source, a pool of inactive cytoplasmic ferritin mRNA is de-repressed and translated (Aziz and Munro 1986). Transferrin receptor gene expression is induced by iron deprivation and it is clear that this regulation, also, is achieved by altering the cytoplasmic stability of the receptor mRNA (Mullner and Kuhn 1988). Transferrin and transferrin receptor expression have been studied in the human gastro-intestinal epithelium by means of fluorescent mono-specific antibodies but no obvious difference between normal and haemochromatotic individuals was identifiable (Banerjee et al. 1986). Transferrin receptor expression has also been studied in biopsy specimens of human liver (Sciot et al. 1987). In patients with hereditary haemochromatosis, transferrin receptor expression was down-regulated compared with controls. This phenomenon was not specific for haemochromatosis and was also observed in patients with secondary iron overload.

It is of interest that transferrin receptor expression has been reported to be increased in blood monocytes from patients with haemochromatosis (Bjorn-Rasmussen et al.

1985). Recently, Testa et al. (1989) have also shown that transferrin receptor expression is stimulated when macrophages are supplemented with iron *in vitro* . This phenomenon is in contrast to other types of cells where iron suppresses receptor synthesis. The relationship between this unusual mode of receptor regulation in macrophages and the increased storage of iron in hereditary haemochromatosis has yet to be clarified but it offers an attractive and promising line of enquiry.

1.8.4 Mononuclear phagocyte system

Macrophages of the mononuclear phagocyte system (MPS) are centrally important in iron metabolism as they are involved in the retrieval of iron from phagocytosed red blood cells. There is some evidence to suggest that these cells may be the site of the primary defect in hereditary haemochromatosis. In secondary iron overload, large deposits of iron are present in the cells of the MPS, particularly of the spleen, bone marrow, and liver, with variable involvement of the parenchymal cells. In hereditary haemochromatosis, the parenchymal cells contain virtually all the haemosiderin deposits, and the MPS very little (Charlton et al. 1973). On the basis of these findings it has been suggested that the cells of the MPS have a common defect leading to reduced iron storage and the delivery of increased amounts of iron via the plasma to hepatocytes and other parenchymal cells (Bothwell et al. 1979).

Some support for this theory has been obtained from a preliminary study of the kinetics of iron release from labelled erythrocytes in hereditary haemochromatosis. In patients with haemochromatosis, an increased fraction of haemoglobin-derived iron was returned directly to the plasma. In normal subjects, less iron was delivered immediately to the plasma and about one third was retained and released over a period of days. The kinetics of haemoglobin iron release in iron-deficient subjects were similar to those of patients with haemochromatosis (Fillet and Marsaglia 1975).

Current information on the function of mononuclear phagocytic cells in haemochromatosis with respect to known proteins of iron metabolism are conflicting. The increased transferrin receptor expression seen in monocytes of patients with haemochromatosis has been discussed above (p 27), but recent experiments that have examined uptake of iron from transferrin, and ferritin biosynthesis by peripheral blood monocytes *in vitro* have not shown any abnormalities in cells obtained from haemochromatosis patients (Jacobs and Summers 1981). However, there is present within mononuclear phagocytic cells a major iron-binding protein of as yet undefined function which may play an important role in the metabolism of iron. This protein is called type 5 acid phosphatase.

1.9 Type 5 Acid Phosphatase

There are multiple forms of human acid phosphatase, four of which - type 5, lysosomal, prostatic and the red cell enzyme - have been studied in detail (see Table 1.1). The distribution of these isoenzymes has been studied mainly by electrophoresis and enzymatic assay of tissue extracts. Five main acid phosphatase zones can be recognized by polyacrylamide gel electrophoresis at pH 4, migrating cathodally and numbered 1 to 5 from the origin (anode) towards the cathode (Yam et al. 1971). Type 5 acid phosphatase is the most cathodal and is completely tartrate-resistant (the red cell acid phosphatase is also tartrate-resistant but does not migrate in this system). The type 5 acid phosphatase is unique in containing iron which gives it a purple colouration (Campbell et al. 1978).

The type 5 isoenzyme has been found in differentiated cells of the human MPS, notably osteoclasts (Hammarstrom et al. 1971; Minken 1982) and alveolar macrophages (Efstratiadis and Moss 1985a) and is also pathologically increased in certain disease states. High levels of type 5 acid phosphatase have been found within the spleen (Robinson and Glew 1980) and monocytes (Troy et al. 1985) of patients

Isoenzymes	Expression	Major identifying properties
Erythrocytic	Widely expressed in cytoplasm of cells as well as in erythrocytes. Both common and rare alleloenzymes exist.	Resistant to inhibition by (+) - tartrate. Low activity on 1-naphthyl phosphate. Low molecular weight (17,000 Da).
Lysosomal	One (or possibly more) forms in virtually all cells.	Inhibited by (+) - tartrate. Relatively high activity on 1-naphthyl phosphate. Molecular weight 100,000 Da.
Prostatic	Essentially confined to prostate with trace expression in other tissues.	Inhibited by (+) - tartrate. Relatively high activity on 1-naphthyl phosphate. Molecular weight 100,000 Da i.e., similar in many respects to lysosomal acid phosphatase but antigenically unique. Micro-heterogenous due to variations in sialic acid content.
Type 5 acid phosphatase	Normally confined to differentiated cells of mononuclear phagocyte system, notably osteoclasts, and alveolar macrophages. Pathological increases in Gaucher cells and 'hairy' cells of hairy cell leukaemia.	Resistant to inhibition by (+) - tartrate. Intermediate reactivity with 1-napthyl phosphate. Intermediate molecular weight (30,000 Da). Iron-containing. Antigenically distinct from tartrate-sensitive isoenzymes.

Table 1.1. Multiple forms of human acid phosphatase - taken from Moss (1986).

with Gaucher's disease, the splenocytes and circulating white cells of patients with hairy cell leukaemia (Yam et al. 1972) and osteoclastic bone tumours (Chen et al. 1979). Increases of type 5 acid phosphatase in the serum have been detected in Gaucher's disease (Robinson and Glew 1980a), malignancies with bone metastases (Tavassoli et al. 1980) and in the serum of children during physiological bone growth (Chen et al. 1979). Increased expression of this isoenzyme is also associated with various B-cell and T-cell leukaemias (Drexler et al. 1986a). In systematic studies of leukaemia cell lines, it appeared that the type 5 isoenzyme was not generally expressed in immature lymphoid cells, but rather by cells arrested in later stages of differentiation (Drexler et al. 1985).

Type 5 tartrate-resistant acid phosphatase has been purified from a number of different sources including bovine (Davis et al. 1981), Gaucher's (Robinson and Glew 1980b) and hairy cell spleen (Ketcham et al. 1985), lung (Efstratiadis and Moss 1985b), placenta (Dipietro and Zengerle 1967, Gieselmann et al. 1984), bovine (Lau et al. 1985) and human bone (Allen et al. 1989) and osteoclastoma tissue (Hayman et al. 1989). Type 5 acid phosphatase is a glycoprotein of molecular weight 30,000-40,000. The pH optima of the type 5 acid phosphatase generally lies between 4.9 and 5.7. The enzyme has an unusual substrate specificity, preferring substrates with strong leaving groups such as nitrophenylphosphate, adenosine triphosphate and pyrophosphate. The enzyme hydrolyses aliphatic phosphates poorly but does hydrolyse the phosphate groups of certain phosphoproteins. Although the type 5 isoenzymes from the above tissues differ from each other in a number of respects, including molecular weight and substrate specificity, they have been reported to cross-react immunologically (Lam et al. 1981; Ketcham et al. 1985).

Immunological and enzymatic studies (Ketcham et al. 1985), as well as protein sequence analysis (Hunt et al. 1987), have shown that the splenic enzyme is closely homologous to uteroferrin, an abundant purple iron-containing acid phosphatase

secreted by the porcine uterus under the influence of progesterone (Roberts and Bazer 1980). The use of immunoaffinity chromatography with antibodies raised against porcine uteroferrin was a critical step in the purification of the human spleen enzyme from a patient with hairy cell leukaemia (Ketcham et al. 1985). In addition, antibodies raised against uteroferrin have been used in the development of a specific immunoassay for type 5 acid phosphatase (Whitaker et al. 1989). Recent studies have indicated that the iron which is essential for the enzymatic activity of the two enzymes is present as a di-iron complex (Davis and Averill 1982). Both enzymes exist in one of two interconvertible forms. In the case of the native, relatively inactive purple (oxidized) form, the iron is believed to exist as a pair of ferric atoms, while in the active pink (reduced) form the iron is represented by a ferrous-ferric iron pair (Antaniatis and Aisen 1983a).

Uteroferrin is a secreted protein, the uterus of the mid-pregnant pig producing up to 2 grams per day, whereas type 5 acid phosphatase is found intracellularly and has been localized to membrane bound organelles (Lam et al. 1976; Shindelmeiser et al. 1987). The mannose-6-phosphate residue of uteroferrin has been shown in biosynthetic labelling experiments to be masked (Baumbach et al. 1984) and this may explain why it is secreted and not directed to the lysosomes. Roberts and Bazer (1980) have proposed that the primary function of uteroferrin is unrelated to its phosphatase activity and that its true rôle may be one of transplacental iron transport during pregnancy. They have argued that the complete inactivity of uteroferrin in the fluid environment where it is found, together with its presence in an amount far exceeding that which could be required for catalytic purposes, argues against an enzymatic rôle. Moreover, using immunochemical and radiotracer techniques, they have shown that uteroferrin can cross the pig placenta, and that its iron is utilized in fetal erythropoeisis (Buhi et al. 1982; Ducsay et al. 1982; Renegar et al 1982). Thus, uteroferrin appears to have a rôle in iron metabolism and it is conceivable that an enzymatic function may be secondary for type 5 acid phosphatase as well. Indeed, ultrastructural studies indicate an

association of the bovine protein with ferritin and haemosiderin aggregates in splenic macrophages, suggesting type 5 acid phosphatase may participate in the retrieval of iron released by erythrophagocytosis (Shindelmeiser et al. 1987).

Since there is evidence that the retrieval of iron by cells of the MPS is defective in hereditary haemochromatosis, the iron-binding type 5 acid phosphatase can be proposed as a candidate protein that may be implicated in the development of this disease.

1.10 Summary and Aims of Work Described in this Thesis

The defect involved in hereditary haemochromatosis has been investigated both with regard to an aberrant protein function and also at the level of DNA. Given that the gene for haemochromatosis maps to the MHC class I region on the short arm of chromosome 6, investigation at the level of DNA is an attractive approach towards the molecular characterization of this disease. The aim of the work described in this thesis was to use pulsed-field gel electrophoresis, in conjunction with MHC probes to examine the structural arrangement of the class I region of the human MHC in haemochromatosis and to search for rearrangements or deletions that may be linked to the disease.

Although the function and expression of the major proteins of iron metabolism have been studied extensively, no primary defect has as yet been found with respect to haemochromatosis. It seems likely, therefore, that an as yet undefined protein of iron metabolism, possibly one expressed in the cells of the mononuclear phagocyte system, is involved in the aetiology of this disease. Thus, a further aim of this work was to investigate the iron-binding purple acid phosphatase of human macrophages in relation to iron metabolism, and to define the chromosomal localization of its gene.

CHAPTER 2: MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Basic Techniques

Standard techniques such as phenol extraction and ethanol precipitation were carried out as described by Maniatis et al. (1982). Unless otherwise stated, microcentrifuge samples were spun at 12,000 x g at room temperature.

2.2 Patients and Control Subjects

In the molecular analysis of the human MHC class I region in hereditary haemochromatosis, six unrelated patients, three affected pedigrees and three healthy control subjects were studied. The patients were diagnosed by Professor Timothy Cox (Hammersmith Hospital, London) and by Dr. Adrian Bomford (Kings College Hospital, London). The diagnosis was based on clinical findings and on the demonstration of abnormal serum parameters of iron status; it was confirmed by histological examination of liver biopsy specimens and staining with Perls' reagent (Scheuer et al. 1962). The HLA type of each individual was determined by the standard NIH technique in the diagnostic hospital tissue typing laboratory (Terasaki et al. 1978).

2.3 DNA Extraction

DNA was prepared from 10 ml of citrated peripheral blood using the urea/SDS lysis method described by Sykes (1983).

The blood was first diluted with one volume of phosphate buffer saline (PBS) and then frozen at -20°C. After thawing, the white cells were harvested by centrifugation at 1,000 x g for 10 minutes at room temperature. The pellet was washed twice with 0.1% Nonidet P40 in PBS and then the cells resuspended in approximately 5 ml of lysis

buffer (300mM NaCl, 10mM EDTA, 10mM Tris. HCl pH7.4, 7M urea). Typically, 0.5 ml volumes of lysis buffer were added until a homogenous and moderately viscous suspension was obtained. To this, 10% w/v sodium dodecyl sulphate (SDS) was added to a final concentration of 2% w/v. After incubation at 37°C for 10 minutes, the solution was extracted with an equal volume of neutralized phenol and the phases separated by spinning at 1,000 x g for 10 minutes. This was repeated until the interface was clear and then followed by two chloroform extractions to remove the phenol. The DNA was precipitated with ethanol, washed in 70% v/v ethanol and resuspended in a final volume of 250 µl TE (see Appendix 1). The final yield of DNA was determined by measuring the optical density (OD) of a 1/100 dilution in water at 260 nm. Typically, about 1 mg was purified from 10 ml of blood using this method.

2.4 Preparation of Encapsulated Genomic DNA

High molecular weight DNA was prepared in agarose inserts essentially as described by van Ommen and Verkerk (1986).

Whole blood was diluted by the addition of an equal volume of PBS and then layered carefully onto an equal volume of Histopaque-1077 (Sigma). After centrifugation at 1,000 x g for 30 minutes, the clearly-visible layer of lymphocytes was recovered by aspiration with a Pasteur pipette. The cells were washed three times in PBS and then mixed with an equal volume of 2% w/v low gelling temperature (LGT) agarose (BRL) (maintained at 40°C) to give a final concentration of 2 x 10^7 cells/ml. The cell suspension was pipetted directly into a cubic insert mould, one side of which had been sealed with tape. Each insert held a volume of approximately 3 x 10^6 cells, equivalent to about 10 µg DNA. After the agarose had set, the tape was removed and the plugs were pushed from the mould into 10 ml NDS buffer (0.5M EDTA, 10mM Tris. HCl, 1% w/v Sarkosyl pH9.5), 1 mg/ml proteinase K (Boehringer Mannheim). After incubation at 50°C overnight, fresh NDS buffer and proteinase K were added and the

agarose inserts incubated for a further 2 days at 50°C. The DNA blocks were then stored in NDS buffer at 4°C.

2.5 RNA Extraction

2.5.1 Preparation of total RNA

RNA was prepared by the caesium gradient-guanidine thiocyanate method (Chirgwin et al. 1979) using ribonuclease-free stock solutions.

Tissue that had been stored at -70°C was ground to a powder in liquid nitrogen and then added to a small volume (approximately 5 ml/g tissue) of fresh GTC solution (5.5M guanidine thiocyanate (Fluka), 25mM sodium citrate, 0.5% sodium lauroyl sarcosine, 0.2M β-mercaptoethanol pH7.0). The volume of homogenate was measured and 0.37 g caesium chloride added per ml. Once dissolved, the mixture was layered onto a 1.2 ml cushion of 5.7M caesium chloride, 25mM sodium citrate, 10mM EDTA pH7.0 in 5 ml ultracentrifuge tubes. These were centrifuged in a Beckman SW55Ti swing out rotor at 30,000 rpm for 22 hours and the RNA pellet taken up in distilled water. The RNA was stored as an ethanol precipitate at -70°C.

2.5.2 Preparation of poly (A)+ RNA

Poly (A)+ RNA was prepared from total RNA by chromatography on oligo (dT)-cellulose (Sigma). The oligo (dT)-cellulose was first equilibrated in sterile loading buffer (20mM Tris. HCl pH7.6, 0.5M sodium chloride, 1mM EDTA, 0.1% SDS) and then used to pour a 1.0 ml column in a Pasteur pipette. The column was then washed with 3 bed-volumes of :-

1. Sterile water
2. 0.1M sodium hydroxide, 5mM EDTA

3. Sterile water

4. Sterile loading buffer

The RNA (1-10 mg) was dissolved in sterile water and heated to 65°C for 5 minutes. An equal volume of 2 x loading buffer was added, the sample cooled and then applied to the column. The flow-through fraction was collected, heated to 65°C, rapidly cooled and reapplied to the column. The column was washed with 5-10 bed-volumes of loading buffer followed by 4 volumes of loading buffer containing 0.1M sodium chloride. The enriched poly (A)+ RNA fraction was eluted with 2-3 bed-volumes of sterile 10mM Tris. HCl pH7.4, 1mM EDTA, 0.05% SDS, ethanol precipitated, washed in 70% ethanol and then resuspended in sterile water.

2.6 Restriction Enzyme Digests

Restriction enzymes were purchased from commercial suppliers and used according to the manufacturers' directions. Reactions were stopped by the addition of 10% Ficoll, 0.006% bromophenol blue, 0.06% xylene cyanol, 0.5% SDS.

Agarose blocks containing DNA for pulsed-field gel electrophoresis were washed three times in 10 ml of 10mM Tris. HCl pH8.0, 0.1mM EDTA, 0.1mM phenyl-methyl-sulfonyl-fluoride for 30 minutes at 4°C, and then equilibrated in the appropriate restriction buffer for 30 minutes at 4°C before digestion. After digestion, in 100 µl volumes, the blocks were incubated in NDS buffer and proteinase K at 1 mg/ml for 2 hours at 50°C followed by equilibration in 100 µl running buffer for 30 minutes at 4°C before loading.

2.7 Gel Electrophoresis

2.7.1 Agarose gel electrophoresis of DNA

Digests containing 5-10 µg DNA were fractionated on horizontal agarose gels (15 cm x 20 cm) ranging from 0.6% to 2.0% w/v, depending on the fragment sizes of interest. The electrophoresis buffer was 1 x Tris-Borate-EDTA (TBE) (see Appendix 1) and gels were usually run overnight at a field strength of 1-3 V/cm. Gels were stained in running buffer containing ethidium bromide (1 µg/ml), visualized on a short-wave ultraviolet transilluminator and photographed using a Polaroid camera.

Size standards were provided by digestion of the plasmid vector pEMBL with *Taq* I restriction enzyme for small fragments, or by *lambda* DNA digested with *Hind* III for large fragments (Northumbrian Biochemicals Ltd.).

2.7.2 Purification of DNA from gels

Restriction fragments were electrophoresed on 'ultra pure' agarose (Bio-Rad) gels and isolated using the freeze-squeeze technique (Tautz and Renz 1983). After staining, the gel was placed on a transilluminator and the relevant bands cut out with a razor blade. The gel slices were placed in 0.5M sodium acetate, 1mM EDTA for 1 hour and then transferred to a 0.75 ml microcentrifuge tube in which holes had been pierced in the top and the bottom, the latter having been plugged with a small amount of siliconized Whatman GF-C paper. This was frozen in liquid nitrogen for 10 minutes and then placed into a capless 1.5 ml microcentrifuge tube and spun for 15 minutes in a microfuge. The liquid that collected in the 1.5 ml tube was precipitated with ethanol and the pellet taken up in TE.

2.7.3 Agarose gel electrophoresis of RNA

RNA was electrophoresed in a gel tank used exclusively for RNA work and of the same dimensions as that described in 2.7.1 above. Typically, 1.5% w/v agarose gels were prepared in 1 x MOPS (Sigma) buffer (see Appendix 1). The agarose was dissolved by heating and then cooled to 50°C before adding 6% v/v formaldehyde (BDH AnalaR 40% v/v). RNA samples containing 10 µg RNA were taken up in 3 volumes loading buffer (66% de-ionized formamide (BDH AnalaR) (see Appendix 1), 13% 10 x MOPS and 20% formaldehyde) and denatured by heating to 65°C for 5 minutes. The samples were then chilled on ice and 5 µl 50% (v/v) glycerol, 0.1 mg/ml bromophenol blue added prior to loading.

Gels were run overnight at 2.5 V/cm and then stained, visualized and photographed as described for DNA (2.7.1). RNA markers with a size range of 0.3-1.6 kb were purchased from Boehringer Mannheim.

2.7.4 Pulsed-field gel electrophoresis (PFGE)

Preliminary studies were carried out using a PFGE apparatus provided by the Medical Research Council (MRC) Mammalian Genome Unit. Later, a 2015 Pulsaphor unit was purchased from LKB. This system consists of an electrophoresis unit, a control unit, a power supply and a thermostatic circulator. Prior to a run the electrophoresis unit was filled with 2.5 litres of 0.1M TBE running buffer (see Appendix 1) and the thermostatic circulator, to maintain a constant running temperature of 13°C, was switched on. Agarose inserts containing 10 µg digested DNA were loaded into the wells of a 1% gel (20 cm x 20 cm). Intact yeast chromosomes or concatamers of *lambda* DNA were used as molecular weight markers. For maximum resolution, only the centre 10 wells were used. Each well was then sealed with LGT agarose to ensure that the inserts stayed in place. The electrodes were positioned in the double inhomogenous mode, and for

resolution of fragments between 40 and 1000 kb, the pulse time was set at 100 seconds.

The DNA was electrophoresed at 330 V for 40 hours, after which the gel was stained, visualized and photographed as described previously (2.7.1).

(i) Preparation of yeast size markers. *Saccharomyces cerevisiae* (strain X2180-1B) was grown on a YPD agar plate (see Appendix 1) overnight at 30°C. Starter cultures were made in 50 ml YPD broth and 5-10 ml used to inoculate 500 ml YPD in 2 litre flasks. These were then placed on an orbital shaker at 30°C and the cells grown until the OD_{600} reached 0.36. The cells were harvested by centrifugation at 1,000 x g, washed in PBS and then resuspended at a final concentration of between 0.5 and 1 x 10^9 cells/ml.

LGT agarose was made up at a concentration of 1% in PBS containing 20mM DTT and 10 µg/ml Zymolase 100-T (Seikagaku Kogyo Co.) and maintained at 37°C. The yeast suspension was warmed to 37°C, mixed with an equal volume of LGT agarose and then pipetted directly into an insert mould. After the agarose had set, the plugs were removed and incubated for 2 hours at 37°C in 10 ml of 20mM DTT, 10 µg/ml Zymolase in PBS. The plugs were then washed in NDS buffer and after incubation overnight at 50°C in NDS and proteinase K (1 mg/ml), fresh NDS and proteinase K was added and the inserts incubated for a further 2 days at 50°C. The blocks were washed in TE and stored in fresh TE at 4°C.

(ii) Preparation of *lambda* oligomers. *Lambda* (λ) DNA was prepared from λc1857Sam7, a lysogenic bacteriophage using the induction method described by Maniatis et al. (1982). Oligomerisation of the DNA was carried out by first embedding the λ virions in agarose plugs. Sufficient virions were used to give a final DNA concentration of 20-30 µg λ DNA/ml. These plugs were incubated in NDS and proteinase K as described for the production of encapsulated genomic DNA (2.4). The

agarose inserts were then equilibrated in 2 x SSC by washing them 3 to 4 times in a 10 fold excess of 2 x SSC and then oligomerisation was allowed to proceed at room temperature for 1-2 weeks. If poor oligomerisation or slight degradation was observed, 10mM EDTA and 10mM Tris. HCl pH7.5 was included in the 2 x SSC.

2.8 Southern Transfer

Restriction fragments were transferred from agarose gels to Hybond N membranes (Amersham) using a capillary blotting method described by Southern (1975). To assist the transfer of large fragments, an initial acidic depurination step was carried out, in which the gel was soaked in 0.25M HCl for 15 minutes. The gel was then given two 30 minute washes in a denaturing solution (1.5M sodium chloride, 0.5M sodium hydroxide), followed by two further 30 minute washes in a neutralizing solution (1.5M sodium chloride, 0.5M Tris HCl pH7.0). The neutralized gel was placed on filter paper soaked in 10 x SSC (see Appendix 1), with wicks in contact with a reservoir also containing 10 x SSC. A sheet of Hybond N was cut to size and placed on the surface of the gel, followed by stacks of absorbant paper towel. After blotting overnight the filter was washed in 2 x SSC for 10 minutes and then left to dry. DNA was fixed to the filter by exposing it to uv light of 254 nm wavelength for 4 minutes.

2.9 Northern Transfer

The transfer was set up as for DNA (2.8) except that the gel was not subjected to treatment with HCl, denaturing or neutralizing solutions.

2.10 Probes

The following probes were used in pulsed-field gel studies :- a general MHC class I probe (pHLA-A2) (Trowsdale et al. 1984), class I-specific genomic probes - HLA-B

(pB250), HLA-C (pC250) (Strachan et al. 1986) and HLA-E (pRS5.10) (Srivastava et al. 1987). An HLA-A locus-specific probe (pA490) was prepared by isolating a 490bp *Pvu* II-*Msp* I fragment from the 3' untranslated region of the class I cDNA clone as previously described by Koller et al. (1984) (see Appendix 3). Other probes used were the tumour necrosis factor α (TNF α) genomic probe (pHGE) (Shirai et al. 1985), human (h) *pim* oncogene cDNA (pHP63) (Dommen et al. 1987), human ferritin heavy (H) chain cDNA (pHF16) (Boyd et al. 1985) and coagulation Factor XIIIa cDNA (pG25) (Weisberg et al. 1987). In addition, MHC class II probes DQα (pDCH1) (Auffray et al. 1984), DQβ (pII-β-1) (Larhammar et al. 1982) and DRβ (pRTV1) (Bidwell et al. 1986) were used in RFLP genotypic analyses.

2.11 Oligolabelling of Probes

Probes were labelled with α-[^{32}P]-dCTP using random oligonucleotide-primed DNA synthesis (Feinberg and Vogelstein 1983). 20 ng aliquots of DNA were labelled using a multiprime DNA labelling kit (Amersham) according to the manufacturers' recommendations. Probes were separated from unincorporated nucleotides by chromatography on Sephadex G50. The Sephadex gel exclusion column was packed in a 10 ml disposable plastic tube (Sterilin) using polymer aquarium filter wool (Interpet) as the base. The reaction mix was applied to the column which was then centrifuged in a bench top centrifuge at 1,000 x g for 4 minutes to separate the free and incorporated label. Typically, final specific activities of 1 x 10^8 cpm/μg DNA were obtained. Probes were denatured (by boiling for 5 minutes followed by 5 minutes on ice) immediately before use.

2.12 Hybridization with Probes

Filter hybridizations were carried out in heat-sealed polythene bags which were placed in a shaking water bath maintained at the appropriate temperature.

2.12.1 DNA filters

Filters were prehybridized in 6 x SSC, 0.1% SDS, 5 x Denhardt's solution (see Appendix 1) and 100 μg/ml of denatured salmon sperm DNA (see Appendix 1) at 65°C for 1-2 hours. The prehybridization solution was then discarded and replaced with 10 ml of fresh hybridization solution (identical to prehybridization solution). The denatured probe was added at a concentration of 0.5-1 x 10^6 cpm/ml and the filter incubated overnight at 65°C. The filters were then washed three times in 2 x SSC for 5 minutes at room temperature and then for 30 minutes at 65°C in each of the following solutions.

1) 2 x SSC 0.1% SDS (2X)

2) 0.2 x SSC 0.1% SDS (2X)

After the final wash, the filters were blotted in order to remove excess liquid but care was taken that they did not dry out completely. The filters were then wrapped in Saran wrap (Dow Chemical Company) prior to autoradiography. In order to remove a probe before rehybridization, filters were washed in 0.4M sodium hydroxide for 30 minutes at 45°C followed by neutralization in 0.1 x SSC, 0.1% SDS, 0.2M Tris. HCl pH7.5 for 30 minutes at 45°C.

2.12.2 RNA filters

Filters were prehybridized in 6 x SSC, 50% deionized formamide, 5 x Denhardt's solution, 0.5% SDS and 100 μg/ml of denatured salmon sperm DNA for 2 hours at 42°C. Filters were hybridized in 10 ml of the same solution to which the denatured probe was added to a final concentration of 0.5-1 x 10^6 cpm/ml. Following overnight incubation at 42°C, the filters were washed once in 10 x Denhardt's, 6 x SSC at 65°C and then given 2 washes in 3 x SSC, 0.1% SDS also at 65°C. After the final wash, the filters were wrapped in Saran wrap prior to autoradiograhy. Stripping of a probe before

rehybridization was carried out by washing the filters in 0.005M Tris. HCl pH8.0, 0.003M EDTA, 0.1 x Denhardt's at 65°C for 1-2 hours.

2.13 Autoradiography

Filters were exposed to films in Kodak cassettes containing Ilford fast tungstate intensifying screens (Genetic Research Instrumentation Ltd.). Either Kodak XAR5 or Fuji RX film was used. Generally the more sensitive Kodak film was reserved for genomic Southern blots, while the Fuji RX film was used for screening libraries. Films were exposed at -80°C and then warmed to 37°C for 30 minutes to minimise condensation before developing. Films were developed automatically in a Fuji RG2 X-ray developing apparatus.

2.14 Plasmid Mini-preps

Cells were grown at 37°C in 1 ml L Broth (LB - see Appendix 1) supplemented with ampicillin at a final concentration of 100 µg/ml. Ampicillin was prepared as a 20 mg/ml stock solution in water, filter sterilized and stored at -20°C. After overnight growth, the culture was transferred to a microfuge tube and centifuged for one minute. The supernatant was discarded and the cell pellet resuspended in 200 µl 50mM glucose, 25mM Tris. HCl pH8.0, 10mM EDTA, 5 mg/ml lysozyme. After 5 minutes incubation at room temperature, 400 µl of 0.2M sodium hydroxide, 1% SDS was added, the mixture inverted gently and then put on ice for a further 10 minutes. After neutralization with 300 µl 5M potassium acetate pH4.8 (5M for potassium and 3M for acetate), the bacterial debris was pelleted by centrifugation. The supernatant was removed and extracted with an equal volume (300 µl) of phenol and chloroform, then precipitated with 1 volume isopropanol. After 5 minutes at room temperature, the mixture was centrifuged in a microcentrifuge (5 minutes) and the supernatant removed. The pellet

was washed in 70% ethanol, dried and resuspended in 30 µl TE. Typically, 3 µl was sufficient for each restriction digest.

2.15 Large-scale Plasmid Preparation

A colony was used to inoculate 1 ml of LB supplemented with ampicillin. After 2-3 hours growth at 37°C, 200 µl of this starter culture was spread onto each of two 15 cm LB plates (see Appendix 1), which were then incubated overnight at 37°C. The colonies were scraped off the surface of the agar and resuspended in 8 ml 50mM glucose, 25mM Tris. HCl pH8.0, 10mM EDTA, 5 mg/ml lysozyme. The suspension was transferred to 40 ml Oakridge tubes and allowed to stand at room temperature for 5 minutes. The cells were lysed by the addition of 16 ml freshly made 0.2M sodium hydroxide, 1% SDS and then left on ice for 10 minutes. The lysate was neutralized by the addition of 12 ml ice cold 5M potassium acetate pH4.8 and incubated for a further 10 minutes on ice. Bacterial debris were removed by centrifugation at 12,000 x g for 20 minutes at 4°C. The supernatant was decanted and the plasmid DNA precipitated with 0.6 volumes of isopropanol at room temperature for 15 minutes. After centrifugation at 12,000 x g for 30 minutes, the pellet was resuspended in 4 ml of TE. The volume of the DNA solution was accurately measured, and for each ml, 1 g of solid caesium chloride (BRL Ultrapure) was added, together with 320 µl of 10 mg/ml ethidium bromide. The solution was transferred to a 5 ml Beckman ultracentrifuge tube which was then sealed using a Beckman tube sealer. Tubes were loaded into the Beckman Vti 65 vertical rotor and centrifuged at 45,000 rpm overnight at room temperature. Two bands of DNA were visible under uv light and the bottom one consisting of closed circular plasmid DNA was removed by puncturing the side of the tube with a 21 gauge hypodermic needle and aspirating the band in a 2 ml syringe. Ethidium bromide was removed from the DNA by extracting five times with an equal volume of n-butanol saturated with water. The salt was then removed by dialysis against TE. The plasmid solution was transferred to a 1.5 ml microcentrifuge tube,

precipitated with ethanol, washed in 70% ethanol and resuspended in a final volume of 500 µl TE. Typically, two LB plates of a bacterial culture containing a high-copy number plasmid would yield 0.25 mg plasmid DNA.

2.16 Preparation of Plasmid Vectors

The plasmid was digested with the desired restriction enzymes and then 1/10 volume of 0.5M Tris. HCl pH9.5 and 2 units calf intestinal phosphatase (Boeringer) added to the reaction mix. After incubation for 30 minutes at 37°C, another 2 units of phosphatase was added and the reaction incubated for a further 30 minutes. The reaction was then extracted twice with an equal volume of neutralized phenol and once with chloroform. The DNA was then purified by centrifugation through G50 sephadex, precipitated and resuspended in TE.

2.17 Ligation and Transformation

Ligations were performed using 100 ng of vector DNA and approximately a three times molar excess of insert DNA. The 20 µl reactions included 4 µl of 5 x ligation buffer (supplied by BRL together with the T4 DNA ligase), 1 µl 10mM ATP and 1 µl of a 1 unit /ml stock of T4 DNA ligase. The ligation mix was incubated overnight at 15°C and then 10 µl used to transform *Escherichia coli* (*E. coli*).

Competent JM101 cells (see Appendix 2) were prepared using a protocol based on the method described by Hanahan (1983).

A single colony was taken and used to inoculate 1 ml LB. After overnight incubation at 37°C, 100 µl of the saturated culture was used to inoculate 30 ml LB, which was shaken vigorously until the OD_{600} was 0.3-0.4. The cells were harvested by spinning at 1,200 x g for 12 minutes at 4°C, resuspended in 10 ml ice cold TFB buffer (10mM

2-N-morpholinoethane sulfonic acid (MES) pH6.2, 100mM rubidium chloride, 45mM manganese chloride.$4H_20$, 3mM hexamine cobalt III chloride, (1M MES stock was adjusted to pH6.2 using potassium hydroxide)) and left on ice for 15 minutes. The cells were then spun down as before and resuspended in 2.2 ml ice cold TFB buffer. After 20 minutes, 200 µl competent cells were added to each ligation mix in chilled microcentrifuge tubes and the mixture placed on ice for a further 30 minutes. The tubes were then transferred to a 42°C water bath and heat shocked for 2 minutes, after which 1 ml LB was added and the cells allowed to recover for 1 hour at 37°C. The cells were then gently spun down and most of the supernatant removed by decantation. The cells were resuspended in the remaining supernatant (about 100 µl) and spread onto an LB plate supplemented with ampicillin. The plates were inverted and incubated at 37°C overnight.

Occasionally the colour test was performed to distinguish between recombinant and non-recombinant pEMBL plasmids. In these cases, 10 µl IPTG (10 mg/ml in distilled water) and 40 µl of 5-bromo-4-chloro-3-indoyl-beta-galactoside (2% in dimethylformamide) were spread onto the surface of the agar plates about 1 hour before use. The colonies that grew, were blue if they contained the re-ligated vector and colourless if they harboured a recombinant plasmid.

2.18 Oligonucleotide Synthesis

Oligonucleotides were synthesised by an automatic DNA synthesiser (New Brunswick: Cyclone synthesiser) using the phosphoramidite method and were supplied still linked to the synthesis columns. The oligonucleotides were extracted from the column as follows:-

1 ml of .880 ammonia solution (BDH AnalaR) was taken up into a disposable 1 ml syringe and inserted into one end of the column with an empty syringe inserted into the

opposite end. The ammonia solution was pushed back and forth through the column five times and then left over the column for 45 minutes at room temperature. This process was repeated twice. Finally the ammonia was sucked completely back into one syringe and then transferred to a screw cap 1.5 ml microcentrifuge tube and incubated at 55°C overnight (in order to remove blocking groups from the oligonucleotides). After cooling to room temperature, 0.1 volumes of 3M sodium acetate pH4.8 and 3 volumes of ethanol were added. The tube was placed on dry ice for 10 minutes then the oligonucleotide precipitate was recovered by centrifugation for 5 minutes. The oligonucleotide was dissolved in 100 µl TE and then precipitated with ethanol again. After washing in 70% ethanol, the final pellet was dissolved in 500 µl TE and stored at -20°C.

The oligonucleotide concentration was determined by measuring the OD_{260} of a 1/40 dilution of the stock solution. 600 µg of a 18-mer oligonucleotide was purified using this method.

2.19 Synthesis of cDNA

5 µg of poly (A)+ RNA was reverse transcribed using a cDNA synthesis kit (Amersham) according to the manufacturers' instructions. After incubation at 42°C for 2 hours, the reaction mixture was chromatographed on a G50 sephadex column to remove unincorporated nucleotides from the RNA/DNA duplexes. The RNA was then hydrolysed by the addition of sodium hydroxide to 66mM and incubating at 65°C for 1 hour. After neutralization with HCl in the presence of 0.2M Tris. HCl pH7.5, the single-stranded cDNA was precipitated with ethanol and resuspended in TE.

2.20 Polymerase Chain Reaction

This method enables DNA sequences to be amplified exponentially starting from as little as a single copy. The target sequence is delineated by two complementary synthetic flanking oligonucleotides which are typically about 20 bases in length. The oligonucleotides anneal to opposite strands and act as primers for DNA synthesis. The amplification consists of cycles of DNA denaturation, annealing of the oligonucleotides to their complementary sequences and then oligonucleotide-primed DNA extension by the *Taq* DNA polymerase. The cycles of denaturing, annealing and extension are controlled by alternating the reaction temperatures between 92°C, 45°C, and 72°C, respectively. The use of thermostable *Taq* DNA polymerase permits rapid extension after specific primer annealing at high temperatures as well as denaturation of the DNA to separate the newly-made strands, without loss of enzyme activity. The amplification reactions were carried out using reagents supplied by Perkin Elmer Cetus.

Each 100 µl reaction contained the following:-

1. 5 µg of each 18-mer primer
2. Reaction buffer: 50mM potassium chloride, 10mM Tris. HCl pH8.3, 1.5mM magnesium chloride, 0.01% gelatin, 200 µM each dNTP (Perkin Elmer)
3. 100 ng of template cDNA
4. Distilled water to a final volume of 100 µl

Samples were heated to 94°C for 1.5 minutes and then 1 unit of *Taq* polymerase (Perkin Elmer) was added. The reactions were carried out in a simple thermocycling apparatus (Foulkes et al. 1988) under the following conditions: 92°C, 2 minutes; 45°C, 2 minutes; 72°C, 2 minutes. After 30 cycles, a final extension of 10 minutes at 72°C was made and an aliquot of the reaction mixture was analysed on a 2% mini-gel. Amplified DNA was gel-purified, and then treated with polynucleotide kinase for 1 hour at 37°C. The reaction was then incubated at 65°C for 1 hour to destroy the kinase before the DNA was ligated into pEMBL 19 cleaved with *Sma* I.

2.21 Screening a cDNA Library

2.21.1 Plating phage

Plating cells were prepared by growing a culture of Y1090 cells (see Appendix 2) to saturation in LB supplemented with ampicillin (100 μg/ml) and magnesium ions (10 mM). Appropriately-diluted phage solutions in SM buffer (see Appendix 1) were mixed with 300 μl of plating cells and then incubated at 37°C for 20 minutes to allow the phage to adsorb. The phage/bacteria mix was then added to 9 ml of 0.7% top agarose (see Appendix 1) at 42°C and quickly poured onto 15 cm LB plates (supplemented with ampicillin and magnesium). After setting, the plates were inverted and incubated at 37°C.

2.21.2 Plaque screening

Circular nylon membranes (Hybond N, Amersham) were placed carefully onto the plates and allowed to soak for 30 seconds in which time three asymmetrical keying marks were made both on the plate and the filter. The filters were lifted off with forceps, allowed to air dry and then placed on a piece of Whatman 3MM paper soaked with 1.5M sodium chloride, 0.5M sodium hydroxide. After 7 minutes, the filters were transferred to paper soaked in 1.5M sodium chloride, 0.5M Tris HCl pH7.0, left for 3 minutes and then finally transferred to another piece of paper soaked in 2 x SSC for 5 minutes. After having been air dried and baked at 80°C for 2 hours, the filters were hybridized to a radioactive probe, as described in 2.12.1.

2.21.3 Plaque purification and small-scale preparation of λ DNA

The tip of a 1 ml disposable pipette tip was cut off to produce a bore of roughly 3 mm diameter. This was used to remove a plug of agar corresponding to the putative

positive hybridization signal from the plate. This plug was placed in 1 ml of SM buffer and left overnight at 4°C in order to allow the phage to diffuse. After a brief vortex, the agar was then spun down and the supernatant removed. A dilution series of the supernatant was re-screened with a radioactive probe. This process was repeated 2-3 times until the plaques were purified to homogeneity. A single well-isolated plaque was picked using a pipette tip into 1 ml SM. A 100 µl of this suspension was plated out using 0.7% top agarose onto 15 cm diameter LB plates (supplemented with 10mM magnesium). After incubation overnight at 37°C the plaques virtually covered the plate. 15 ml SM was added directly to the plate and the phage eluted by gentle orbital shaking for 2 hours. The SM was then transferred to Oakridge tubes and the remaining *E. coli* cells lysed by the addition of 50 ml of chloroform, followed by vortexing. The bacteria were removed by centrifuging at 10,000 x g for 10 minutes and the supernatant was removed. The phage were then harvested from the bacterial supernatant by centrifugation in a Beckman SW40 rotor at 25,000 rpm for 1 hour and resuspended in 500 µl SM to which RNase A and DNase 1 were each added to a final concentration of 1 mg/ml. After incubation at 37°C for 2 hours, 5 µl 10% SDS , 5 µl 0.5M EDTA was added and the phage solution further incubated at 68°C for 20 minutes. Proteins were removed by extraction with phenol and choloroform and then the DNA precipitated by addition of an equal volume of isopropanol. After washing in 70% ethanol, the pellet was taken up in 30 µl TE and half this volume used for digestion with restriction enzyme.

2.22 DNA Sequencing

2.22.1 Preparation of single-stranded templates

A colony containing the clone of interest within a pEMBL plasmid was used to inoculate 2.5 ml of LB (supplemented with ampicillin and magnesium). This culture was grown at 37°C until the OD_{550} was 0.4-0.5. 15 µl phage F1 and 2.5 ml LB were

then added and the cells grown for a further 5 hours. The culture was then poured into two 1.5 ml microcentrifuge tubes and the cells were recovered by centrifugation for 5 minutes. The supernatant was poured into two more microcentrifuge tubes, taking care not to disturb the cell pellet, and to each tube 250 µl of 2.5M sodium chloride containing 20% w/v polyethylene glycol (PEG) 6000 was added. The mixture was vortexed, left to stand at room temperature for 5 minutes and then centrifuged for 5 minutes. The supernatant was decanted and then all remaining traces removed by further centrifugation and careful aspiration with a fine glass pipette. The pellets were resuspended in 100 µl TE, combined and 200 µl neutralized phenol added. The mixture was vortexed for 10 seconds, and then spun for two minutes. The aqueous layer was removed and then extracted in the same way with chloroform. The DNA was precipitated with ethanol, washed with 70% ethanol and resuspended in 12 µl TE, 2 µl of which was run on a mini-gel. If the preparation was successful two bands could be seen after staining, the higher one being the F1 DNA and the lower one the single-stranded DNA.

2.22.2 DNA sequencing

Single-stranded DNA preparations were sequenced using the dideoxynucleotide chain termination method (Sanger et al. 1980). Typically, 1-2 µg (7 µl) of the single-stranded DNA preparations were sequenced using the DNA polymerase "Sequenase" (United States Biochemical Co-orperation) according to the manufacturers' recommendations. Sequencing reactions were electrophoresed on 6% acrylamide, 8M urea vertical gels. Urea (72 g), acrylamide (8.55 g) and bis-acrylamide (0.45 g) were dissolved in approximately 120 ml of 1 x TBE by heating to 40°C. The final volume was then adjusted to 150 ml, and the solution de-gassed and filtered.

After addition of 1.5 ml 10% ammonium persulphate and 25 µl TEMED, the mixture was poured between siliconised glass plates to form a 160 mm x 350 mm x 0.5 mm

gel. Wells were formed with shark tooth combs (BRL) and the gel allowed to set overnight. Immediately before electrophoresis, samples were heated in a boiling water bath for 3 minutes. The slots of the gel were washed out with 1 x TBE just before loading and then 5 µl of each sequencing reaction was added per slot in a fixed order (e.g., left to right T, C, G, A). When all the samples had been loaded, the gel was electrophoresed with a current of 28 mA until the bromophenol blue dye had migrated to the edge of the gel.

After dismantling the gel apparatus, the glass plates were separated leaving the gel attached to one plate. Gels were fixed by immersion in 10% acetic acid, 10% methanol for 45 minutes. A piece of Whatman 3MM paper was then placed on top of the gel and lifted up with the gel attached. The gel was covered with Saran wrap and dried down at 80°C using a Biorad 283 vacuum gel dryer. When completely dried, the Saran wrap was removed, and the gel exposed to Fuji RX film at room temperature overnight.

2.22.3 Growth of F1 phage

For the preparation of a new stock solution, a single colony of JM101 cells was grown in 1 ml LB until the cells reached exponential phase (OD_{550} 0.4-0.5). 50 µl of the previous F1 stock solution (approximately 10^{12} phage/ml) was added and the culture shaken for another 20 minutes to allow the phage to adsorb. This culture was then poured into 100 ml of pre-warmed LB which was then shaken gently overnight at 37°C. The cells were removed by centrifugation in 350 ml Sorvall tubes for 15 minutes at 6,000 x g. The supernatant was poured into a fresh tube and converted to 4% w/v PEG 6000, 0.5M sodium chloride by addition of the appropriate solids. After these had dissolved, the mixture was spun at 6,000 x g for a further 15 minutes. All the supernatant was removed and the PEG pellet was resuspended in 2 ml TE. The titre of the stocks was determined by using 1 µl, 5 µl, 20 µl and 50 µl aliquots to infect cells

and the minimum amount of input phage needed to produce the maximum amount of single-stranded plasmid was estimated.

2.23 Isolation of Human Peripheral Blood Monocytes and their Transformation into Macrophages by Culture in Autologous Serum

All tissue culture procedures were carried out in a class III microbiological cabinet using strict sterile precautions.

Human mononuclear cells were separated from peripheral blood by a combination of dextran sedimentation and centrifugation through a discontinuous plasma-Percoll gradient as described by Savill et al. (1989). Typically, 500 ml of fresh citrated blood was aliquoted into 50 ml sterile polypropylene tubes (Falcon Plastics) and centrifuged at 300 x g for 20 minutes. The platelet-rich plasma was aspirated and centrifuged at 2,000 x g for 10 minutes to generate platelet-poor plasma (PPP) or recalcified (20mM final concentration calcium chloride (13.4% w/v) added to 40 ml) to prepare platelet-rich, plasma-derived serum (PRPDS). To the residual cells, 6 ml 6% dextran (500,000 mol. wt., Sigma) in 0.9% w/v saline was added, and the contents made up to 50 ml with 0.9% w/v saline, mixed gently, and then allowed to stand for 30 minutes to sediment the red cells. The leucocyte-rich plasma was then aspirated and centrifuged at 300 x g for 6 minutes. Mononuclear cells were then separated from neutrophils and remaining red cells by centrifugation through a Percoll density gradient. Typically, four such gradients were set up per 500 ml of whole blood. For each gradient the leucocytes were resuspended in 2 ml of PPP and transferred to a 10 ml polystyrene tube (Sterilin). The leucocyte suspension was then underlaid with 2 ml of 42% stock Percoll (Sigma) (9:1 v/v Percoll-0.9% w/v saline) in PPP, followed by 2 ml 51% stock Percoll in PPP. The gradients were centrifuged at 300 x g for 10 minutes and then the layer of mononuclear cells was aspirated from the underlying layer of neutrophils. After washing in PPP and Hanks (Flow), the cells were resuspended at 4 x 10^6/ml in

Dulbecco's Modified Eagles Medium (DMEM) (Gibco) (to which penicillin and streptomycin had been added at 100 IU/ml). One ml of the cell suspension was then added to each well of a 24-well tissue culture plate (Flow). After 1 hour incubation at 37°C and 5% CO_2, the wells were washed and the adherent monocytes cultured for 3 days in DMEM in the presence of 10% autologous PRPDS.

CHAPTER 3

A GENOTYPIC ANALYSIS BY CONVENTIONAL SOUTHERN BLOTTING OF MHC CLASS I AND CLASS II GENES IN TWO FAMILIES AFFECTED BY HEREDITARY HAEMOCHROMATOSIS

Chapter 3: A Genotypic Analysis by Conventional Southern Blotting of MHC Class I and Class II Genes in Two Families Affected by Hereditary Haemochromatosis.

3.1 Introduction

Two families affected by hereditary haemochromatosis formed the basis of initial investigations of the molecular genetics of this disease. The diagnosis of haemochromatosis was based on demonstration of abnormal serum parameters of iron status and confirmed by histological examination of liver biopsy specimens. In view of the strong linkage between hereditary haemochromatosis and the MHC class I locus, HLA typing of family members was carried out to identify further homozygotes and heterozygote carriers of the disease.

Family HH4 consisted of four members (see Figure 3.1). HLA typing showed that the propositus was homozygous for the HLA-A3 allele and the B7 antigen, with which it is in linkage disequilibrium. The sister of the propositus had inherited only one HLA-A3-B7 haplotype from her mother and was thus a heterozygous carrier of haemochromatosis. In this family the parents were first cousins and therefore the two haemochromatosis genes inherited by the propositus were likely to have been identical by descent from a single grandparent.

Family RH7 consisted of a mother and six offspring, three male and three female. The eldest son had presented with impotence and evidence of abnormal liver function. Clinical examination showed pigmentation, hypogonadism and signs of liver enlargement. Investigations showed a raised serum iron of 40.5 μmol/l (normal less than 30 μmol/l), transferrin saturation of 77% (normal less than 60%) and a markedly raised serum ferritin concentration of 4700 μg/l (normal less than 250 μg/l for males). Histological examination of the liver biopsy showed cirrhosis with heavy deposition of

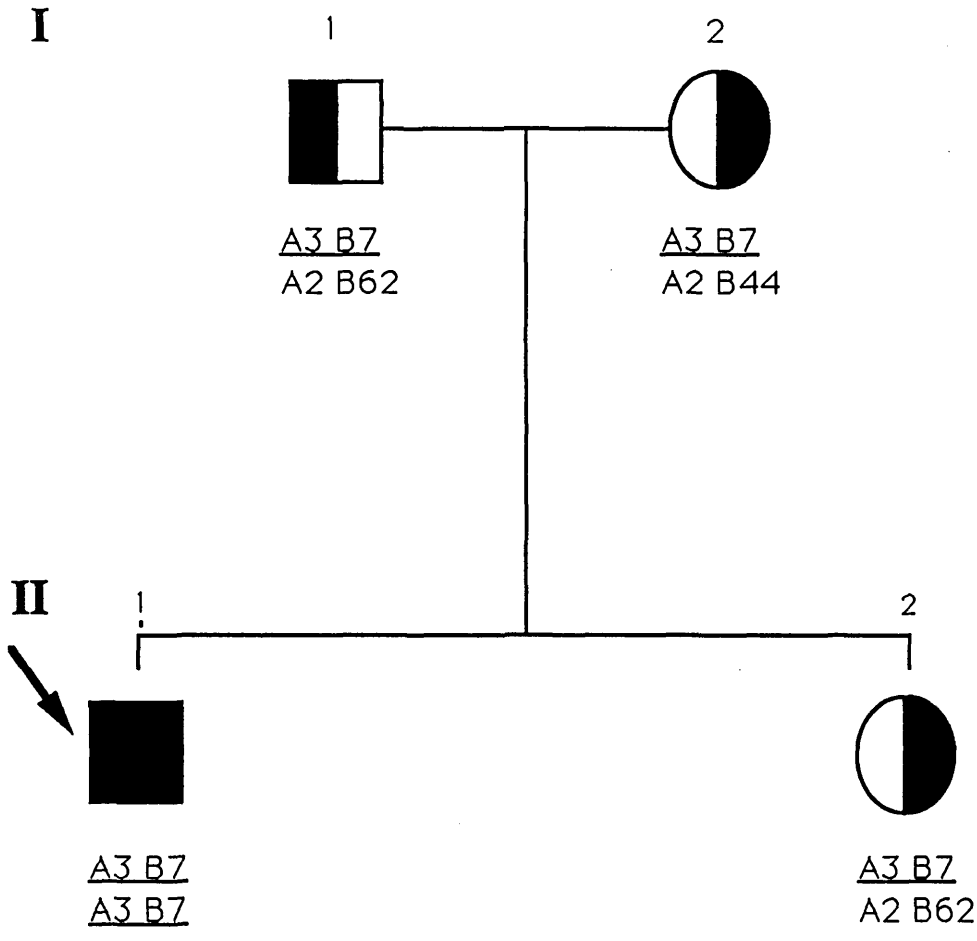


Figure 3.1. Family tree of HH4. The HLA haplotypes are shown beneath the corresponding family members. The propositus is indicated by an arrow. The HLA haplotypes carrying the haemochromatosis gene are underlined. Closed symbols and half-closed symbols indicate the homozygous and heterozygous state respectively.

iron in all cell types, indicating haemochromatosis. The HLA tissue type was - A1, A3; B8, B14; DR3, DR7; DRw52, DRw53; DQ2. The father of the propositus died in 1964 with liver failure, ascites, diabetes and terminal bronchial carcinoma but investigation had shown a raised serum iron and pigment cirrhosis; he too had been diagnosed as having haemochromatosis. The mother, now aged 88, has been in good health and exhibits no signs or symptoms of iron-storage disease. She had normal serum parameters of iron status. In this pedigree the occurrence of disease in two generations may be accounted for by a homozygous/heterozygous mating (see Figure 3.2). The other siblings must therefore be at least heterozygous for the haemochromatosis gene and have had a 50% chance of inheriting two disease-predisposing alleles. However, attempts to identify family members at risk by carrying out HLA typing were unavailing since the results of HLA typing by the NIH serological technique were identical in the propositus's mother and also in his five asymptomatic siblings i.e A1,A3; B8,B14; DR3,DR7; DRw52,DRw53; DQ2. A pedigree study of MHC class III genes by serological complement typing likewise failed to identify an extended haplotype that segregated informatively in this family. Although it is possible that the six siblings have inherited the same two haplotypes from their mother and father and are homozygous for the haemochromatosis gene, it is more likely that they have inherited different sets of parental chromosomes which cannot be distinguished by conventional serological analysis. If this were the case, since all six offspring possess the same serological HLA type, it is likely that their deceased father was also A1,A3; B8,B14; DR3,DR7; DRw52,DRw53; DQ2. As discussed later on p 71, the identification of homozygous affected and heterozygous individuals amongst the offspring of the heterozygous/homozygous mating confirms the assumption that the parents possesed HLA types that are serologically indistinguishable.

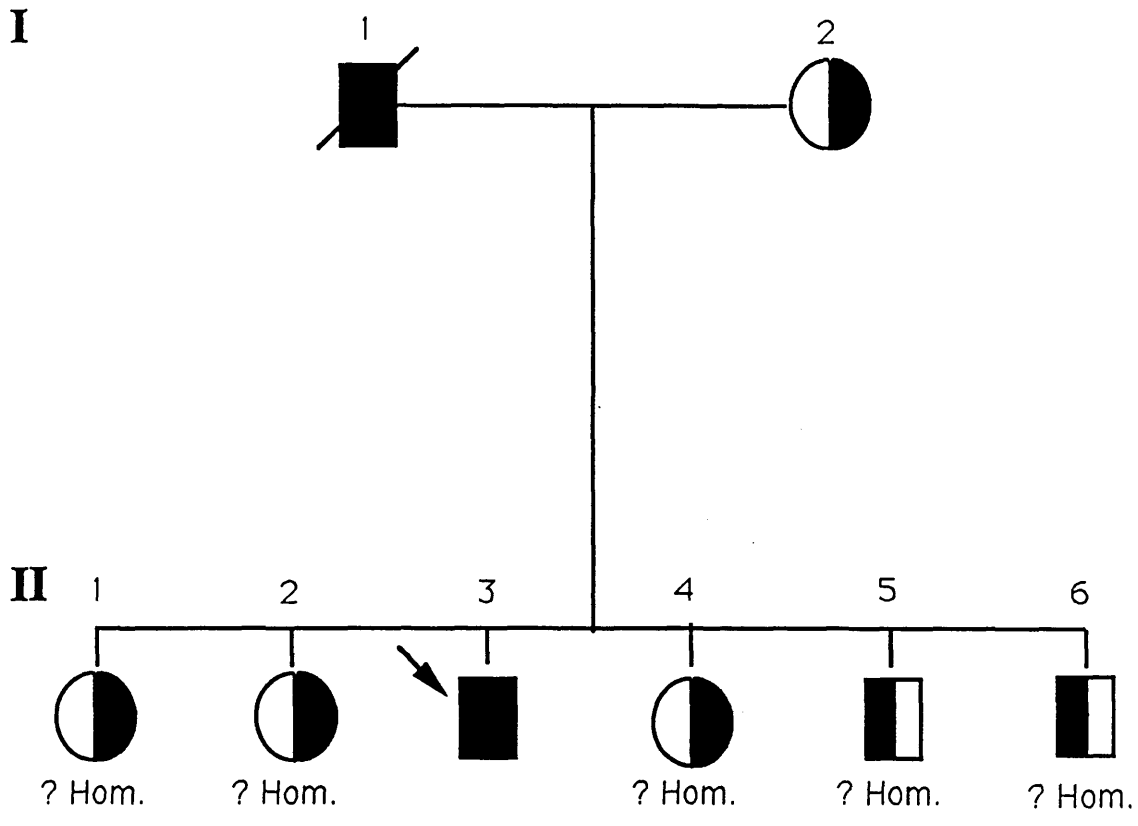


Figure 3.2. Family tree of RH7. The result of a homozygous/heterozygous mating. The propositus is marked with an arrow. The other siblings must be at least heterozygous for the haemochromatosis allele. The results of HLA typing, however, were identical for each family member, and therefore uninformative with regard to the inheritance of the haemochromatosis gene. (?Hom. = ? Homozygous)

The availability of DNA probes for HLA genes has permitted the evaluation of HLA polymorphisms at the genomic level. Indeed, the potential value of RFLP analysis in genotyping class II HLA-DR and DQ alleles was examined soon after cDNA clones became available (Andersson et al. 1984) and RFLP analysis has now become a useful tool in assessing allelic polymorphism at these loci. Typically, exon specific DRβ, DQβ and DQα probes are hybridized to *Taq* I-digested genomic DNA (Bidwell 1988). Using this system, the probes reveal specific hybridization patterns for most serologically-defined alleles and also resolve several splits of individual serological specificities.

MHC class I probes are not used to the same extent in genotypic analyses. However, hybridization of a full-length class I probe to Southern blots of genomic DNA has revealed 15 to 25 restriction fragments depending on the enzyme used (Orr et al. 1982). This is consistent with 15-20 related class I sequences - a much greater number than that predicted by serological analysis, which only defines the classical transplantation antigens A, B and C. Thus, this approach has the advantage of permitting potential polymorphisms at a large number of loci to be screened for simultaneously. On the other hand, the serological approach is restricted to polymorphisms that affect the coding regions of the class I HLA-A, B and C genes. In some cases it has been possible to correlate a particular RFLP with a serological specificity (Cann et al. 1983; Cohen et al. 1983). However, use of locus-specific class I probes has not yet allowed a more refined analysis of serologically-defined alleles to be achieved.

Thus RFLP analysis of class II genes, and to a lesser extent class I genes, is a useful adjunct to HLA typing that may assist in determining the segregation of HLA haplotypes. The method was, for this reason, used to investigate further the segregation of haemochromatosis genes in family RH7.

This chapter describes a genotypic analysis of family RH7 using *Taq* I restriction enzymes and MHC class I and class II probes. The analysis was also extended to include the gene encoding the 'a' subunit of FXIII, the urea-stabilizing factor of blood coagulation. The gene for FXIIIa has been mapped to chromosome 6 at p24-25 (Board et al. 1988) and recent analysis of genomic clones has indicated the presence of a polymorphism detectable with *Eco*R I and *Bam*H I restriction enzymes (Ichinose and Davie 1988). Thus the probe for FXIIIa was used as an additional polymorphic marker for the investigation of the parental origin of the short arm of chromosome 6.

The additional class I sequences detected by Southern hybridization are thought to represent pseudogenes as well as related functional genes (Strachan 1987). The latter may be equivalent to the murine Qa or T1a cluster and may be considered as candidate genes involved in the causation of haemochromatosis. Accordingly, this chapter further describes the segregation of *Hind* III class I hybridizing fragments within families RH7 and HH4.

3.2 Results

3.2.1 Genotypic analysis of family RH7

Genomic DNA from family RH7 was digested with *Taq* I restriction enzyme and then electrophoresed on a 0.8% agarose gel. After transfer to a nylon membrane, the DNA restriction fragments were hybridized to a general class I probe. This digest was then repeated and the resultant filter hybridized successively to class II probes, DRβ, DQα and DQβ.

The class I probe revealed 14 restriction fragments ranging in size from 0.8 kb to 6.5 kb (see Figure 3.3). However, this hybridization pattern was identical for each of the seven family members.

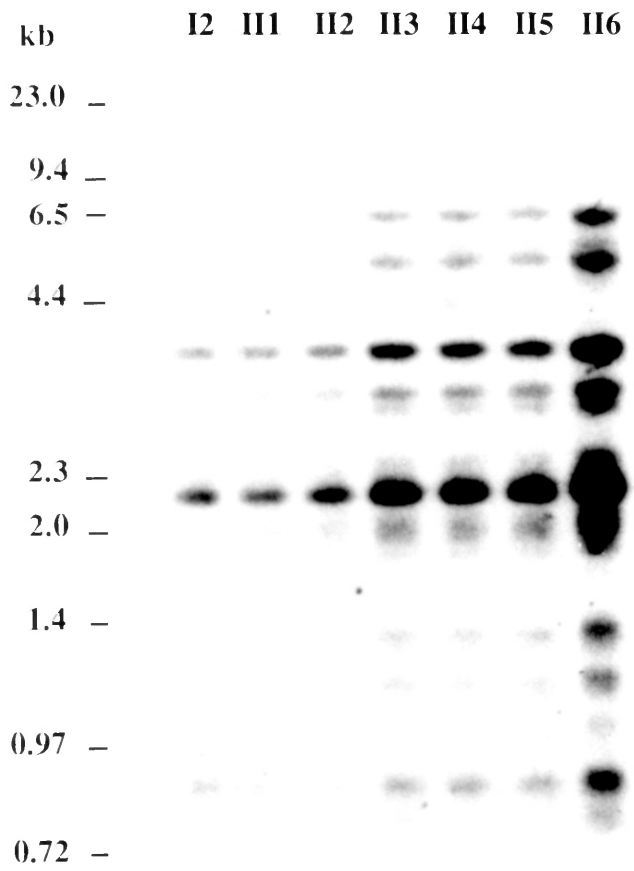


Figure 3.3. Southern blot of *Taq* I-digested DNA from family RH7 hybridized with the general class I probe. Size markers indicate fragments obtained from a mixture of *Hin*d III-digested *lambda* DNA and pEMBL plasmid cleaved with *Taq* I.

Hybridization with the class II probes proved also to be uninformative. The DRβ probe identified the same eight restriction fragments for each individual (see Figure 3.4a). The bands labelled 7 (1), (2), (3) and (4) correlate with the DR7 DRw53 serological allele. The restriction fragments identified as 3 (1), (2) and (3) are associated with DR3 DRw52 serological specificity. Hybridization with the DQα probe also revealed identical hybridization patterns for each family member (see Figure 3.4b). The restriction fragment numbered 2 (1) is characteristic of DQw2 DR7 and the band identified as 2 (2) is associated with DQw2 DR3. The cross-hybridizing, non-polymorphic DX gene signal is also shown. The DQβ probe was also used. This probe, which gave high background was also in accordance with the results using DRβ and DQα probes; no differences in hybridization pattern between individual family members was observed.

DNA from family RH7 (note sibling II1 was not included in this analysis) was also digested with *Bam*H I and *Eco*R I restriction enzymes. After electrophoresis on a 0.8% agarose gel, the DNA was transferred to a nylon membrane and hybridized to the FXIIIa probe (see Figure 3.5).

The *Bam*H I digest revealed two fragments of 18 kb and 5.5 kb which were present in the DNA of each family member. However, the *Eco*R I digest gave a faint doublet at approximately 1 and 1.2 kb, a single band of 8 kb and a polymorphic fragment at >23 kb. This latter fragment was absent in the mother (I2) and sibling II4, but present in the remaining offspring.

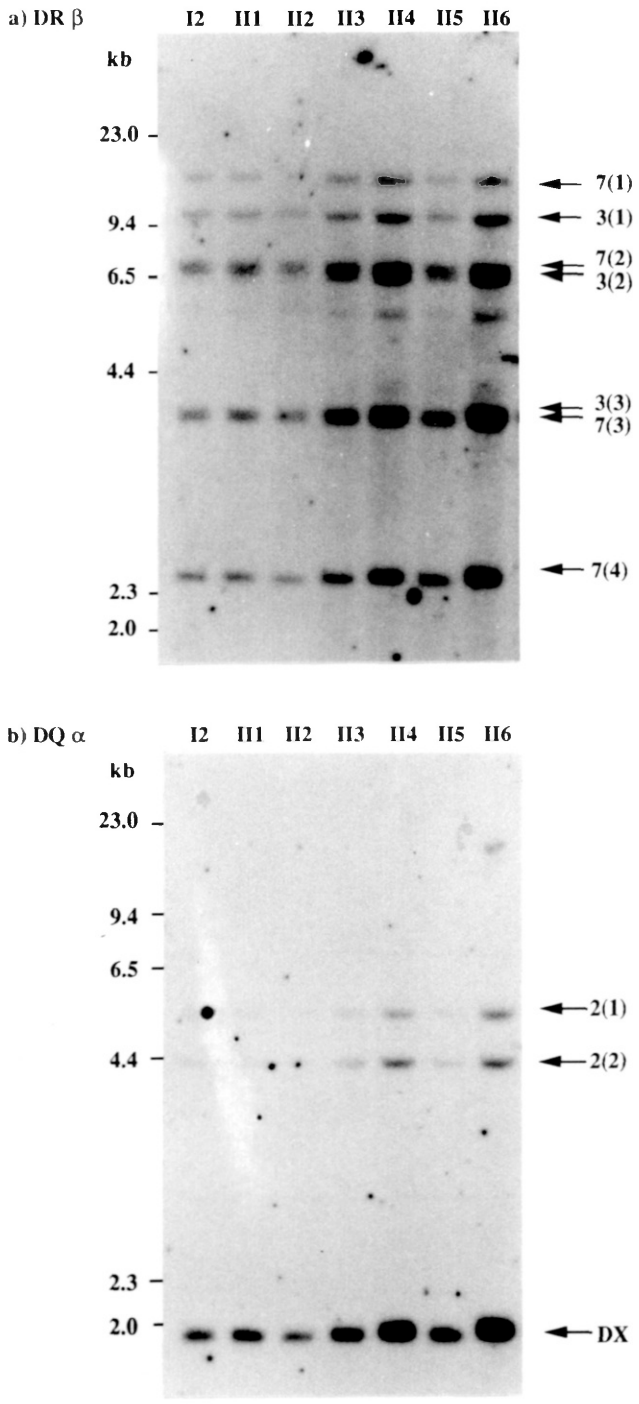


Figure 3.4 Southern blot of *Taq* I-restricted DNA from family RH7 hybridized with a) the MHC class II DR β probe and b) the DQ α probe. Size markers indicate fragments obtained from *Hin*d III-digested *lambda* DNA.

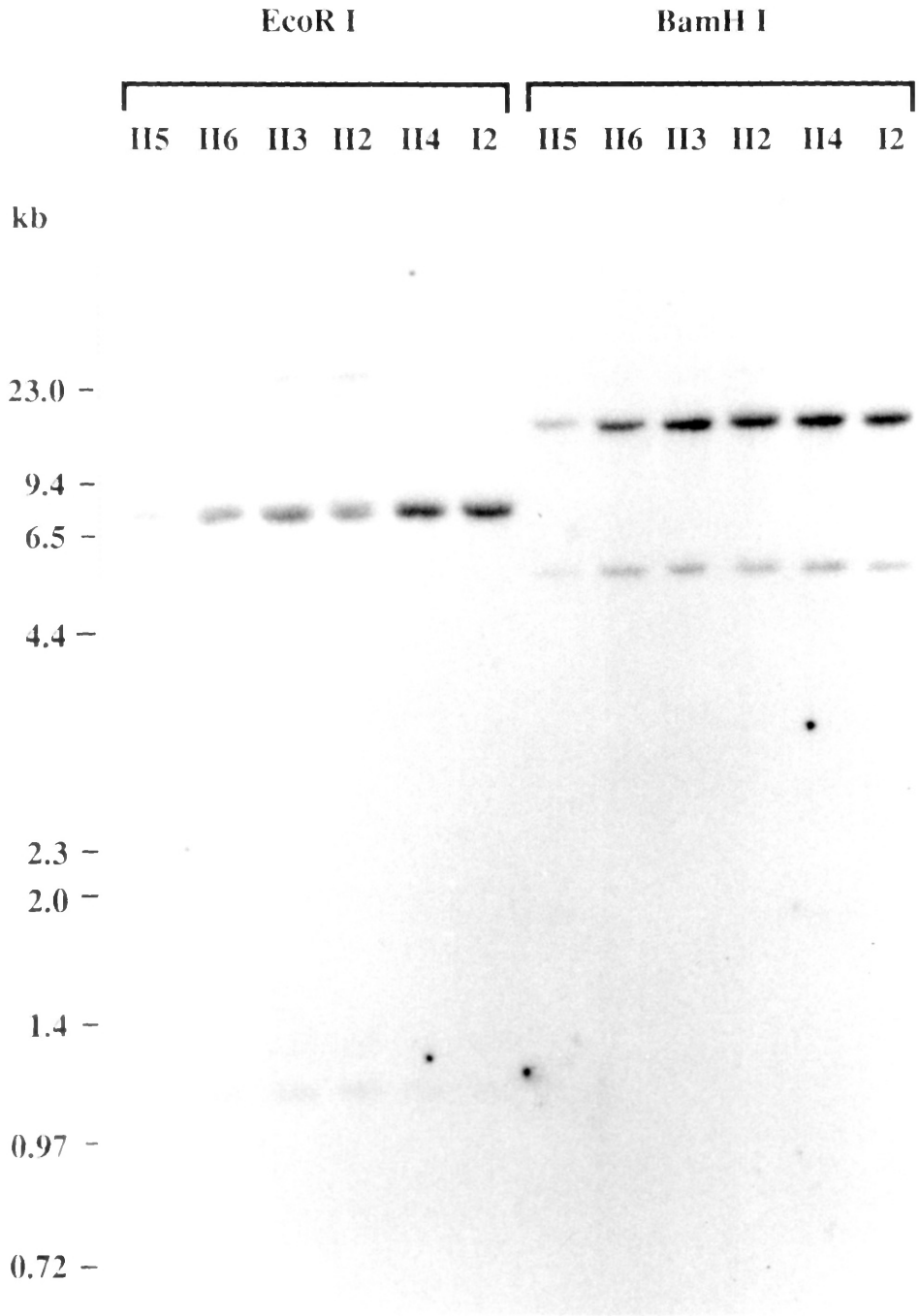


Figure 3.5. Southern blot of *Bam*H I and *Eco*R I-restricted DNA from family RH7 hybridized with the FXIIIa probe. The size markers are fragments obtained from a mixture of *Hin*d III-digested *lambda* DNA and pEMBL plasmid cleaved with *Taq* I.

3.2.2 Class I hybridizing fragments in DNA from families RH7 and HH4 using *Hind* III restriction enzyme.

Genomic DNA from members of both families RH7 and HH4, was digested with *Hind* III endonuclease, electrophoresed on a 0.8% agarose gel for 36 hours at 40 V to resolve the high molecular weight *Hind* III fragments and then transferred to a nylon membrane. Hybridization with the general class I probe revealed approximately 14 fragments between 4 kb and 23 kb in size.

This hybridization pattern was identical for each of the individuals in family RH7 that were studied (note sibling II1 and II5 were not included in this analysis) (see Figure 3.6a). However, differences in high molecular weight restriction fragments were seen among individuals in family HH4 (see Figure 3.6b). Two hybridizing fragments labelled *a* and *b* in Figure 3.6b were present in the DNA of the parents (I1 and I2) and the unaffected sibling (II2), but only the lower molecular weight fragment *b* was present in the DNA of the propositus (II1). However, band *b* was also present in the DNA of an HLA-matched control subject C1 (HLA A3,31; B7,B40). The *a* fragment was present in the DNA of control subject C2 (HLA A2,A2; B44,B44). Thus, in family RH4, the *a* band most probably segregates with the A2-B63/44 haplotype and the *b* band with the A3-B7 haplotype (see Figure 3.1). No other patterns specific for a given HLA type were observed.

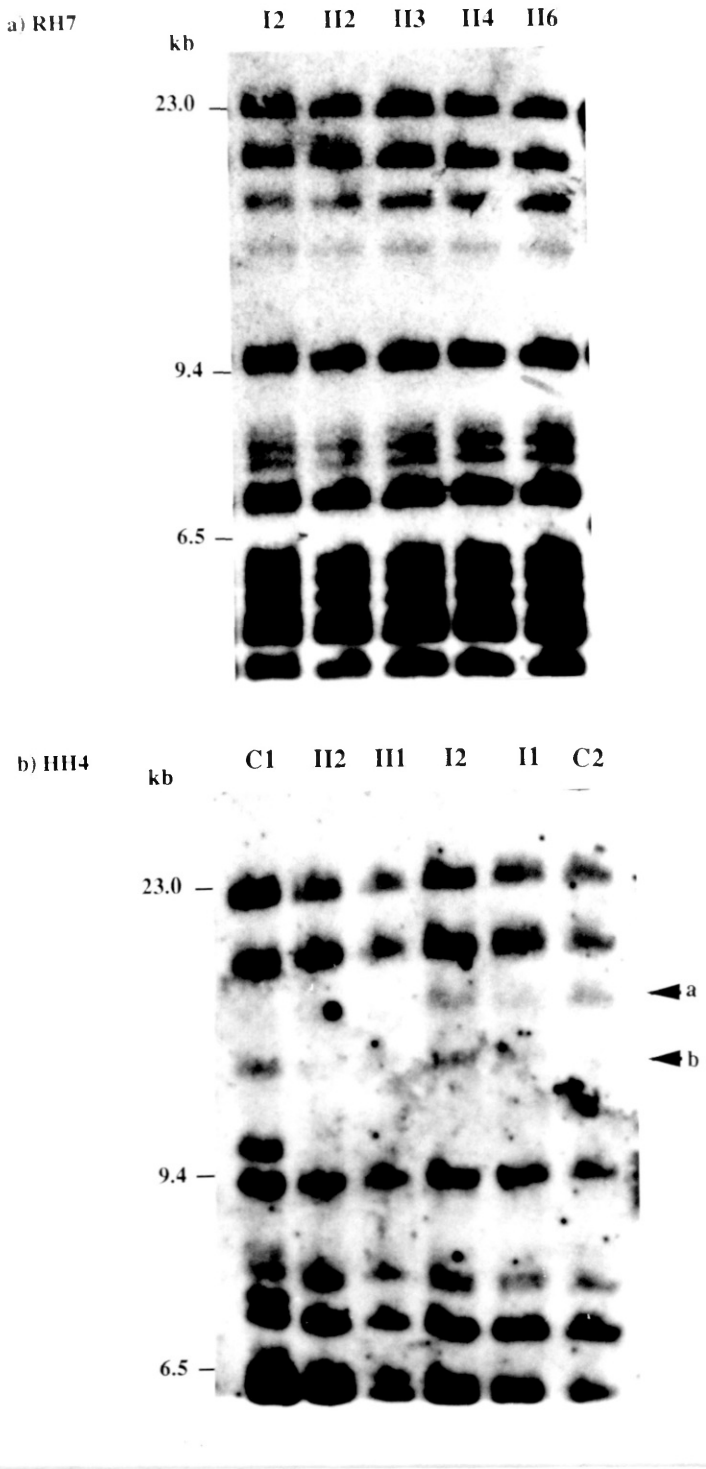


Figure 3.6. MHC class I-*Hind* III restriction fragments in DNA from a) family RH7 and b) family HH4. The two polymorphic fragments identified in family HH4 are indicated by an arrow. These fragments remained very faint even after extended exposure of the film. *Hind* III-digested *lambda* DNA was used as a size marker.

3.3 Discussion

Liver disease, often complicated by cancer, is a frequent cause of death in patients with haemochromatosis (for reviews see Bothwell, Charlton and Moltulsky 1989; Cox and Lord 1989). However, it is now known that individuals without cirrhosis who are depleted of iron by venesection have a normal life expectancy; it has also become clear that removal of iron does not prevent the development of cancer in the cirrhotic liver (Neiderau et al. 1985). These considerations emphasise the importance of identifying asymptomatic individuals with haemochromatosis in the pre-cirrhotic stage. The linkage of the haemochromatosis gene to the HLA-A allele means that HLA typing studies on families affected by haemochromatosis is often beneficial in the identification of asymptomatic individuals homozygous for the haemochromatosis gene. However, in this present study, family RH7 was found to be remarkable in that all family members were HLA identical by conventional serology. Their HLA type was A1,3; B8,14; DR3,7; DRw52,53; DQ2.

In an attempt to dissect the HLA serological specificities and thus determine the segregation of the haemochromatosis gene within family RH7, genotyping by RFLP analysis was carried out using MHC class I and class II probes.

The class II DRβ, DQα and DQβ probes are known to reveal specific hybridization patterns for most serological specificities. Of particular interest in this study is the fact that the DRβ probe detects 'splits' of the serological specificities DR3 and DR7. Thus DR3 and DR7 are each characterized by a specific RFLP. However, on digestion of DNA from family RH7 with *Taq* I and hybridization with the DRβ probe, the same hybridization pattern characteristic of the DR3 and DR7 alleles was observed in each family member. Likewise, no difference in hybridization patterns among individual family members was observed with the DQα and DQβ probes. Hybridization of a class

I probe to *Taq* I-restricted DNA revealed 14 cross-hybridizing class I sequences. This hybridization pattern was also identical for each member of family RH7.

In conclusion, genotypic analysis of HLA was not helpful in identifying HLA haplotypes that co-segregate with the haemochromatosis gene within family RH7, since the HLA types that were catagorized by serological methods could not be further resolved at the genomic level. This was unexpected, particularly when the diversity of the class I genes is considered and where complex hybridization patterns relating to 20-30 genes are also taken into account.

It remained possible that the siblings in family RH7 are truly HLA identical, and are all homozygous for the haemochromatosis gene. This prompted further analysis of asymptomatic siblings of the propositus. Clinical examination showed no overt signs of liver or endocrine dissorder, but in two sisters of the propositus (II2 and II4) the serum ferritin was elevated to 670 and 640 μg/l, respectively (normal less than 150 μg/l for women). These two individuals were further investigated for iron-storage disease by nuclear magnetic resonance (NMR) spectroscopy. Owing to the paramagnetic properties of iron, the NMR image (Leung et al. 1984) displays an altered signal intensity and the liver appears darker in shade in patients with increased tissue stores of iron. Because the magnetic spectroscopy scans strongly suggested that siblings II2 and II4 of the propositus were indeed affected by significant tissue iron overload, these individuals underwent liver biopsy. In both instances, marked deposition of stainable iron was detectable histochemically, and the histological appearances were characteristic of pre-cirrhotic hereditary haemochromatosis. These two women were treated by a programme of weekly venesection treatment to remove iron. In each case, 35 units (17 litres) of blood were removed without ill-effect and with complete restoration of iron parameters to normal. The removal of 8-9 g of iron without the development of significant iron deficiency in middle-aged women confirms the presence of greatly increased tissue iron stores in these patients. Thus, the

transmission of haemochromatosis in family RH7 deduced at the time of writing is depicted in Figure 3.7.

Needle biopsy of the liver is a procedure that has obvious risks. Thus a non-invasive method for detecting iron overload offers distinct advantages for the management of members of affected pedigrees at risk from the development of pre-malignant disease. In the unique pedigree reported here, intensive HLA studies in this regard have been non-contributory and it is proposed to utilise NMR spectroscopy in the further follow-up of family members who remain at risk from the development of haemochromatosis.

Analysis of family RH7 with the FXIIIa probe did reveal an RFLP with the *Bam*H I restriction enzyme. However, the polymorphic >23 kb fragment was absent in the mother and sibling II4, but present in the other offspring, and so clearly did not segregate with haemochromatosis in this family. It therefore appears that the FXIIIa gene is sufficiently far from the haemochromatosis locus and MHC complex to be frequently separated from them by recombination events.

In the further investigation of class I genes as candidates for the abnormal gene in haemochromatosis, DNA samples from members of pedigrees RH7 and HH4 were digested with *Hin*d III restriction endonuclease. Hybridization with the class I probe revealed 8-14 restriction fragments. This hybridization pattern was identical in each member of family RH7 - findings that resemble the results of probing with class I genes using restriction enzyme analysis with *Taq* I. Family RH7 will therefore be particularly valuable for the further molecular analysis of haemochromatosis because its members provide internal controls of low heterozygosity in the class I region.

In the study of HH4, two polymorphic *Hin*d III fragments *a* and *b* (see Figure 3.6b) were observed. Fragment *b* was found to segregate with the haemochromatosis gene in this family but analysis of control subjects showed it to be associated with the A3-

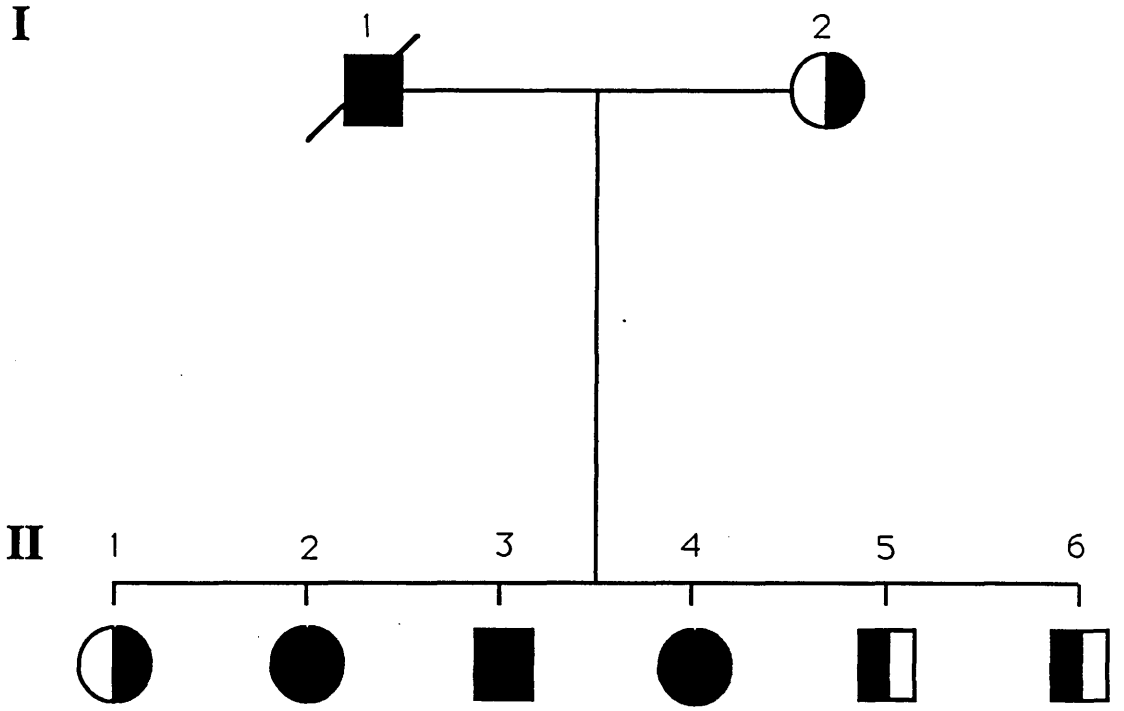


Figure 3.7. Emended family tree of RH7 based on the discovery of raised serum ferritin levels in two asymptomatic siblings (II2 and II4) of the propositus. Iron overload was confirmed by magnetic resonance spectroscopy and liver biopsy. It is noteworthy however, that the other siblings remain at possible risk from the development of haemochromatosis and further follow-up studies are required at 2-5 year intervals.

B7 haplotype and not to be specific for haemochromatosis. Fragment *a* appeared to correlate with the A2-B62/44 haplotype. The primary associations of these polymorphic fragments is most probably with the HLA-A3 and A2 serological alleles as the class I probe used in this study has been shown to be mainly specific for HLA-A antigens (Cragg et al. 1988). The non-variable fragments may represent pseudogenes or sequences corresponding to the mouse Qa or T1a cluster which may be less polymorphic than the classical HLA genes (Strachan 1987).

Lucotte and Coulondre (1986) have described an association between a 10 kb *Pvu* II class I restriction fragment and the haemochromatosis susceptibility gene. However, this fragment too was found to be primarily associated with the A3 serological allele and did not prove to be specific for the chromosome bearing the haemochromatosis allele. In a study carried out by Cragg et al. (1988) in which class I sequences in the DNA of 12 unrelated haemochromatosis patients and 85 control subjects were examined with a panel of restriction enzymes, certain hybridization patterns that correlated with specific HLA-A antigens were identified but no restriction fragments specific for haemochromatosis were detected. In a recent more detailed study Hansen and Kushner (1989) used class I probes to analyse RFLP's in 38 unrelated haemochromatosis patients. Differences in RFLP allele frequencies were identified between the control and haemochromatosis groups but none were found to be statistically significant.

Thus, RFLP studies of families HH4 and RH7, as well as those carried out by other groups, have been unable to detect a deletion or rearrangement of class I genes in haemochromatosis patients. Nonetheless, the class I probe is able to distinguish fragments that are associated with certain serological specificities. Indeed, the RFLP's associated with the A3 serological allele emphasize the tight linkage of haemochromatosis to the HLA-A locus and it is possible that RFLP analysis may prove to be useful as an additional method for identifying subjects at risk from developing the

disease. It must be noted, however, that the cross-hybridization that attends the use of non-specific probes means that there is uncertainty as to whether the RFLP derives from the gene encoding the serologically-derived allele or a neighbouring gene that is in strong linkage disequilibrium. Attempts have been made to obtain locus-specific probes (Grumet et al. 1983; Koller et al. 1984; Davidson et al.1985; Strachan et al. 1986) and they should simplify studies of HLA-A, B and C alleles as genetic markers of disease susceptibility.

Another possible candidate for the abnormal gene in haemochromatosis that has not been investigated here, but which merits consideration, is the gene for the H subunit of ferritin. Ferritin H chain sequences are present on at least ten different human chromosomes (Cragg et al 1985; McGill et al. 1987) and a ferritin H gene has apparently been mapped near the MHC locus at 6p21.3. RFLP analyses of the H ferritin gene have been carried out, but have revealed no polymorphisms that are directly linked to the putative haemochromatosis gene (Cragg et al. 1988; David et al. 1989). The currently prevailing view is that the ferritin gene on chromosome 6 is probably a pseudogene. Studies carried out by Costanzo et al. (1986) have suggested that only one gene is responsible for the synthesis of apoferritin H chain mRNA in a number of tissues. This is believed to be the gene on chromosome 11 that directs the biosynthesis of human ferritin in mouse-human or hamster-human hybrid cells (Cragg et al. 1985; Cragg et al. 1988).

In conclusion, there appears to be little evidence that class I genes, or indeed the gene for ferritin H chain, are implicated at the putative disease locus for the gene that predisposes to the development of haemochromatosis. However, the haemochromatosis locus is known to be within 1 cM of the HLA-A locus on the short arm of chromosome 6 and the next goal must be to refine its sub-chromosomal localization. RFLP studies using HLA probes have only been successful in reaffirming the tight linkage of the haemochromatosis gene to the class I region. An alternative

approach toward the molecular characterization of the haemochromatosis gene is to search for rearrangements and deletions within the MHC class I region that may be linked to the disease. Given the genetic data such a chromosomal mutation may be as much as several megabases physically distant from any class I gene. To investigate the sub-chromosomal arrangement of genes in this region of the human genome, a physical technique for the separation of large fragments of DNA would be required. The new technique of pulsed-field gel electrophoresis permits the resolution of DNA fragments up to two megabases in size and the further investigation of the putative disease locus in haemochromatosis in this study was based on this procedure.

CHAPTER 4

A PULSED-FIELD GEL ANALYSIS OF THE MHC CLASS I REGION IN a) TWO FAMILIES RH7, AND HH4, AFFECTED BY HAEMOCHROMATOSIS AND b) A FURTHER SIX UNRELATED PATIENTS AND THREE HLA-MATCHED CONTROL SUBJECTS

Chapter 4: A Pulsed-field Gel Analysis of the MHC Class I Region in a) Two Families RH7, and HH4, Affected by Haemochromatosis and b) A Further six Unrelated Patients and Three HLA-matched Control Subjects

4.1 Introduction

In the study of the genetic structure of mammalian chromosomes there exists a 'resolution gap' between molecular cloning experiments and meiotic linkage analysis. The technique of pulsed-field gel electrophoresis, which is capable of separating DNA molecules of up to 2000 kb, effectively bridges this gap and enables large-scale restriction site mapping of chromosomes to be carried out.

Pulsed-field gel electrophoresis (PFGE) was first described by Schwartz and Cantor (1984). Using this technique, the resolution of large molecules was achieved by a method whereby the DNA was subjected alternately to two approximately perpendicular fields (one uniform, the other non-uniform). A modification of this method was published by Carle and Olson (1984). They too used perpendicular fields but both were non-uniform. This arrangement made the DNA run through the gel in a more symmetrical pattern but the outer tracks were strongly curved. Several efforts have since been made to improve the geometry of the DNA tracks. An electrophoretic apparatus, designed by Southern et al. (1987), rotates the gel through an obtuse angle at each switching interval. This results in straight tracks running from cathode to anode. Field inversion gel electrophoresis (FIGE) (Carle and Olson 1986) is also capable of producing straight tracks of DNA. Separation of large DNA molecules by this system is achieved simply by periodically inverting a uniform electric field in one dimension.

The original technique of Schwartz and Cantor (1984) was based on the concept that when molecules in a gel matrix are subjected to an electric field perpendicular to their

direction of migration, they will reorientate themselves along the new field and move forward. Smaller molecules will have a higher mobility because they will be able to reorientate more quickly than larger ones. It is now becoming clear that the resolution of large fragments by PFGE requires fields which are at obtuse angles to one another and the exact basis of the separation is still open to speculation. The electrophoretic mobility of the DNA depends strongly on the pulse time (duration of applied field) which can be adjusted to optimize fractionation in a given size range. Typically, a longer pulse time is required for the effective resolution of larger DNA molecules.

In some lower eukaryotes, such as yeast and trypanosomes, whole chromosomes are small enough to be resolved as individual bands. Thus, PFGE has been used to separate and analyse yeast chromosomes (Schwartz and Cantor 1984) and also used to examine the chromosomes of trypanosomes (van der Ploeg et al. 1984).

In higher eukaryotes such as mammals, chromosomes are too large to be separated as individual bands, since the smallest human chromosomes have been estimated to be approximately 30 Mb. However, large fragments of mammalian DNA can be generated by restriction enzymes that cleave infrequently. The enzymes that have found the widest application are those that have an 8 bp recognition sequence, e.g., *Not* I. In addition, enzymes may be chosen with one or more CpG dinucleotides in their recognition sequence, e.g., *Mlu* I, *Pvu* I and *Nru* I. Such recognition sites are rare in the mammalian genome because the CpG dinucleotide occurs infrequently and is often methylated at the cytosine, which inhibits cleavage by most of these enzymes (Brown and Bird 1986).

Pulsed-field gel electrophoresis, in conjunction with infrequently-cutting enzymes, has been used in the construction of physical maps of large stretches of mammalian DNA (Brown and Bird 1986; Nguyen et al. 1987). Of particular relevance to the study of

haemochromatosis has been the application of PFGE to construct a physical map of the human Major Histocompatability Complex (MHC).

The human MHC complex is located on the short arm of chromosome 6 in the 6p21.31 - 6p21.33 region (Spring et al. 1985) and consists of three major linked gene clusters - class I, class II and class III. The class I genes include those that encode the major transplantation antigens HLA-A, B and C. These genes encode a highly polymorphic M_r 44,000 heavy chain that is associated non-covalently on the cell surface with an invariant M_r 12,000 β_2 microglobulin chain. These classical class I molecules are present on most somatic cells and function in the recognition of target cells by cytotoxic T lymphocytes. As assessed by Southern blotting, the MHC class I gene family contains 15-20 highly related genes, many more than can be accounted for by the HLA-A, B and C genes (see Chapter 3). Several alleles of the HLA-A, B and C loci have been cloned and characterized but less is known about the structure and function of the additional non-A, B, C class I genes. Recently a number of these genes have been isolated and sequenced (LN-IIA (Biro et al. 1982), HLA 12.4 (Malissen et al. 1982), HLA-E (Srivastava et al. 1987; Koller et al. 1988), HLA-6.0 (Geraghty et al. 1987), HA2 (Paul et al. 1987) and Cda12 (Ragoussis et al. 1989)). The LN-IIA and HLA-12.4 are thought to represent pseudogenes but the others have been shown to encode an HLA class I heavy chain after DNA-mediated gene transfer studies. In the case of HA2, HLA-E, HLA-6.0, and Cda12, the heavy chain has been found in association with β_2 microglobulin at the cell surface.

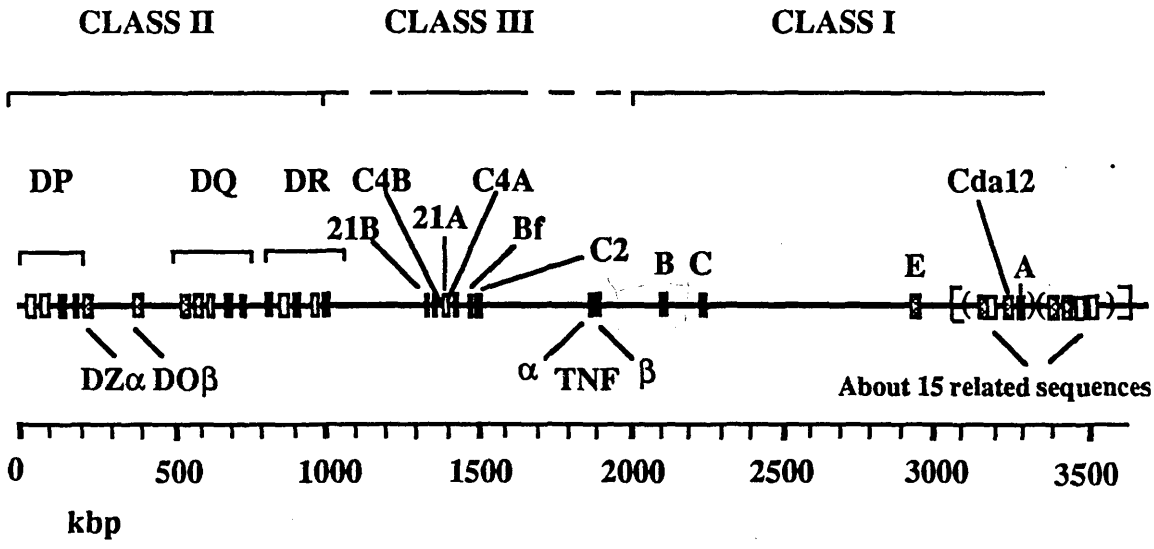
The class II genes are clustered in at least three sub-regions DP, DQ, and DR. Each sub-region contains a minimum of one α and β chain pair of genes which encode the polymorphic chains of the class II heterodimers (Trowsdale et al. 1985a). Two more genes, DOβ (Tonelle et al. 1985) and DZα (Trowsdale and Kelly 1985b), are also in the class II region. The class III region contains the genes encoding the serum complement proteins , factor B and the second and fourth components of complement

(C2 and C4, respectively) as well as two copies of the steroid 21-hydroxylase genes (Carroll et al.1984; Carroll et al.1985).

Genetic analysis of recombinant MHC haplotypes established that the gene order within the HLA complex was 6 centr: HLA class II loci: class III loci: HLA-B: C: A: 6pter (Lamm and Olaisen 1985). Within the class II region, the DP sub-region mapped on the centromeric side of DQ and DR (Kavathas et al. 1981; Shaw et al. 1981). The class III genes were located beween the HLA-DR and HLA-B loci, but the orientation and distance of these genes relative to the class I and II genes was not determined owing to the absence of informative recombination events within the region. Clusters of overlapping cosmid clones were isolated from the sub-regions of the class II loci (Trowsdale 1987) and from the class III region (Carroll et al. 1984) but were not linked completely by mapping studies.

A knowledge of the arrangement of MHC genes and the physical distances between them is desirable for a complete understanding of several important properties of the MHC, especially the association of haplotypes with a variety of diseases. For these reasons, a number of groups have used PFGE to construct a long-range restriction map of the human MHC.

Using PFGE, Hardy et al. (1986) established the order of the HLA-D sub-region. Physical linkage between the class II and class III loci has also been demonstrated (Ragoussis et al. 1986; Lawrance et al. 1987). Further studies by Dunham et al. (1987) and Carroll et al. (1987) have defined the position and orientation of the complement genes in the class III region. In addition, the loci for the TNF α and β genes have been mapped between the class III and class I regions (Carroll et al. 1987; Dunham et al. 1987; Inoka and Trowsdale 1987; Ragoussis et al. 1988) (see Figure 4.1 for composite map). So far, however, the available physical mapping data of the class I region is tentative and has not yet been definitively extended to HLA-A. This is partly due to the



Expressed genes: black; pseudogenes: white; genes of undefined status: cross-hatched.

Figure 4.1. Composite map of the human MHC.

fact that analysis of the class I region by PFGE is made difficult by the close similarity of the class I sequences. Locus-specific probes do exist for some class I genes, including HLA-B, C, E and Cda12, but a degree of of cross-reactivity occurs. HLA-E and Cda12 have both been mapped by PFGE between HLA-A and HLA-C (Carroll et al. 1987; Ragoussis et al. 1989). The position of the other related class I genes is not known with certainty, although it appears that the majority of them are telomeric to the HLA-A gene (Orr et al. 1983; Koller et al. 1989).

This chapter describes the use of PFGE in the examination of the structural arrangement of class I genes in hereditary haemochromatosis. Several rare-cutting restriction endonucleases were used to obtain large fragments of genomic DNA for separation by PFGE and hybridization to a panel of probes. These probes included those that hybridized within the class I region as well as those that hybridized to sites that flanked it, thereby enabling a search for rearrangements or deletions that may be linked to the disease.

4.2 Results

4.2.1 Section A: A pulsed-field gel analysis of the class I region in families RH7 and HH4

Preliminary studies were carried out using a PFGE apparatus provided by the MRC mammalian genome unit. Subsequently, a 2015 Pulsaphor unit, purchased from LKB, was employed (results typed in bold - see tables). Both machines were based on the design of Schwartz and Cantor (1984) but used non-uniform electric fields. High molecular weight DNA samples from families RH7 and HH4 were digested with the infrequently cutting enzymes, *Mlu* I, *Not* I, *Nru* I and *Pvu* I, and the digested samples were separated on PFGE gels at a pulse time set to optimize resolution of the fragment

size of interest (typically 50-1000 kb). Restriction enzyme fragments were transferred to Hybond N membranes which were hybridized successively with probes from the short arm of chromosome 6. The probes used were those within the region of the MHC mapped by PFGE including the TNF α probe, the HLA cross-hybridizing class I probe, class I-specific genomic probes HLA-E (RS 5.10) and B250, C250 for the serologically defined HLA-B and C loci respectively. In addition, a probe (A490) that has been reported to be A locus-specific was prepared from the 3' untranslated region of the cross-hybridizing class I probe, as previously described by Koller et al. (1984). Other probes outside the region mapped by PFGE were also used and included human (h)-*pim* oncogene cDNA, coagulation factor XIIIa and ferritin H chain cDNA. The genes encoding *h-pim* and FXIIIa have been mapped by somatic cell hybrid techniques to 6p21 (Nagarajan et al. 1986) and 6p24-25 (Board et al. 1988), respectively. The ferritin H chain gene is part of a multigene family and has been mapped to a number of human chromosomes, including the short arm of chromosome 6 at 6p12-21.3 (Cragg et al. 1985; McGill et al. 1987).

(i) Family RH7. The restriction fragments observed for family RH7 are shown in Table 4.1. The hybridization patterns were the same in each family member studied (the mother I2, and siblings II2, II3, II4, II6 - see Figure 3.2 p 61, for family tree) for all the enzymes and probe combinations. It is noteworthy, however, that in some cases the migration of restriction fragments initially appeared to differ between family members (see Figure 4.3a p 88 and Figure 4.4 (family HH4) p 93). However, these differences were consistently observed on hybridization of the same filter with different probes. Thus they appeared to be artifacts, due in some cases to differential loading of DNA in the gel tracks (as in Figure 4.4) and did not represent a true molecular weight difference.

	Class I	A490	B250	C250	TNF α	**HLA-E**	**h-PIM**	**FXIIIa**
Not I	740 650 500 400	740 650 500 400	NR	NR	370	**360**	**500** **320**	**NR**
Mlu I	340	340	340 100	340	130	**600**	**910** **800**	**580**
Nru I	>980 630 525	>980 630 525	630	630	50	**785**	**630** **550**	**NR**
Pvu I	>980 730	>980 730	730	730	730	**NR**	**550**	**NR**

Table 4.1. Restriction fragments (Sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Not* I, *Mlu* I, *Nru* I and *Pvu* I-digested DNA from family RH7. Results in plain text were obtained using the PFGE apparatus provided by the MRC mammalian genome unit; results typed in bold were obtained using the LKB 2015 Pulsaphor machine. NR = No restriction fragments observed.

The MHC class I restriction fragments observed (Table 4.1) were in general agreement with the composite long-range restriction map of this region (Carroll et al. 1987; Dunham et al. 1987) shown in Figure 4.2. However, some differences were noted:

Digestion of genomic DNA with *Not* I and hybridization with the general class I probe revealed four fragments of 740, 650, 500 and 400 kb (see Figure 4.3a). The multiplicity of fragments seen with *Not* I is most likely due to cross-hybridization of this probe with related class I sequences although partial digestion cannot be excluded. The genes of the class I region cross-hybridize by virtue of sequences encoding the β_2 microglobulin-binding domain (exon 4) as well as other sequences that are highly conserved in class I genes (Srivastava et al. 1985).

I was unable to assign the HLA-B or C genes to any one of the *Not* I fragments described above, as a result was not obtained for the class I-specific B250 and C250 probes. These probes are small and for some digests including *Not* I, it was difficult to obtain suitable signals on pulsed-field gel blots. In addition, the A490 probe was unhelpful in localizing the HLA-A gene to a distinct fragment. This probe has been reported to be specific for the HLA-A locus (Koller et al. 1984) but has been found in these experiments and by others (T. Strachan personal communication) to cross-hybridize with fragments detected by the general class I probe. The level of sequence divergence between the HLA-A genes and the class I pseudogenes LN-IIA, HLA12.4 is particularly low (9.7%) compared with a value of 17.0% and 15.8% for the B and C genes, respectively (Strachan 1987). This creates particular difficulties for the production of an A locus-specific probe and it is likely that the A490 probe contains sequences which cross-hybridize with the class I pseudogenes. The TNF α and HLA-E probes were found to hybridize to single fragments of 370 and 360 kb, respectively.

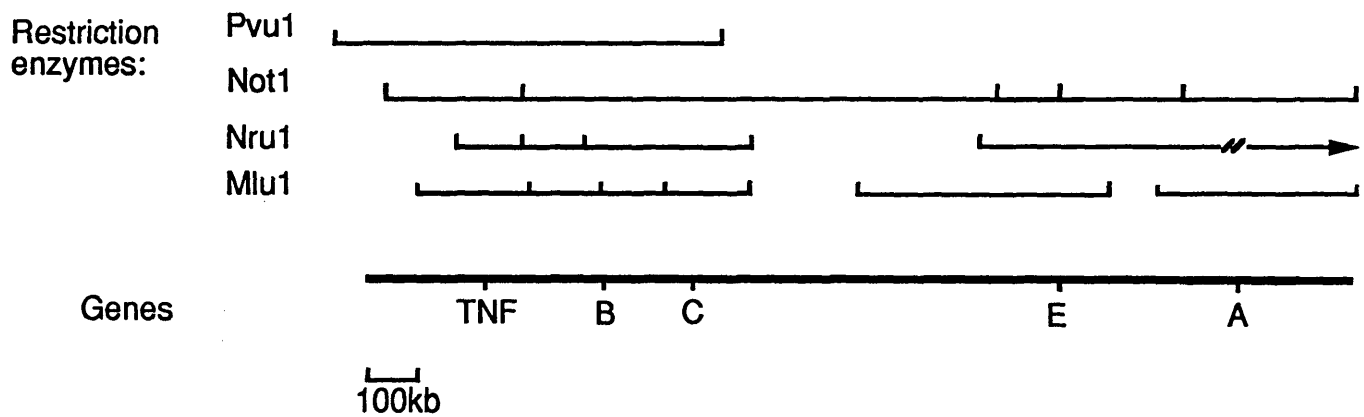


Figure 4.2. Composite long-range restriction map of the class I region of the human MHC derived from Dunham et al. (1987); Carroll et al. (1987). Open spaces correspond to a region where contiguous fragments have not been identified. For convenience the 1200 kb *Nru* I fragment hybridizing to the HLA - E and HLA - A locus probes is not drawn to scale.

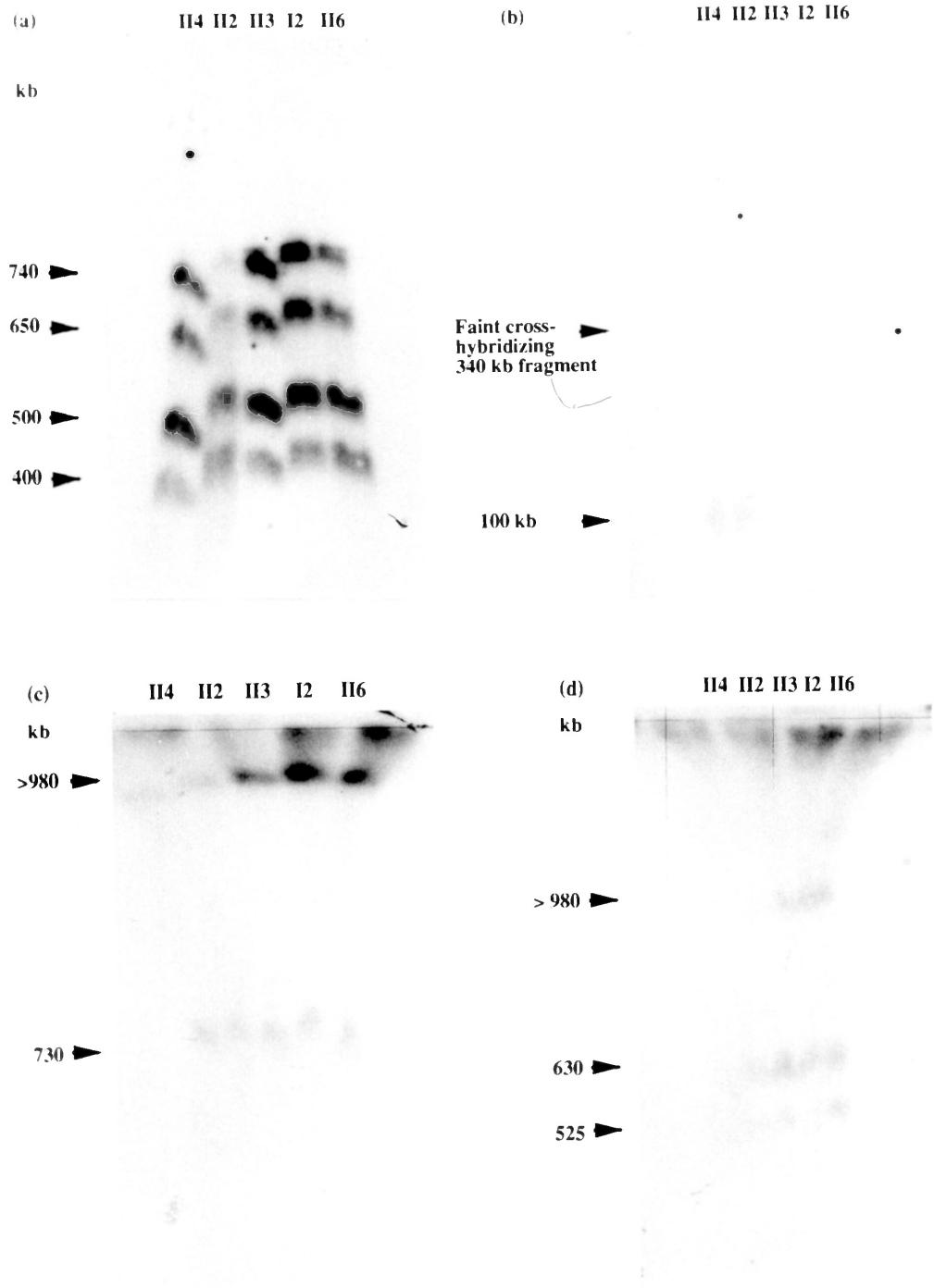


Figure 4.3. PFGE analysis of genomic DNA from members of family RH7 a) *Not* I digest hybridized with the general class I probe b) *Mlu* I digest hybridized with the B250 probe c) *Pvu* I digest and d) *Nru* I digest hybridized with the general class I probe. Yeast (*Saccharomyces cerevisiae)* chromosomes and concatamers of *lambda* c1857S7 provided markers up to 980 kb. The pulse time was set to optimize resolution of fragment between 40-1000 kb.

Carroll et al. (1987) were also able to assign the HLA-E gene to a separate *Not* I fragment (see Figure 4.2). In addition, Dunham et al. (1987) and Carroll et al. (1987) were able to map the HLA-B and C genes to a common *Not* I fragment of approximately 900 kb and the HLA-A gene to a separate 350 kb fragment (Carroll et al. 1987). A recent analysis by Ragoussis et al. (1989) has made use of improved methods for separating large DNA molecules (Smith et al.1987; Vollrath and Davies 1987) and have detected a 1400 kb *Not* I fragment linking HLA-A and HLA-E genes (not shown in Figure 4.2).

Digestion of DNA from family RH7 with *Mlu* I restriction enzyme maps the A and C genes to a 340 kb fragment. The same fragment was observed by Chimini et al. (1988) and probably reflects co-migration of fragments rather than linkage of the A and C genes within 340 kb. The B250 probe cross-hybridized weakly with the 340 kb fragment but also detected an additional 100 kb band (see Figure 4.3b). The TNF α and HLA-E probes also hybridized to distinct fragments of 130 kb and 600 kb, respectively. Thus, the fragments containing the HLA-A, B, C, E and TNF α genes appear to be separated by *Mlu* I digestion.

These *Mlu* I fragments are in general agreement with the map shown in Figure 4.2. However, a 170 kb band detected by the C locus probe in a number of haplotypes (Carroll et al. 1987; Chimini et al. 1988; Ragoussis et al. 1989) was not seen in association with the same haplotypes here. It must also be noted that Ragoussis et al. (1989) have been able to link the HLA-A and HLA-E genes within a 1500 kb *Mlu* I fragment. Furthermore, using a cross-hybridizing class I probe, they have detected an additional 800 kb *Mlu* I fragment which they suggest maps telomeric and contains non-classical (i.e.non-HLA -A, B, C) class I genes.

The *Pvu* I digest of family RH7 mapped the HLA-B, C and TNF α genes to a 730 kb fragment. A linking *Pvu* I fragment of similar size has been observed by Dunham et al.

(1987) (see Figure 4.2). The class I and A490 probes cross-hybridized with this fragment but also recognized an additional large fragment > 980 kb (see Figure 4.3c). This fragment can therefore be mapped telomeric to the 730 kb fragment and probably contains the non-classical class I genes. It is likely that the HLA-E gene is also present on the 980 kb *Pvu* I fragment but that it was not resolved by the LKB Pulsaphor machine used for this particular experiment (see legend to Table 4.1).

Digestion of DNA from family RH7 with *Nru* I and hybridization with the class I probe revealed three fragments - a fragment larger than 980 kb, and two further bands at 630 and 525 kb (see Figure 4.3d). The A490 probe cross-hybridized with each of these three fragments but the C250 and B250 probes were used successfully to map the B and C genes to the 630 kb fragment. Thus, the 980 kb and 525 kb fragments can be mapped telomeric to this fragment. The TNF α and HLA-E probes recognized a 50 kb and 785 kb fragment, respectively. The latter result was obtained by hybridization to a different filter (see legend to Table 4.1). Subsequent studies using the same filter (see Section B) have shown that the HLA-E, B and C probes in fact detect a fragment of the same size. This may reflect co-migration of bands or genetic linkage of these genes. Linkage is possible as the analysis of a deletion mutant suggests that the HLA-E gene lies centromeric to HLA-A (Koller et al. 1988). The above results represent a divergence from the composite map depicted in Figure 4.2 which places the HLA-E and A genes on a 1200 kb fragment and the B and C genes on a common 325 kb fragment.

The difference between fragment sizes observed here and those shown in Figure 4.2 may be explained by haplotype-specific restriction fragment length polymorphisms or by insertions or deletions of DNA. There could also be CpG dinucleotide methylation differences between DNA in the cell line used by Dunham et al. (1987) and Carroll et al. (1987) to construct the map and the source of genomic DNA used here. Small

differences in apparent molecular size are more likely to be due to differences in the estimation of sizes of a given fragment.

In combination with data presented by others (Carroll et al.1987; Dunham et al. 1987; Ragoussis et al. 1989), the most likely arrangement of the class I genes is presented in Figure 4.2. However, the present map of the class I region telomeric to HLA-C should remain tentative as contiguity of fragments has not been confirmed. The order in which the HLA-E and A genes are shown with respect to HLA-C is based on analysis of a deletion mutant (Koller et al. 1988). However, the detection of a *Nru* I fragment linking B, C and A genes in this study provides additional evidence for this arrangement. If no additional *Not* I fragments are present within the class I region, the minimum distance separating HLA-C and A is about 1000 kb (Carroll et al. 1987; Ragoussis et al. 1989), a region sufficiently long to contain many, as yet, uncharacterized genes - some of which may be associated with disease. Further studies will undoubtedly be devoted to obtaining an exact map of the entire class I region so as to establish unequivocally the position of the additional class I genes and pseudogenes that map there.

(ii) Family HH4. The restriction fragments obtained for family HH4 are shown in Table 4.2. The molecular weight of a given fragment was found to vary by as much as 10% between different filters but the hybridization patterns were generally indistinguishable from those previously observed for family RH7.

A difference in fragment size between family HH4 and family RH7 was seen however, using the cross-hybridizing class I probe and the *Not* I restriction enzyme. In family HH4, the class I probe recognized four *Not* I fragments, between 380 and 810 kb (see Figure 4.4). In family RH7 the same probe and enzyme combination revealed four fragments between 400 and 740 kb (see Figure 4.3a). Subsequent studies (see

	Class I	A490	B250	C250	TNF α	**HLA-E**	**h-PIM**	**FXIIIa**
Not I	810 700 450 380	810 700 450 380	NR	NR	320	**360**	**280**	**NR**
Mlu I	388	388	388 97	388	170	**650**	**910**	**600**
Nru I I1 I2 II1 II2	2 x >980, 930, 2 x >980 2 x >980 2 x >980, 930,	660, 530 660, 530 660, 530 660, 530	660, 450 660, 660, 660, 450		97	**730**	**630** **525**	**NR**
Pvu I	>980 730	>980 730	730	730	730	**NR**	I1 490, 360 I2 490 II1 490 II2 490,360	**NR**

Table 4.2. Restriction fragments (sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Not* I, *Mlu* I, *Nru* I and *Pvu* I-digested DNA from family HH4. Results in plain text were obtained using the PFGE apparatus provided by the MRC mammalian genome unit; results typed in bold were obtained using the LKB 2015 Pulsaphor machine. Restriction fragment length polymorphisms were seen among family members on digestion with *Nru* I and *Pvu* I and hybridization with HLA-A,B,C probes and the *h-pim* probe respectively. NR = No restriction fragments observed.

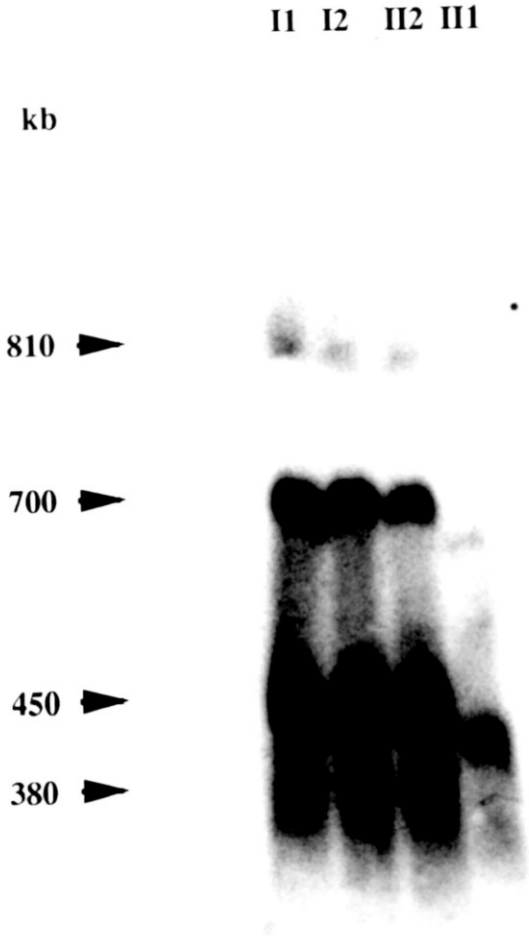


Figure 4.4. PFGE analysis of *Not* I-digested DNA from members of family HH4 using the general class I probe. The DNA in the far-right lane has electrophoresed faster than the other DNA samples. This is due to unequal loading of DNA in the gel tracks and does not represent a true molecular weight difference.

Section B), in which the propositi of families RH7 and HH4 were run on the same gel, have shown that the 650 kb band seen in family RH7 is absent in HH4 and is in fact replaced by the 810 kb fragment. This variation in fragment size in RH7 and HH4 probably reflects the different HLA haplotypes segregating within these two families. The *h-pim* probe also detected a difference in hybridization pattern between the two families. Digestion of DNA from family RH7 with *Not* I and *Mlu* I and hybridization with the *h-pim* probe revealed two restriction fragments for each enzyme (see Table 4.1). In contrast, the *h-pim* probe recognized only the lower molecular weight *Not* I fragment (280 kb) and the higher molecular weight *Mlu* I fragment (910 kb) in the DNA of family HH4. Thus it appears that the *h-pim* probe detects polymorphic sites for *Not* I and *Mlu* I restriction enzymes in these two families.

Further analysis of family HH4 has also revealed differences in hybridization patterns among family members. Digestion of DNA with the *Nru* I restriction enzyme and hybridization with the general class I and A490 probes revealed four fragments (two > 980 kb, one of 660 kb and a faint band at 530 kb) that were present in each individual and also a faint polymorphic 930 kb fragment (see Figure 4.5a). The 930 kb fragment was present in the father (I1) and unaffected sibling (II2) but absent in the mother (I2) and propositus (II1). The father and unaffected sibling are the only members of this family to have the A2-B62 haplotype (see Figure 3.1 p 59). Thus, the presence or absence of the 930 kb band appears to be associated with the possession of this haplotype and not with the incidence of haemochromatosis. The B250 and C250 probes recognized a 660 kb *Nru* I fragment and also a weakly hybridizing polymorphic 450 kb band (Figure 4.5b). This 450 kb fragment segregated within family RH4 in the same way as the 930 kb fragment.

The *h-pim* probe also showed heterozygosity in family HH4. After digestion of DNA with *Pvu* I, the *h-pim* probe revealed a 490 kb fragment present in each individual and

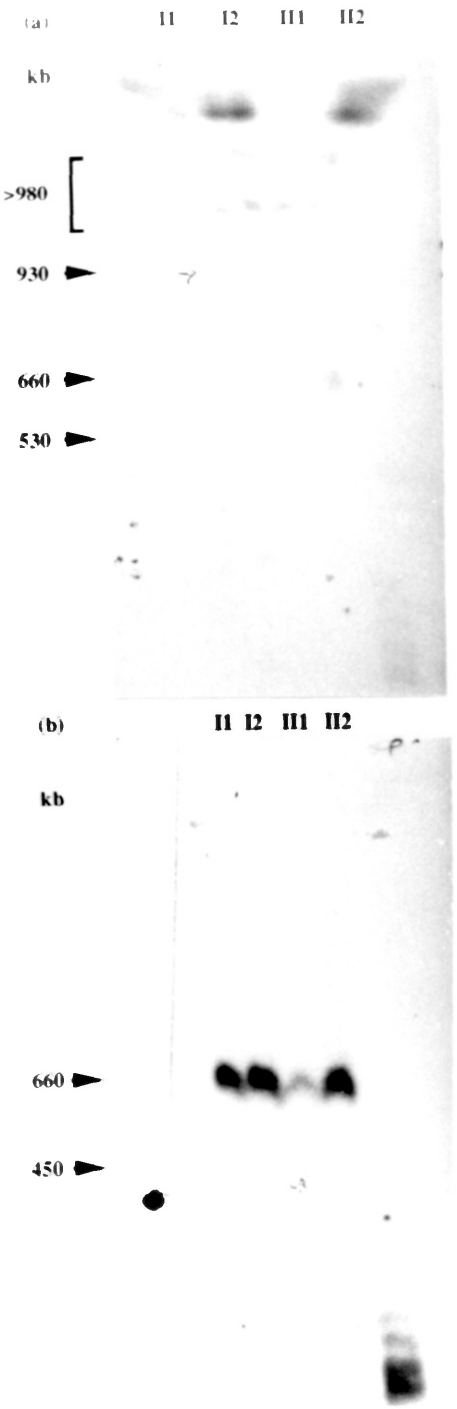


Figure 4.5. PFGE analysis of *Nru* I-digested DNA from members of family HH4 using a) the general class I probe and b) the C250 probe. The polymorphic 930 kb and 450 kb fragments recognized by the general class I and C250 probes, respectively, remained very faint even after exposure of the film for 3 weeks. Yeast (*Saccharomyces cerevisiae)* chromosomes and concatamers of *lambda* c1857S7 provided markers up to 980 kb.

also a polymorphic 360 kb fragment.This fragment showed the same familial distribution as the 930 and 450 kb bands described above, being present only in the father and unaffected daughter. Thus it appears that the 360 kb *h-pim* fragment also segregates with the A2-B62 haplotype within this family. After digestion with *Nru* I restriction enzyme, the *h-pim* probe recognized two fragments of 630 and 525 kb in the DNA of family HH4. Restriction fragments of similar size were also detected in family RH7. In both cases, the lower molecular weight fragment appeared to result from a partial digestion as it was not present consistently in any one individual on repeated *Nru* I digestion. Partial restriction is caused by the sensitivity of rare-cutting enzymes to CpG methylation in their recognition site. Although such partial digestion often results in complicated patterns, it can add important information since more sites can be analysed.

In the study of both families, HLA-E and FXIIIa restriction fragments were consistently not detected after digestion with certain enzymes (see Tables 4.1 and 4.2). This is likely to be due to the generation by these enzymes of fragments outside the resolution range (50-1000 kb) used in these experiments.

The ferritin H chain probe was also used in the pulsed-field analysis of RH7 and HH4. However, this probe gave consistently complex hybridization with a very high background. These problems are probably a reflection of the presence of genes encoding the ferritin H chain on a number of human chromosomes.

Pulsed-field gel electrophoresis is not without technical difficulties. The migration of DNA was found to be influenced by factors other than molecular weight, especially by the loading of DNA in the gel tracks. Thus, in the analysis of data from each filter, it was important to compare the banding patterns that were obtained with different probes. Consistent differences in migration patterns indicate a loading artifact rather than differences in true molecular size. It was also noted that the apparent molecular

size for a given band could vary by as much as ± 10% for different filters. A similar estimation of the variation in size determinations of large DNA fragments using different techniques (FIGE, Rotaphor) was reported by Ragoussis et al. (1989). It is clear that such variation in a fragment of several hundred kilobases might easily preclude detection of deletions that affect genes of sizes 10-20 kb. Thus, for mapping studies where fragments were to be compared, it was important to study the DNA samples of interest on the same filter.

4.2.2 Section B: A pulsed-field gel analysis of the class I region in a further six unrelated haemochromatosis patients and three HLA-matched control subjects

High molecular weight DNA samples from the six unrelated haemochromatosis patients, propositi from families RH7 and HH4 and three HLA-matched control subjects (C1-C3) were digested with infrequently cutting enzymes *Mlu* I, *Not* I, *Nru* I and *Pvu* I. The propositi from families RH7 and HH4 were included in the analysis so that direct comparisons could be made between the banding patterns described in Section A and unrelated patients with the disorder. Southern filters of DNA separated by the LKB PFGE apparatus were hybridized with the same panel of probes as in the family study (Section A) except that the ferritin H chain probe was not used. The HLA type of these individuals are set out in Table 4.3.

(i) *Mlu* I restriction fragments. The restriction fragments obtained after digestion with *Mlu* I endonuclease are illustrated in Table 4.4. The same *Mlu* I fragments were observed as previously described in the study of RH7 and HH4. In addition, several new restriction fragments associated with specific haplotypes were detected. The cross-hybridizing class I probe recognized between two and four fragments that ranged from 70-450 kb (see Figure 4.6a). Stripping and re-probing of the same filter with the A490 probe demonstrated that the strong hybridizing 355 kb fragment present in each of the

	RH7 (II3)	HH4 (II1)	P1	P2	P3	P4	P5	P6	C1	C2	C3
A	1 3	3 3	1 3	1 23	1 2	2 3	1 1	3 10	1 1	3 31	2 2
B	8 14	7 7	7 17	8 44	17 44	5 7	44 44	7 44	8 8	7 40	44 44

Table 4.3. HLA class I serological typing of three healthy control subjects (C1-3), six unrelated patients (P1-6) and propositi from families RH7 and HH4.

	RH7 II3	HH4 (II1)	P1	P2	P3	P4	P5	P6	C1	C2	C3
Class I	355 145	355 70	355 70	355 315 145 70	355 70	355 70	355 70	355 70	355 145	450 355 70	355 70
A490	355	355	355	355 315	355	355	355	355	355	450 355	355
B250	145	70	70	145 70	70	70	70	70	145	70	70
C250	355	355	355	355	355	355	355	355	355	355	355
TNF α	240	240	240	240	240	240	240	240	240	240	240
h-PIM	910 825	910	910 825	910 825	910 825	910 825	910	910 825	910 825	910 825	910 825
HLA-E	645	645	645	645	645	645	645	645	645	645	645
FXIIIa	590	590	590	590	590	590	590	590	590	590	590

Table 4.4. Restriction fragments (sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Mlu* I-digested DNA from three control subjects (C1-3), six unrelated haemochromatosis patients (P1-6) and propositi from families RH7 and HH4. Serological class I HLA typing of the subjects studied is shown in Table 4.3.

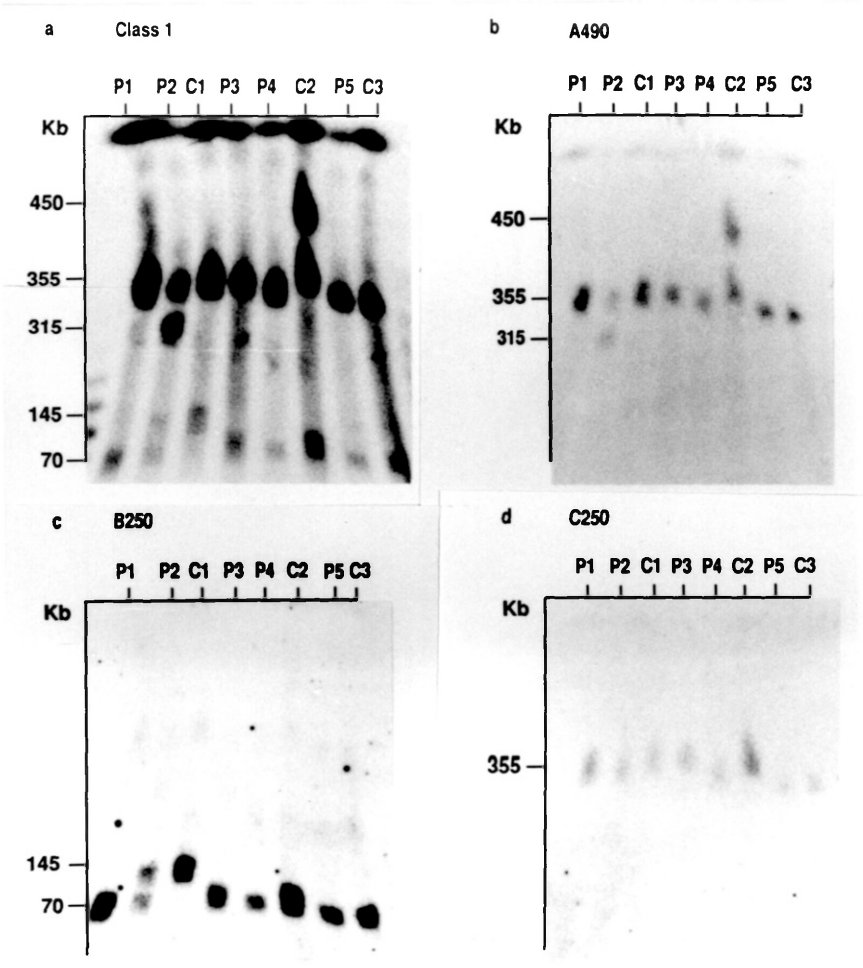


Figure 4.6. PFGE analysis of *Mlu* I-digested DNA from three control subjects and five of the six unrelated haemochromatosis patients. The same filter was hybridized with the general class I probe (a) and then sequentially with the A490 (b), B250 (c) and C250 (d) locus-specific probes. Yeast (*Saccharomyces cerevisiae*) chromosomes and concatamers of *lambda* c1857S7 DNA were used as markers.

patients and controls contains the HLA-A gene (see Figure 4.6b). The additional 315 kb band detected in patient number two (P2) has recently been described by Chimini et al. (1988) in association with the A24 haplotype. Patient number two possesses an A23 haplotype which is a serological split of A24. It thus seems likely that the 315 kb fragment is associated with the related A23/A24 serological haplotypes and is not specifically linked with haemochromatosis. In addition, the 450 kb band present in control subject two (C2) can by assigned to the A31 haplotype of this individual. The weakly hybridizing 70 kb and 145 kb were recognized by the HLA-B locus-specific probe (see Figure 4.6c). Both these fragments were present in control subjects, the 145 kb fragment appearing to associate with the B8 haplotype. The HLA-C locus-specific probe hybridized to a 355 kb fragment that was apparently identical to that revealed by the A locus probe (see Figure 4.6d), but did not recognize the A23 and A31 specific fragments. This confirms the presence of two different co-migrating fragments rather than linkage between A and C genes within 355 kb. As in the PFGE analysis of RH7 and HH4, the additional HLA-C 170 kb fragment that has been seen by other groups was not detected. Hybridization with TNF α, HLA-E, and FXIIIa probes revealed single fragments of 240, 645 and 590 kb, respectively, in both patients and controls. The *h-pim* probe revealed a 910 kb fragment in two out of the eight patients and a 910 kb and 825 kb fragments in the remainder, including the controls.

(ii) *Not* I restriction fragments. The hybridizing fragments observed after digestion with *Not* I restriction enzyme are shown in Table 4.5. The cross-hybridizing class I probe recognized either three or four fragments that ranged from 280 to 770 kb. Four class I fragments were previously detected in the DNA of HH4 (II1) (see Section A). It is likely that the missing 810 kb, at the limit of resolution of the LKB Pulsaphor machine (20-1000 kb) was not resolved. Thus, there appear to be two hybridization patterns detectable by the class I probe, both of which were present in control subjects. The 770 kb band present in control subject two (C2) appeared to be associated with

	RH7 (II3)	HH4 (II1)	P1	P2	P3	P4	P5	P6	C1	C2	C3
Class I	690 600 450 280	690 450 280	690 450 280	NR	690 450 280	690 450 280	690 450 280	690 600 450 280	690 600 450 280	770 690 450 280	690 450 280
A490				AS	FOR	HLA-A2					
B250						NR					
C250						NR					
TNF α	360	360	360	360	360	360	360	360	360	360	360
h-PIM	480 280	280	280	480 280	280	280	280	280	280	280	480 280
HLA-E	360	360	360	360	360	360	360	360	360	360	360
FXIIIa						NR					

Table 4.5. Restriction fragments (Sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Not* I-digested DNA from three control subjects (C1-3), six unrelated haemochromatosis patients (P1-6) and propositi from families RH7 and HH4. NR = No restriction fragments observed. The A490 probe cross-hybridized with each of the restriction fragments detected by the general class I probe.

A31-B40 and is the only fragment that could be assigned to a single haplotype. The A490 probe cross-hybridized with all the fragments recognized by the class I probe. As in Section A, the HLA-B and C locus-specific probes were not helpful in assigning these genes to specific *Not* I fragments. The TNF α and HLA-E probes both detected a 360 kb fragment. In the light of the mapping data discussed in Chapter 4, it is likely that this represents the co-migration of two similarly sized fragments. The *h-pim* probe hybridized either to a 480 kb and 280 kb *Not* I fragment or to a 280 kb *Not* I fragment alone. Two out of six unrelated patients had both bands as did one of the control subjects.

(iii) *Nru* I restriction fragments. The cross-hybridizing class I probe detected two *Nru* I fragments of 780 and 720 kb (see Table 4.6), equivalent to the 630 and 525/530 kb fragments described in the study of families RH7 and HH4. However, the additional larger fragments (>980 kb) previously detected were not separated by the LKB Pulsaphor apparatus. The A490 probe cross-hybridized with the 780 and 720 kb fragments but stripping and re-probing of the same filter demonstrated, as before, that the larger of these two fragments contain the B and C genes. The HLA-E probe was also found to hybridize to this 780 kb band. This most probably reflects linkage of the HLA-E, B and C genes within 780 kb as previously discussed (see Section A). The TNF α probe hybridized to a 145 kb *Nru* I fragment. The *h-pim* probe recognized one *Nru* I fragment at 645 kb and a weaker band at 525 kb.

	RH7 (II3)	HH4 (II1)	P1	P2	P3	P4	P5	P6	C1	C2	C3
Class I	780 720	780 720	780 720	780 720	780 720	780 720	780 720	780 720	780 720	780 720	780 720
A490				AS	FOR	HLA-A2					
B250	780	780	780	780	780	780	780	780	780	780	780
C250				AS	FOR	B250					
TNF α	145	145	145	145	145	145	145	145	145	145	145
h-PIM	645 525	645 525	645 525	645 525	645 525	645 525	645 525	645 525	645 525	645 525	645 525
HLA-E	780	780	780	780	780	780	780	780	780	780	780
FXIIIa						NR					

Table 4.6. Restriction fragments (sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Nru* I-digested DNA from three control subjects (C1-3), six unrelated haemochromatosis patients (P1-6) and propositi from families RH7 and HH4. NR= No restriction fragments observed. The A490 probe cross-hybridized with each of the two restriction fragments recognized by the class I probe; the B250 and C250 probes recognized only the 780 kb fragment.

(iv) *Pvu* I restriction fragments. The results obtained using the *Pvu* I restriction enzymes are shown in Table 4.7. The class I and TNF α probes were found to hybridize to a common 780 kb *Pvu* I fragment as seen in the family study. However, the larger (>980 kb) fragment detected previously by the general class I and A490 probes was not separated by the LKB Pulsaphor machine. The *h-pim* probe recognized a single fragment of 560 kb.

4.2.3 Family DH2

In addition to the six unrelated haemochromatosis patients, two related patients DH1 and DH2 were also studied. They are, respectively, a father and son both of whom were affected by xerocytosis and haemochromatosis. The co-inheritance of these two disorders raised the possibility of a large genetic lesion (e.g., a deletion) involving two loci which might thus be detectable by PFGE. However, using the same enzymes and probes as above, no difference in banding pattern was detected between DH1 and DH2 and control subjects. The fragment sizes that were obtained were consistent with those previously observed (see above).

	RH7 (II3)	HH4 (II1)	P1	P2	P3	P4	P5	P6	C1	C2	C3
Class I	780	780	780	780	780	780	780	780	780	780	780
A490				AS	FOR	HLA-A2					
B250				AS	FOR	HLA-A2					
C250				AS	FOR	HLA-A2					
TNF α				AS	FOR	HLA-A2					
h-PIM	560	560	560	560	560	560	560	560	560	560	560
HLA-E						NR					
FXIIIa						NR					

Table 4.7. Restriction fragments (sizes in kb) detected by probes from the short arm of chromosome 6 after PFGE analysis of *Pvu* I-digested DNA from three control subjects (C1-3), six unrelated haemochromatosis patients (P1-6) and propositi from families RH7 and HH4. NR= No restriction fragments observed. The class I, A490, B250, C250 and TNF α probes recognized the same 780 kb fragment.

4.3 Discussion

Pulsed-field gel electrophoresis has been applied successfully to investigate genetic lesions that are responsible for inherited disease in man. Kenwrick et al. (1987) have used this technique to define the end points of deletions responsible for Duchenne muscular dystrophy. This chapter describes the use of PFGE in the investigation of the genetic defect in haemochromatosis, a condition in which the molecular lesion has been mapped to within 1cM of the HLA-A locus on the short arm of chromosome 6. DNA samples from three affected families, RH7, HH4 and DH2, and a further six unrelated patients were analysed using a panel of probes from this region.

In the analysis of the three families, the association of a deleted or rearranged haplotype with haemochromatosis was not seen. Indeed, the hybridization patterns were identical in each member of family RH7 for all the probe and enzyme combinations. Heterozygosity of restriction fragments was identified in family HH4 using the *Nru* I restriction enzyme and the cross-hybridizing class I, C250/B250 probes and the *Pvu* I enzyme and *h-pim* probe (see p 110 for further discussion of *h-pim* polymorphism). However, the observed RFLP's segregated with the A2-B62 haplotype and were not associated with haemochromatosis.

Similarly, no evidence for a genetic deletion or rearrangement associated with haemochromatosis was obtained in the analysis of the six unrelated patients. However, differences in MHC class I fragment sizes were observed using *Not* I and *Mlu* I restriction endonucleases. It is noteworthy that the same *Mlu* I fragments which correlated with the A23 and B8 haplotypes in this study have recently been reported in association with the A24 (a serological split of A23) and B8 haplotypes by Chimini et al. (1988). Such variation in restriction fragments can be explained by haplotype-specific polymorphisms involving restriction sites or by insertions and deletions of DNA. In this regard, there is indirect evidence from RFLP studies that suggests that the

copy number of class I genes may vary among different haplotypes (Cohen et al. 1985). In particular, cloning experiments have shown that in one A3 haplotype, two class I genes flank the A3 gene, whereas in the A24 haplotype only one flanking gene was found (Nguyen et al. 1985). Here, a specific *Mlu* I and *Not* I fragment was found to be associated with the A31 haplotype, and it would be of interest to determine and compare the organization of this gene with that of HLA-A23/24 genes.

Apart from the limited variations described here, the general conservation of rare-cutting sites in the highly polymorphic MHC class I region was striking. Conservation of the long-range organization of the class II and class III regions of the human MHC has also been noted elsewhere (Tokunaga et al. 1988; Dunham et al.1989a), although, as in this study, variation in fragment sizes between haplotypes was also observed. These authors were able to attribute this variation to specific insertions or deletions of DNA. Some of these differences could also be accounted for by the number of C4 genes present (Dunham et al. 1989b). In some cases, size variation seen in the class II region was considered to be possibly related to differences in the number of DRβ genes (Dunham et al. 1989a), which may vary from one to four, depending on the DR specificity (Bohme et al. 1985; Bell et al. 1987). In addition, Tokunaga et al. (1988) associated a 40 kb class I deletion between TNF and HLA-B with the A1-B8-DR3 haplotype. However, in the present study of individuals from the British population this deletion was not observed. The extent to which these rearrangements are conserved between the same haplotypes, and in different populations, is at present, somewhat unclear.

The conservation of rare-cutting sites seen within the MHC region may be the product of a clustering of non-methylated CpG dinucleotides in C + G rich 'islands' (Bird 1986). These so-called 'HTF islands' or *Hpa* II tiny fragment islands, can be detected in chromosomal DNA as clustered sites for several infrequently cutting restriction enzymes. It has been estimated, from studies of the mouse, that the mammalian

genome should contain approximately 30,000 HTF islands (Bird 1986). If so, their spacing appears to vary among different regions. Five HTF islands have so far been found within the human MHC (Dunham et al. 1987; Ragoussis et al. 1989) but the same number has been detected within 35 kb of the α globin gene complex (Fischel-Ghodsian et al. 1987). HTF islands have been generally associated with expressed genes, particularly with the region where transcription begins (Bird 1987). However, it must be noted that not all vertebrate genes possess unmethylated CpG-rich domains (Bird 1986) and many are partially or heavily methylated (Cooper 1983), as appears to be the case for many class I genes (see below).

CpG-rich regions with many sites for rare-cutter enzymes have been found at the 5' end of all class I human genes hitherto sequenced, including pseudogenes (Chimini et al. 1988), and it is conceivable that other, as yet, unsequenced class I genes may display similar features. If all these CpG regions were unmethylated, the enzymes used in this study would have been expected to cut the class I region, which is believed to contain at least 20 genes, into fragments of about 20-50 kb. In fact, the relatively large fragments that were obtained reflect either an absence of CpG-rich regions, which appears unlikely, or the fact that many of the CpG-rich regions are methylated. Methylation of cytosine frequently leads to its deamination and subsequent transition to thymine (Bird 1987) and it has been suggested that some form of sequence exchange may occur to restore or conserve the CpG dinucleotide in the class I region (Chimini et al. 1988).

There has been some evidence, particularly convincing in the mouse system, that homologous exchange of genetic material occurs frequently. Geliebter and Nathenson (1988) have recently shown that spontaneous *in vivo* mutants of the murine class I k^b histocompatibility gene are generated by a 'microrecombination' process that results in the transfer of short segments of DNA from donor genes to k^b. An analogous process is also believed to operate in the human MHC class I region (Lopez de Castro et al.

1982; Seemann et al. 1986). This exchange of blocks of nucleotides may account for the high degree of sequence conservation between non-allelic class I genes as well as the extreme polymorphism seen at some loci. The mechanism responsible for these effects is most probably either unequal cross-over or gene conversion (Baltimore 1981). The interaction that takes place between two genes during gene conversion involves a non-reciprocal alteration in their structure in which both genes retain their integrity and physical location. In contrast, unequal cross-over results in reciprocal alterations in the two genes. The overall conservation of the long-range organization of the MHC class I region suggests that gene conversion events represent the primary mechanism responsible for the generation of polymorphism. It is noteworthy, however, that the restricted, haplotype-related variation in class I fragments that has been observed appears to be due to variation in the copy number of certain class I, II or III genes and is thus consistent with the occurrence of sporadic unequal cross-over events at meiosis.

With the exception of probes for the HLA genes, the only other probe that revealed restriction fragment length polymorphisms was the human *h-pim* cDNA. Combined observations in this work (from Section A and B) suggests that the *h-pim* probe detects polymorphic sites for *Not* I, *Mlu* I and *Pvu* I restriction enzymes. These polymorphic fragments were found to be present in family members unaffected by haemochromatosis and in control subjects. They were therefore not specifically associated with the disease. However, of particular interest is the *Pvu* I polymorphism that was found to segregate with HLA type in family HH4 (see Section A). The *h-pim* probe has been localized to the 6p21 region of the short arm of chromosome 6 by analysis of somatic cell hybrids (Nagarajan et al. 1986) but its position relative to the MHC complex is not known. I was not able to link the *h-pim* locus physically to any one HLA gene (i.e., no common hybridizing fragment was detected) but it did appear that the *h-pim* gene was close enough to the MHC complex to escape separation by recombination events in family HH4. It would be of considerable interest to determine

the precise localization of the *h-pim* gene as there is some evidence for its rôle in human haematopoiesis and possibly in the development of leukaemia. (Nagarajan et al. 1986).

In conclusion, using PFGE, I was unable to detect any major lesion in the vicinity of the class I region on chromosome 6, from a total of nine unrelated patients affected by haemochromatosis. This suggests that the mutation responsible for haemochromatosis results from a relatively discrete structural lesion. The lesion could be a point mutation, or perhaps a small intragenic deletion or rearrangement that is beyond the current resolving power of the PFGE technique. Thus, a further advance on the disease locus will call for techniques of greater sophistication and at present this would include procedures for extensive chromosome walking or complete sequence analysis of the region in several affected patients and suitable control subjects.

An alternative approach is the isolation and characterization of a gene that may be implicated in human iron metabolism and may thus be considered as a candidate involved in the development of haemochromatosis. Such an approach has become particularly attractive with the development of the polymerase chain reaction that allows genes for which a partial or conserved amino acid sequence is known to be cloned rapidly. Once cloned, the candidate gene(s) can be tested directly for a causal role in haemochromatosis by the determination of its chromosomal localization. Accordingly, the second half of this thesis describes the cloning and characterization of the gene encoding a human iron-binding protein in macrophages, the type 5 (tartrate-resistant) acid phosphatase. The cellular and ultra-structural location of this enzyme, together with its unique iron-binding properties, indicate that it may be involved in the aetiology of hereditary haemochromatosis.

CHAPTER 5

THE SEQUENCE, PATTERNS OF TISSUE EXPRESSION AND CHROMOSOMAL LOCALIZATION OF HUMAN TYPE 5 TARTRATE-RESISTANT ACID PHOSPHATASE

Chapter 5: The Sequence, Patterns of Tissue Expression and Chromosomal Localization of Human Type 5 Tartrate-resistant Acid Phosphatase.

5.1 Introduction

The function and expression of the major proteins of iron metabolism - transferrin, ferritin and the transferrin receptor have been studied extensively, but as yet no primary defect in their structure or expression has been found with respect to haemochromatosis. It seems likely therefore, that another undefined protein of iron metabolism is involved in the causation of this disease. A possible candidate that has been subject to detailed study in this work, is the iron-containing type 5 tartrate-resistant purple acid phosphatase.

Type 5 acid phosphatase is a lysosomal enzyme that has been purified from a number of sources, including bovine and human spleen (Davis et al. 1981; Ketcham et al. 1985). The protein is expressed by cells of the human mononuclear phagocytic system but the function of type 5 acid phosphatase within these cells is largely unknown. However, immunochemical and enzymatic studies (Ketcham et al. 1985), as well as protein sequence analysis (Hunt et al. 1987), have shown that the splenic phosphatase is closely homologous to uteroferrin, a purple iron-binding protein abundant in the uterine secretions of pregnant sows (Roberts and Bazer 1980). Physiological studies *in vivo* have implicated uteroferrin in the delivery of maternal iron to the developing piglet (Buhi et al 1982; Renegar et al 1982; Ducsay et al. 1982) and it is possible that the human type 5 acid phosphatase plays a comparable rôle in iron metabolism. Indeed, ultrastructural studies of bovine spleen (Shindelmeiser et al. 1987) have shown an association of the protein with ferritin and haemosiderin aggregates in littoral macrophages, suggesting that it may participate in the retrieval of iron released physiologically by erythrophagocytosis. Thus, it is possible that a defect in type 5 acid

phosphatase may be responsible for the reduced accumulation of iron that has been previously noted within the mononuclear phagocytes of patients suffering from haemochromatosis (Charlton et al. 1973; Fillet and Marsaglia 1975).

To investigate further the structure and functional expression of the purple acid phosphatase in relation to iron metabolism, the human phosphatase cDNA was cloned and sequenced. Section A of this chapter describes the isolation of full-length acid phosphatase from a human cDNA library. The DNA probe used to screen the library was obtained by a novel procedure that utilizes the polymerase chain reaction. Section B describes the use of the human acid phosphatase cDNA to a) investigate the pattern of tissue expression of type 5 acid phosphatase within cells of the mononuclear phagocyte system and b) to determine the chromosomal localization of the acid phosphatase gene.

5.2 Results

5.2.1 Section A: The cloning and sequencing of human type 5 tartrate-resistant acid phosphatase

(i) The polymerase chain reaction. Published amino acid sequences for porcine uteroferrin and acid phosphatase from bovine spleen (Hunt et al. 1987) were used to derive oligonucleotide primers for use in the polymerase chain reaction (PCR). Oligonucleotides were synthesized to provide all possible sequences coding for two regions conserved between porcine uteroferrin and bovine spleen phosphatase (see Figure 5.1). Sense oligonucleotides were made to FQETFED and antisense to GHYPVW. Each pool of oligonucleotide primers for the PCR was highly degenerate and contained 256 individual sequences. In uteroferrin, the distance between these sequences is 115 amino acid residues, but attempts to amplify the human gene from

```
T A P T P I L R F V A V G D W G G V P N A P F H T A R E M   Prot (A)
T P A P M _ L R F V A V G D W G G V P N A P F Y S A --E M   Prot (B)

A N A K A I A T T V K T L G A D F I L T L G D N F Y F T G   Prot (A)
A N A K A X A --T V K X X G A D F V X S --G D N F Y F S --  Prot (B)

V H D A K D K R F Q E T F E D V F S D P S L R N V P W H V   Prot (A)
--------------- F Q E T F E D V F S A S P X R S V P W ----  Prot (B)

L A G N H D H L G N V S A Q I A Y ----K K I L K R --X S K   Prot (A)
X A G N H D H X G N V S ------------A K K X X --------S K   Prot (B)

I S K R W N F P S P Y Y R L R F K I P R P R ------X F M L   Prot (A)
X S K R W K F P S P Y Y R X R F K X P R S D T R V X F M L   Prot (B)

D T V T L C G N S D D F V S Q Q P E R N L A L A R T Q L A   Prot (A)
D T V T X C G N S D D F V --------------------A R T Q X A   Prot (B)

W I K K Q L A A A K E D Y V L V A G H Y P V W S I A E H G   Prot (A)
W X K K Q X A A A K E D Y V X V A G H Y P V W S X A E H G   Prot (B)

P T H C L V K Q L L P L L T T H K V T A Y L Q G H D H N L   Prot (A)
V V H C X V K Q X X P X X N A H K V T A Y X C G H D H N X   Prot (B)

Q Y L Q --------------V L S G A G N F M D P S K K H L R K   Prot (A)
Q Y X Q Q E N G X G F V X S G A G N F M D P S K K H ----Q   Prot (B)

V P N G Y L R F H F G A E N S L G G F A Y V E I T P K E M   Prot (A)
V P D G Y X R F H Y G A E N S X G G F A Y V E X S P K E M   Prot (B)

S V T Y I E A S G K S L F K T K L P R R A R S               Prot (A)
S V T Y X E A S A N S X F K T R X P R
```

Figure 5.1. Comparison of the partial amino acid sequence data for porcine uteroferrin (Protein A) and bovine spleen acid phosphatase (Protein B) taken from Hunt et al. (1987). Missing sequence information is indicated by (------). (X) indicates that the residue was identified by mass spectroscopy as either leucine or isoleucine. (____) indicates that adjacent residues are connected in the sequence. The two regions that were used to derive oligonucleotides primers for the PCR reaction are underlined.

genomic DNA were unsuccessful, presumably because of the presence of one or more introns in this region. Thus single-stranded cDNA was used as the template for the reaction. The polyadenylated messenger RNA used for reverse transcription was obtained from the spleen of a patient with Gaucher's disease - a disorder in which there is abundant expression of type 5 acid phosphatase.

The oligonucleotides which anneal to complementary and opposite strands of the cDNA delineate the target sequence and act as primers for DNA synthesis. The first round of DNA synthesis by the polymerase results in conversion of single-stranded cDNA to double-stranded DNA. Thereafter, the amplification consists of cycles of DNA denaturation, annealing of the oligonucleotides to their complementary sequences and then oligonucleotide-primed DNA synthesis by the *Taq* DNA polymerase. The reactions were carried out in a simple thermocycling apparatus (Foulkes et al. 1988) (see Figure 5.2) which controlled the cycles of denaturing, annealing and extension by alternating the reaction temperatures between 92°C, 45°C and 72°C, respectively.

Analysis of the resulting reaction products on a 2% agarose mini-gel after amplification, showed a major fragment of 330 bp, which was approximately that of the predicted size. The additional minor bands probably represented non-specific amplified products (Mullis and Falloona 1987). The 330 bp DNA was cloned to yield the plasmid pPCR330, the insert of which was sequenced. The deduced amino acid sequence of this clone was found to be almost identical to the corresponding sequences of porcine uteroferrin and bovine spleen acid phosphatase (see Figure 5.3). Overall, there was 85% identity between PCR330 and uteroferrin.



Figure 5.2. The thermocycling apparatus. A Histokinette (a tissue-embedding machine) was adapted for performing the PCR reaction. A steel rack was suspended from the mechanical arm. During one cycle, the arm pivots about the central axis and sequentially dips the rack into the three thermostatically controlled water baths. In the figure, the arm is raised in the process of transferring the tube rack between water baths.

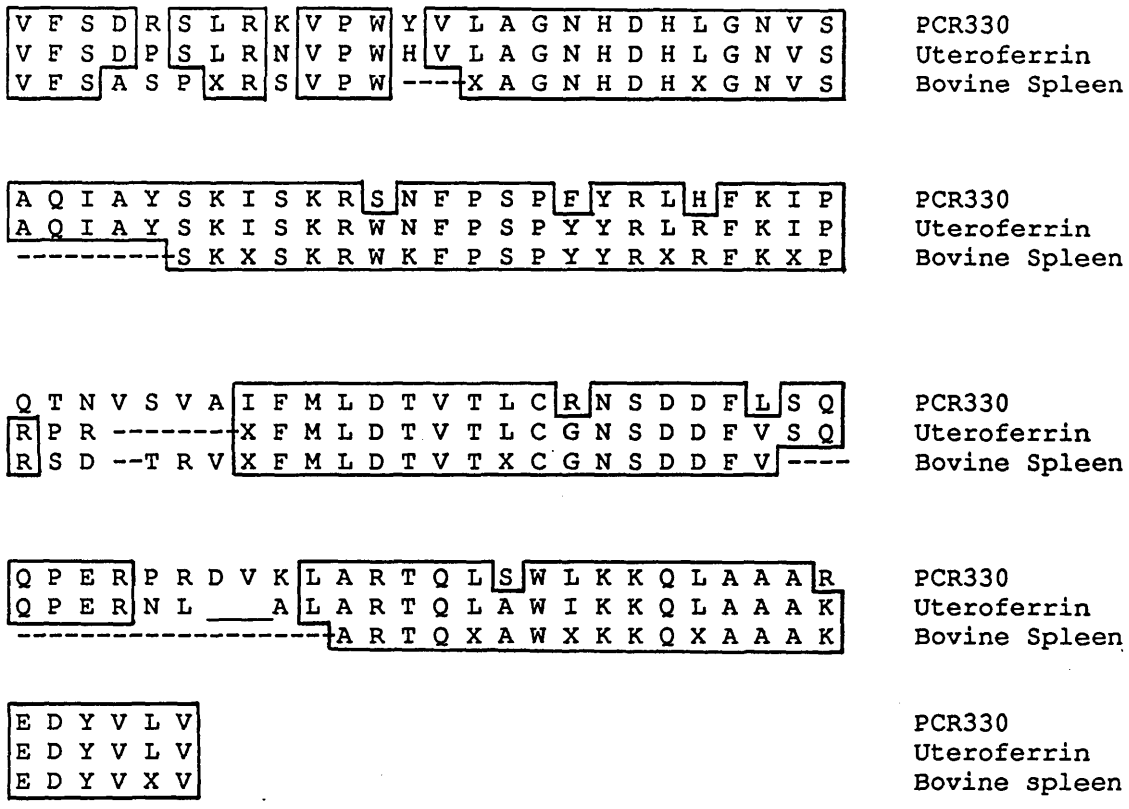


Figure 5.3. Comparison of deduced amino acid sequence of PCR330 with the corresponding amino acid sequence of porcine uteroferrin and bovine spleen acid phosphatase. Missing sequence information is indicated by (----). (X) stands for leucine or isoleucine, and for purposes of comparison with the bovine and porcine sequence, these residues are considered interchangeable. (___) indicates that adjacent residues are connected in the sequence. Sequences that are identical in two or three proteins are boxed.

(ii) Screening of a human placental cDNA library. PCR330 was used to screen 1.25 x 10^5 plaques of a human placental cDNA library (Clontech) that had been generated in *lambda* gt 11. Three putative clones were plaque-purified, digested with *Eco*R I and found to contain 1.3 - 1.4 kb inserts. The insert from the largest of these designated *lambda* HP3 was subcloned to give pHP3, and sequenced following the strategy shown in Figure 5.4. Figure 5.5 shows the 1359 bp nucleotide sequence and deduced amino acid sequence of HP3. This nucleotide sequence is identical to that of the probe PCR330, except for the replacement of C residues at positions 424 and 519 by G. Thus, the 1.4 kb cDNA appears to be an authentic clone for type 5 acid phosphatase. The discrepancy between the spleen and placental sequences may represent allelic variations or result from occasional replication errors by *Taq* polymerase during the polymerase chain reaction.

The full-length acid phosphatase cDNA is composed of a 35 bp 5' untranslated region followed by two possible in-frame initiator methionines both of which lie downstream of an in-frame stop codon at nucleotide 12-14. It cannot be assumed that initiation occurs exclusively at the first ATG codon, and it is possible that both sites are employed. If the first ATG codon is used, the open reading frame is 975 bp and encodes 325 amino acids which would correspond to a polypeptide of molecular mass 36571 Da. The 3' non-coding region is 346 bp long with a potential polyadenylation signal 19 bp upstream of the poly A tract. The 5' end of the smaller cDNA inserts were sequenced and found to be identical to HP3, but lacked nucleotides at the 5' end.

The amino-terminal region of human acid phosphatase contains many hydrophobic residues that are characteristic of a signal peptide. The assignment of a cleavage site between residues -1 and +1 is based on von Heijne's (-3,-1) rule (von Heijne 1986). The residue in position -1 is the preferred alanine, and the serine at position -3 is the preferred small, neutral amino acid. Cleavage at this point would provide a signal sequence of 17-19 amino acids, depending on which initiation codon was employed.

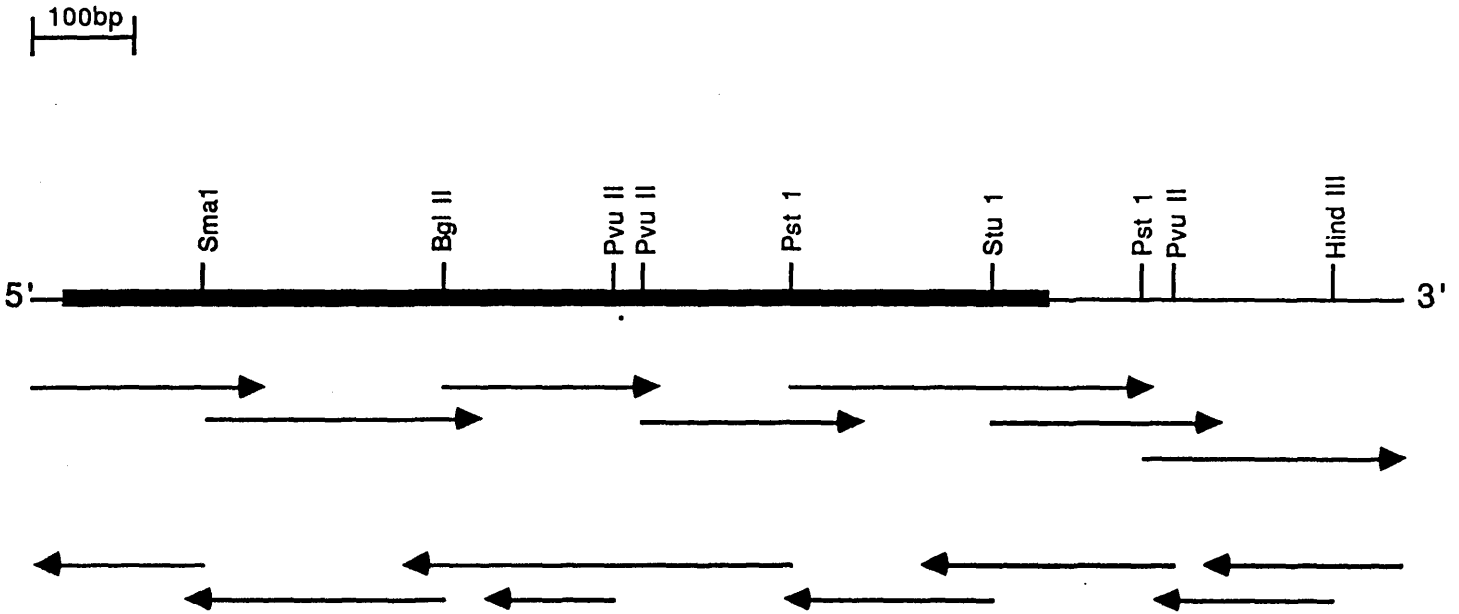


Figure 5.4. Restriction map and sequencing strategy for the tartrate-resistant acid phosphatase cDNA clone HP3. The position of restriction sites used in sequencing are indicated. The solid box represents the coding region, the lines designate the 5' and 3' untranslated regions. Arrows show the direction and the extent of each sequence determination.

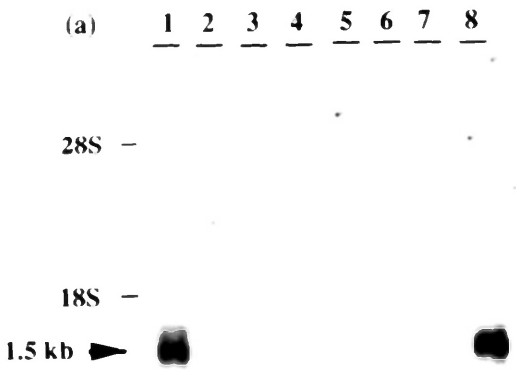
Figure 5.5. Nucleotide and deduced amino acid sequence of human tartrate-resistant acid phosphatase cDNA. The signal peptidase cleavage site follows amino acid -1 and the +1 amino acid is assigned to the first residue of the mature protein. The two possible N-linked glycosylation sites (Asn-Val-Ser) are at residues 97-99 and 128-130. The termination codon is indicated by an asterisk and the putative polyadenylaton signal in the 3′ untranslated region is underlined.

```
                                AGAGCCTCCGGTGACTGGCCTGTGTCTCCCCCTGG   35
-19                                        -10
Met Asp Met Trp Thr Ala Leu Leu Ile Leu Gln Ala Leu Leu Leu
ATG GAC ATG TGG ACG GCG CTG CTC ATC CTG CAA GCC TTG TTG CTA  80
            -1  +1                                      10
Pro Ser Leu Ala Asp Gly Ala Thr Pro Ala Leu Arg Phe Val Ala
CCC TCC CTG GCT GAT GGT GCC ACC CCT GCC CTG CGC TTT GTA GCC  125
                                    20
Val Gly Asp Trp Gly Gly Val Pro Asn Ala Pro Phe His Thr Ala
GTG GGT GAC TGG GGA GGG GTC CCC AAT GCC CCA TTC CAC ACG GCC  170
            30                                          40
Arg Glu Met Ala Asn Ala Lys Glu Ile Ala Arg Thr Val Gln Ile
CGG GAA ATG GCC AAT GCC AAG GAG ATC GCT CGG ACT GTG CAG ATC  215
                                    50
Leu Gly Ala Asp Phe Ile Leu Ser Leu Gly Asp Asn Phe Tyr Phe
CTG GGT GCA GAC TTC ATC CTG TCT CTA GGG GAC AAT TTT TAC TTC  260
            60                                          70
Thr Gly Val Gln Asp Ile Asn Asp Lys Arg Phe Gln Glu Thr Phe
ACT GGT GTG CAA GAC ATC AAT GAC AAG AGG TTC CAG GAG ACC TTT  305
                                    80
Glu Asp Val Phe Ser Asp Arg Ser Leu Arg Lys Val Pro Trp Tyr
GAG GAC GTA TTC TCT GAC CGC TCC CTT CGC AAA GTG CCC TGG TAC  350
            90                                          100
Val Leu Ala Gly Asn His Asp His Leu Gly Asn Val Ser Ala Gln
GTG CTA GCC GGA AAC CAT GAC CAC CTT GGC AAT GTC TCT GCC CAG  395
                                    110
Ile Ala Tyr Ser Lys Ile Ser Lys Arg Trp Asn Phe Pro Ser Pro
ATT GCA TAC TCT AAG ATC TCC AAG CGC TGG AAC TTC CCC AGC CCT  440
            120                                         130
Phe Tyr Arg Leu His Phe Lys Ile Pro Gln Thr Asn Val Ser Val
TTC TAC CGC CTG CAC TTC AAG ATC CCA CAG ACC AAT GTG TCT GTG  485
                                    140
Ala Ile Phe Met Leu Asp Thr Val Thr Leu Cys Gly Asn Ser Asp
GCC ATT TTT ATG CTG GAC ACA GTG ACA CTA TGT GGC AAC TCA GAT  530
            150                                         160
Asp Phe Leu Ser Gln Gln Pro Glu Arg Pro Arg Asp Val Lys Leu
GAC TTC CTC AGC CAG CAG CCT GAG AGG CCC CGA GAC GTG AAG CTG  575
                                    170
Ala Arg Thr Gln Leu Ser Trp Leu Lys Lys Gln Leu Ala Ala Ala
GCC CGC ACA CAG CTG TCC TGG CTC AAG AAA CAG CTG GCG GCG GCC  620
            180                                         190
Arg Glu Asp Tyr Val Leu Val Ala Gly His Tyr Pro Val Trp Ser
AGG GAG GAC TAC GTG CTG GTG GCT GGC CAC TAC CCC GTG TGG TCC  665
                                    200
Ile Ala Glu His Gly Pro Thr His Cys Leu Val Lys Gln Leu Arg
ATA GCC GAG CAC GGG CCT ACC CAC TGC CTG GTC AAG CAG CTA CGG  710
            210                                         220
Pro Leu Leu Ala Thr Tyr Gly Val Thr Ala Tyr Leu Cys Gly His
CCA CTG CTG GCC ACA TAC GGG GTC ACT GCC TAC CTG TGC GGC CAC  755
                                    230
Asp His Asn Leu Gln Tyr Leu Gln Asp Glu Asn Gly Val Gly Tyr
GAT CAC AAT CTG CAG TAC CTG CAA GAT GAG AAT GGC GTG GGC TAC  800
            240                                         250
Val Leu Ser Gly Ala Gly Asn Phe Met Asp Pro Ser Lys Arg His
GTG CTG AGT GGG GCT GGG AAT TTC ATG GAC CCC TCA AAG CGG CAC  845
                                    260
Gln Arg Lys Val Pro Asn Gly Tyr Leu Arg Phe His Tyr Gly Thr
CAG CGC AAG GTC CCC AAC GGC TAT CTG CGC TTC CAC TAT GGG ACT  890
            270                                         280
Glu Asp Ser Leu Gly Gly Phe Ala Tyr Val Glu Ile Ser Ser Lys
GAA GAC TCA CTG GGT GGC TTT GCC TAT GTG GAG ATC AGC TCC AAA  935
Glu Met Thr Val Thr Tyr Ile Glu Ala Ser Gly Lys Ser Leu Phe
GAG ATG ACT GTC ACT TAC ATC GAG GCC TCG GGC AAG TCC CTC TTT  980
Lys Thr Arg Leu Pro Arg Arg Ala Arg Pro  *
AAG ACC AGG CTG CCG AGG CGA GCC AGG CCC TGA ACTCCCATGACTGCC 1028
CAGCTCTGAGGCCCGATCTCCACTGTTGGGTGGGTGGCCTGCCGGGACCCTGCTCACAGG 1088
CAGGCTTTTCCTCCAACCTGTGGCGCTGCAGCAGGGCAGGAAGGGGAAACACAGCTGATG 1148
AACTGTGGTGCCACATGACCCTTGTGGCACAGATGCCCACGTATGTGAAACACACATGGA 1208
CATGTGTCCCAGCCACAGTGTTATGCTCTGTGGCTGGCTCACCTTTGCTGAGTTCCGGGG 1268
TGCAATGGGGGAGGGAGGGAGGGAAAGCTTCCTCCTAAATCAAGCATCTTTCTGTTACTG 1328
ATGTTCAATAAAAGAATAGTTGCCAAGGCTGAAAAAAAAAAAAAAAAAAAAAAAAAAAA   1359
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Eukaryotic signal sequences typically range from 15-25 residues with 19 amino acids as the average length (von Heijne 1985). Since the amino-terminal sequence of the human type 5 acid phosphatase has not been determined, this assignment remains tentative and is based on data derived from the cDNA sequence. The latter predicts two possible glycosylation sites (Asn - Val - Ser) for asparagine-linked oligosaccharides at residues 97 and 128.

5.2.2 Section B: Pattern of tissue expression and chromosomal localization of human type 5 tartrate-resistant acid phosphatase

(i) Patterns of tissue expression of type 5 acid phosphatase. Total RNA was prepared from a range of human tissues and cells including the spleen of a patient with Gaucher's disease, normal spleen, liver, bone marrow and reticulocytes. RNA was also prepared from peripheral blood mononuclear cells and monocyte-derived macrophages that had been cultured for 3 days in the presence of 10% autologous serum (see Chapter 2 (2.23)). The RNA was electrophoresed on a 1.5% denaturing-agarose gel and then transferred to Hybond N membranes, which were hybridized to the full-length acid phosphatase insert from pHP3. Type 5 acid phosphatase-specific RNA was found to be expressed in large amounts in Gaucher's spleen and in monocyte-derived macrophages (see Figure 5.6a). On further exposure of this filter, a weak signal was seen in mononuclear cells and liver but none was detected in total RNA from control spleen, bone marrow or reticulocytes (see Figure 5.6b). The length of the RNA transcript from Gaucher's spleen was determined to be 1.5 kb using commercial RNA molecular weight markers. The greater abundance of acid phosphatase RNA in macrophages, compared with monocytes from which these cells were derived, correlated closely with the measured enzymatic activity (see Table 5.1). The latter was determined by Dr Maria Bevilacqua in our laboratory using a specific enzymatic immunoassay (Whitaker et al. 1989). We observed very low activities of acid phosphatase in normal peripheral blood mononuclear cells and a more than



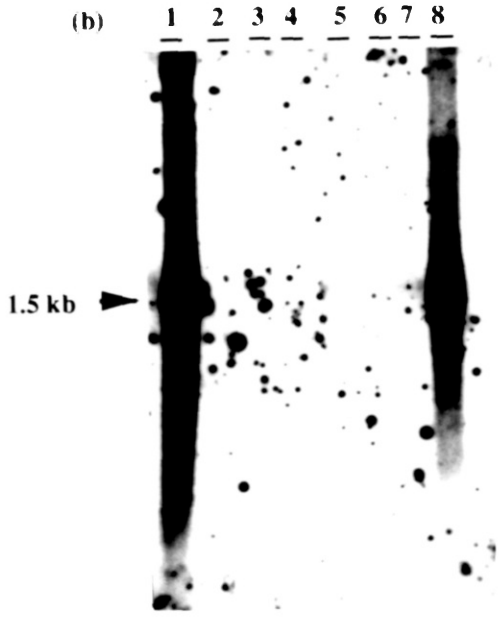


Figure 5.6. a) Northern blot analysis of 10 μg aliquots RNA from human tissues and cells using full-length acid phosphatase cDNA as a probe. Total RNA was isolated from Gaucher's spleen (lane 1), normal spleen (lane 2), bone marrow (lane 3), reticulocytes (lane 4), liver (lane 5 and 6), monocytes (lane 7) and monocyte-derived macrophages (lane 8). A 1.5 kb mRNA for acid phosphatase is seen in samples from Gaucher's disease spleen and macrophages. Markers at the right of the figure show the position of 28 S and 18 S rRNA. The exact size of the acid phosphatase transcript was determined using RNA calibration standards (BRL). (b) A longer exposure of the same filter detects a weak signal in liver (lanes 5 and 6) and mononuclear cells (lane 7).

Cells	Enzyme Activity (μmol.min.$^{-1}$mg protein^{-1})
Peripheral blood mononuclear cells (day 0)	0.21 (0.07)
Monocyte-derived macrophages (day 3)	4.73 (2.2-8.8)

Table 5.1. Type 5 acid phosphatase activity in human monocytes and macrophages. Peripheral blood mononuclear cells from a healthy individual were prepared as described in Materials and Methods (Chapter 2, 2.23) and cultured with 10% autologous serum at 37°C. Adherent mononuclear cells were harvested at the indicated times and lysed in 50mM Hepes/2% w/v Triton x-100 pH7.5. Acid phosphatase activity in 12,000 x g supernatant fractions of the cell lysates was determined in duplicate by the immunoenzymatic procedure (Whitaker et al. 1989) and is expressed as micromoles of 4-nitrophenol phosphate hydrolysed per minute per milligram of protein. Day 0 cells were obtained from ten subjects and day 3 macrophages were derived from five separate individuals. The mean value is given with the range in parenthesis.

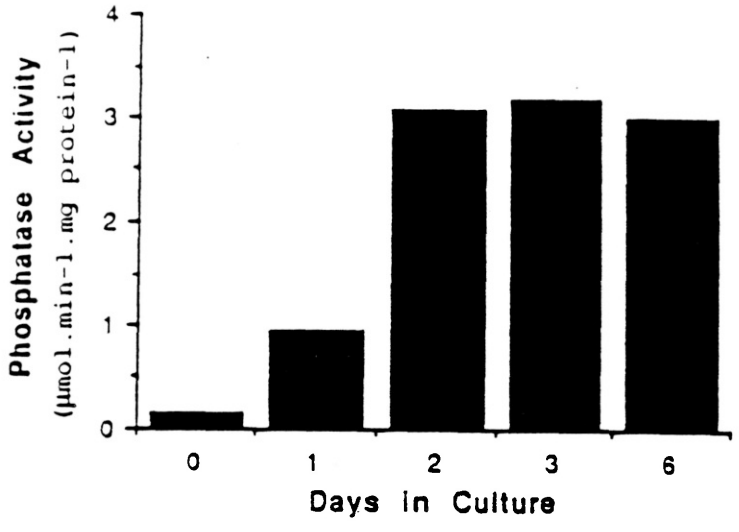


Figure 5.7. Time course of induction of type 5 acid phosphatase in human monocyte-derived macrophages in culture. The data are representative of four similar experiments carried out with cells obtained from different individuals.

twentyfold increase after the cells had been cultured for three days in the presence of autologous serum. A time course (see Figure 5.7) shows that the expression of type 5 acid phosphatase activity reaches a peak at three days. It was found that the type 5 acid phosphatase activity constituted about one half of the total acid phosphatase activity of the monocyte-derived macrophages.

To study further the patterns of tissue expression of type 5 acid phosphatase, RNA was prepared from the spleen of a patient with hairy cell leukaemia, pulmonary alveolar macrophages obtained from a bronchial lavage and osteoclastoma tissue. The latter was a generous gift from the laboratory of Professor Timothy J Chambers (Department of Histopathology, St Georges Hospital Medical School, London). These RNA samples, together with RNA from Gaucher's spleen (a positive control), were electrophoresed on a 1.5% denaturing gel. The RNA was transferred to Hybond N membranes and hybridized with the insert from pHP3. A single 1.5 kb acid phosphatase transcript was detected in each of the tissues examined (see Figure 5.8).

(ii) Genomic representation and chromosomal localization of type 5 acid phosphatase.

Human genomic DNA was digested with *Bam*H I, *Eco*R I and *Xba* I restriction endonucleases and probed with HP3. The pattern of hybridization observed in Southern blots of *Bam*H I and *Eco*R I digests, consisting of single fragments of approximately 12 and 20 kb, are consistent with the presence of a single copy gene. Digestion wih *Xba* I revealed two allelic restriction fragments of 8 kb and 1.4 kb (see Figure 5.9). The least frequent 8 kb fragment was detected in three out of eleven unrelated individuals. (DNA was kindly donated by Dr Steven Durham, Department of Allergy, Brompton Hospital, London).

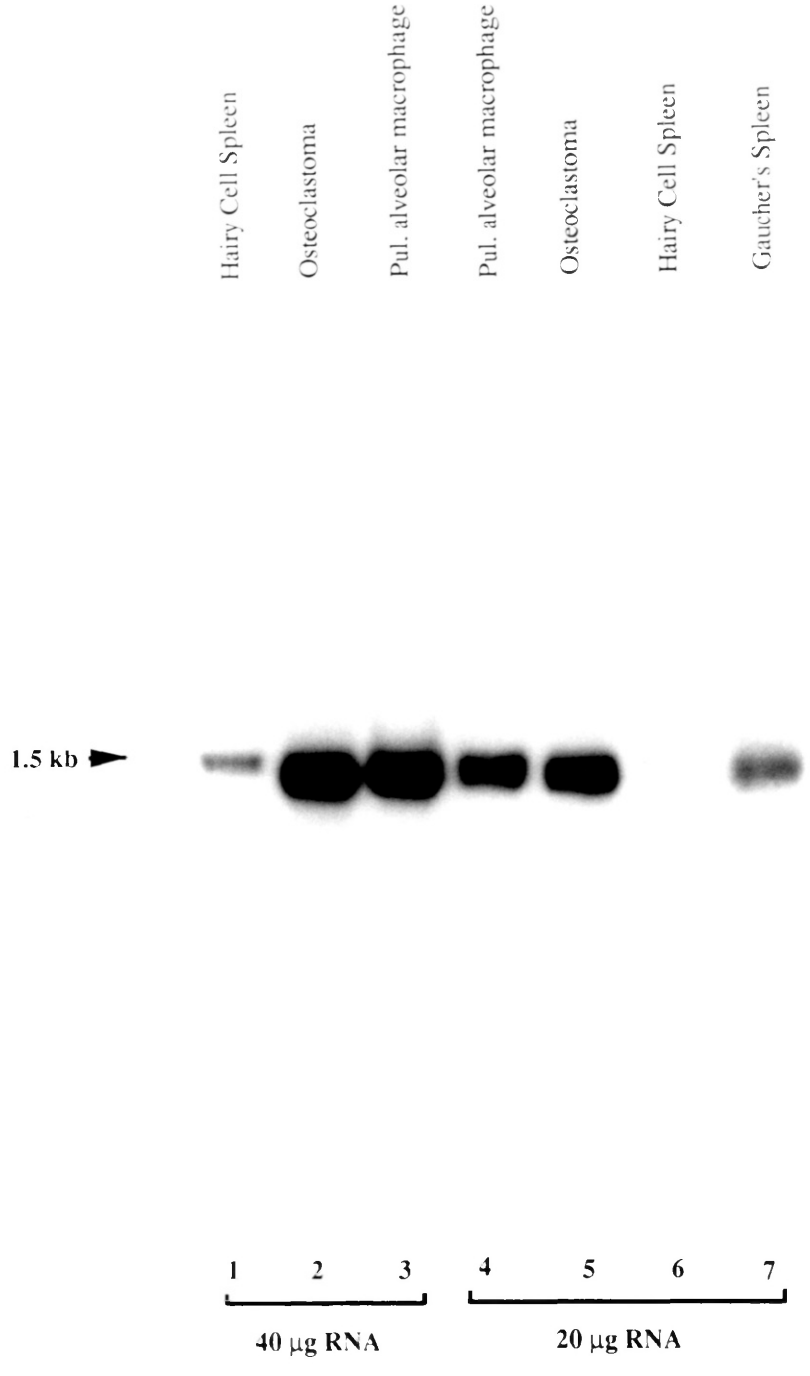


Figure 5.8. Northern blot analysis of RNA prepared from hairy cell spleen, Gaucher's spleen, osteoclastoma tissue and pulmonary alveolar macrophages using full-length acid phosphatase cDNA as a probe. A 1.5 kb mRNA for acid phosphatase is seen in each of the tissues examined.

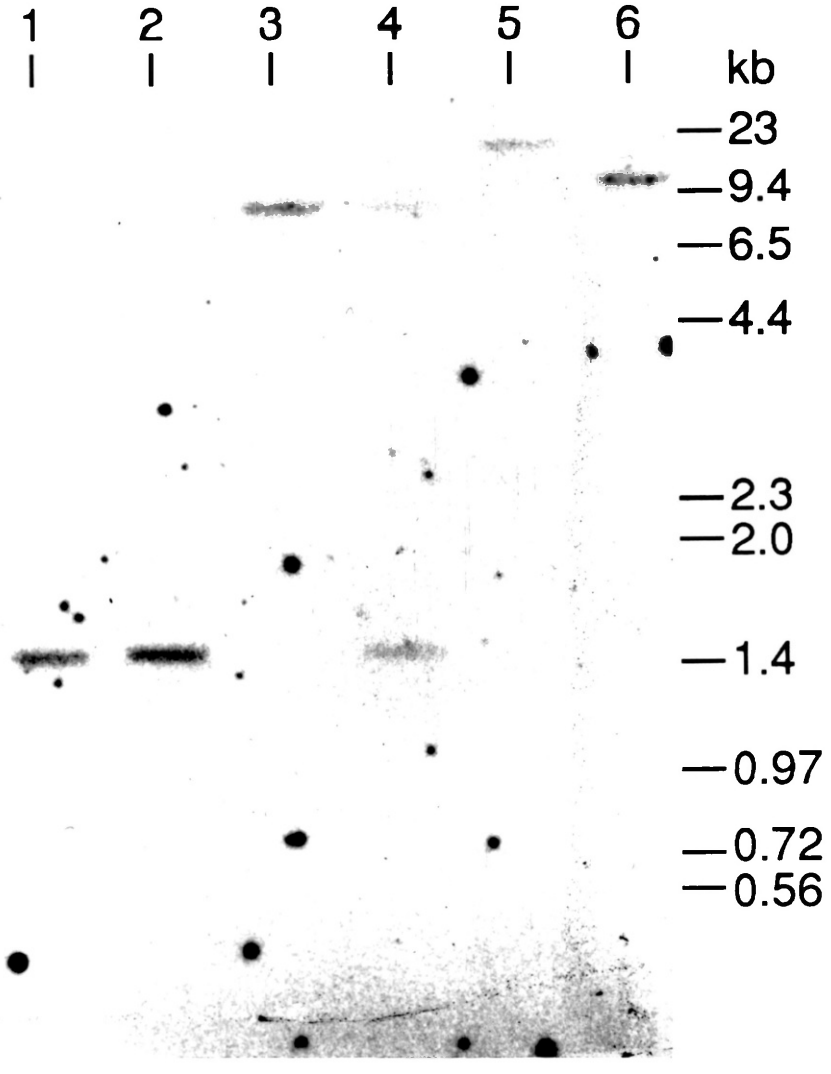


Figure 5.9. Genomic representation of type 5 acid phosphatase. Human genomic DNA was digested with *Xba* I (lanes 1-4), *Eco*R I (lane 5), *Bam*H I (lane 6) and probed with HP3. Lanes 1-4 depict two allelic restriction fragments. The DNA samples in lanes 3 and 4 were obtained from individuals that are homozygous and heterozygous, respectively, for the least frequent allele that is associated with the 8 kb band. Size markers indicate fragments obtained from a mixture of *Hin*d III-digested *lambda* DNA and pEMBL plasmid cleaved with *Taq* I.

DNA prepared from one rat (SIF15) and two mouse (DT124, STP418) somatic cell hybrids, generously provided by Dr Michael Lafan (Department of Haematology, RPMS, London) was examined (see Table 5.2 for chromosome complement of hybrids). These DNA samples, together with genomic DNA from a human control, were digested with the *Hin*d III restriction enzyme and separated on a 0.8% agarose gel. The resulting fragments were then transferred onto Hybond N membranes and the filter hybridized with the full-length acid phosphatase cDNA (HP3). A 6 kb band was detected in the control DNA but was not present in the hybrid DNA's (see Figure 5.10a). This suggests that the type 5 acid phosphatase gene is present on one of the following chromosomes: 1, 4, 5, 8, 9, 12, 13, 16, 17, 19, or 22 and does not in fact map to chromosome 6. To check the chromosome 6 complement of the hybrids, the filter was stripped and rehybridized with an MHC class I probe (see Figure 5.10b). It can be seen that SIF15 and STP418 possess chromosome 6 which is in agreement with Table 5.2. Thus, the human type 5 acid phosphatase gene was not localized to the same chromosome as the predisposing disease locus for haemochromatosis.

Further mapping of the type 5 acid phosphatase gene was carried out in collaboration with S.H. Rider, P.A. Gorman, V.A. Groves and D. Sheer at the Imperial Cancer Research Fund laboratories, Lincoln's Inn Fields, London. The somatic cell hybrids and ***in situ*** hybridization techniques are described in detail in Lord et al. (1990). The HP3 probe for the human type 5 acid phosphatase gene hybridized with a 6.7 kb *Bam*H I fragment of mouse genomic DNA. Similar signals were detected in rat (at 4.0 kb) and hamster (at 4.3kb and 3.3 kb) digests, indicating that the acid phosphatase sequences are conserved between the human and rodent genomes.

	HUMAN CHROMOSOME																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
SIF15	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	+
DT124	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	+	-	+
STP418	-	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+

Table 5.2. Chromosome complement of the rat (SIF15) and two mouse (DT124, STP418) somatic cell hybrids. (+) and (-) indicate, respectively, the presence or absence of a given chromosome.

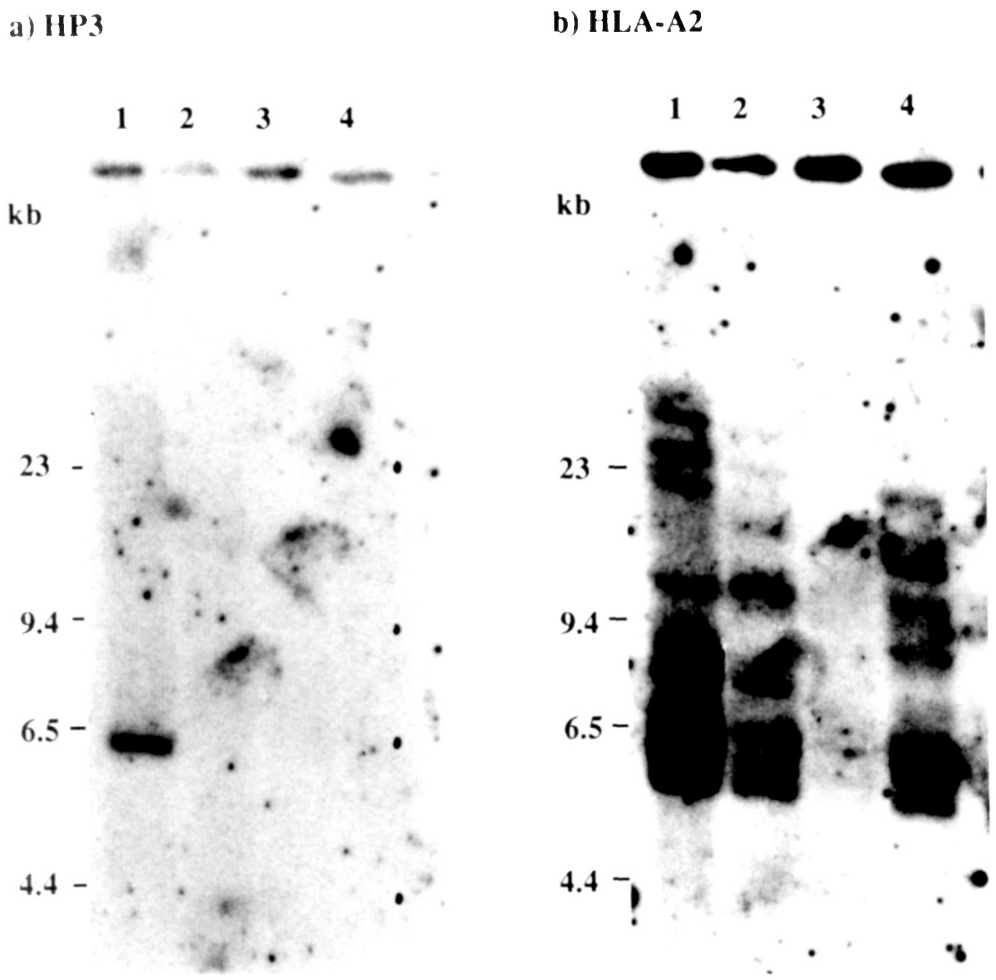


Figure 5.10. Southern blot analysis of three somatic cell hybrids using full-length acid phosphatase cDNA as a probe. a) DNA prepared from the three somatic cell hybrids (see Table 5.2) and a human control was digested with *Hin*d III and probed with HP3. A 6 kb acid phosphatase band present in human control DNA (lane 1) was not detected in STP418 (lane 2), DT124 (lane 3) or SIF15 (lane 4). b) To check the chromosome complement of the hybrids, the same filter was stripped and rehybridized with an MHC class I probe. Chromosme 6 was present in STP418 (lane 2) and SIF15 (lane 4) in agreement with Table 5.2.

DNA prepared from a panel of 42 interspecific somatic cell hybrids was examined and the segregation of the human 12 kb *Bam*H I fragment in these hybrids indicated that the type 5 acid phosphatase gene was localized on chromosome 19 with zero discordance. These data are summarized in Table 5.3. *In situ* hybridization was carried out using the probe HP3 to confirm independently and refine the localization of the type 5 acid phosphatase gene on human chromosome 19. In all, 199 silver grains were counted from 100 metaphase spreads and 24 grains were found on chromosome 19 ($X^2 = 25$; $p < 0.001$). Of these 24 grains, 20 were present on chromosome bands 19p 13.2-13.3 with a peak at 19p13.2 (Figure 5.11).

	HUMAN CHROMOSOME																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordance -/-	28	31	22	31	28	27	27	30	31	24	23	29	28	20	26	27	27	22	35	26	25	22	15
Concordance +/+	6	3	5	3	4	3	3	3	1	4	4	4	4	5	3	3	4	5	7	2	5	3	5
Discordance -/+	7	4	13	4	7	8	8	5	4	11	12	6	7	15	9	8	8	13	0	9	10	13	20
Discordance +/-	1	4	2	4	3	4	4	4	6	3	3	3	3	2	4	4	3	2	0	5	2	4	2

Table 5.3. Concordance table for mapping HP3.

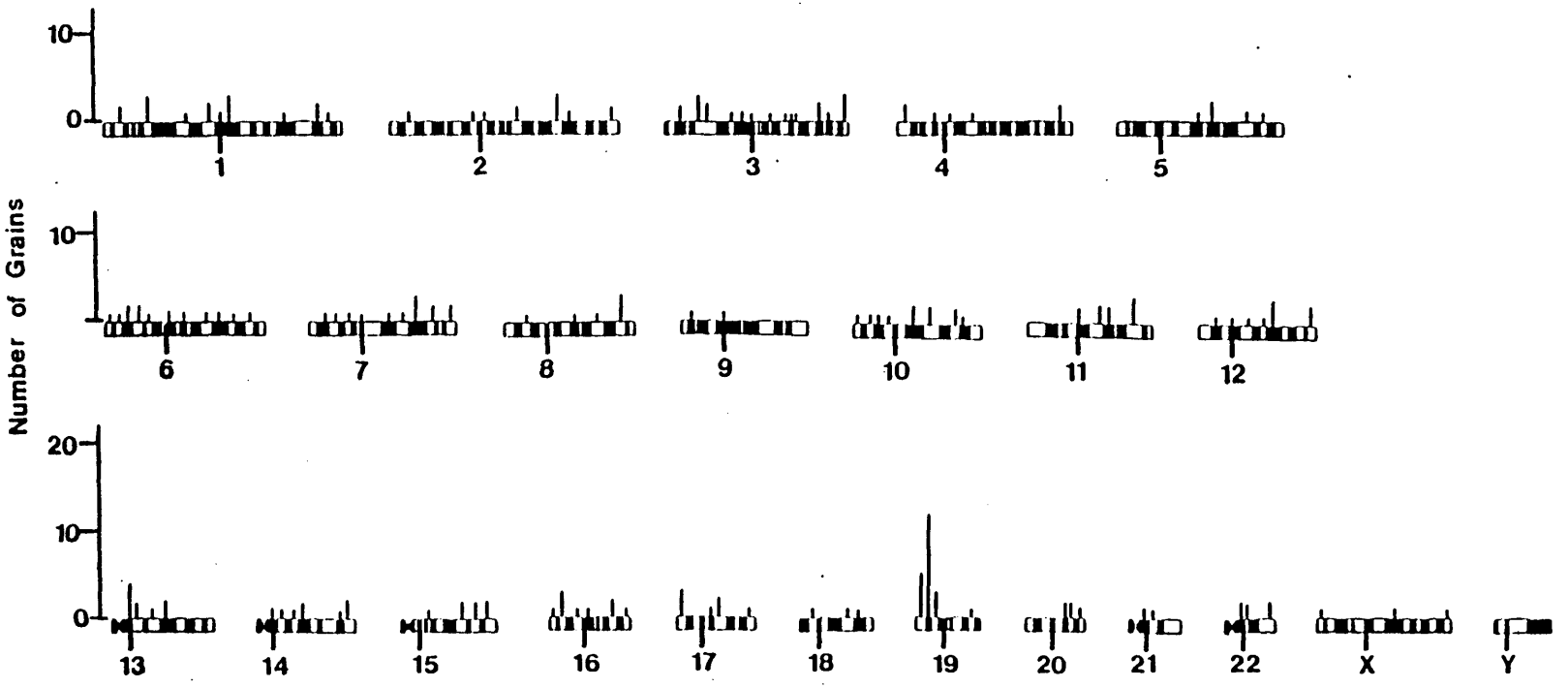


Figure 5.11. Diagrammatic representation of frequency distribution of radioautographic grains over human chromosomes in 100 metaphase spread preparations. The peak frequency is at 19p 13.2-13.3.

5.3 Discussion

5.3.1 The cloning and sequencing of human type 5 tartrate-resistant acid phosphatase

This chapter describes the successful cloning of the human type 5 acid phosphatase by a novel procedure. A specific cDNA probe was generated by the PCR when highly degenerate oligonucleotides derived from amino acid sequence were used as primers. The probe produced by this mixed oligonucleotide primed amplification of cDNA (MOPAC) (Lee et al. 1988) was then used to isolate the full-length clone from a human cDNA library. The MOPAC procedure has the advantage over direct use of degenerate oligonucleotide probes in that screening of the cDNA library can be carried out at maximum hybridization stringency. Thus MOPAC should have a general application for the cloning of genes whose amino acid sequence is known.

The close identity of the deduced amino acid sequence of the clone isolated from the human placental library to the partial amino acid sequence of bovine spleen acid phosphatase (Hunt et al. 1987) and to the complete sequence of porcine uteroferrin (Ketcham et al. 1989) suggests that it is authentic for type 5 acid phosphatase (see Figure 5.12). Overall, the amino acid sequence of the human enzyme has 85% identity to porcine uteroferrin and 82% to the corresponding regions of the partial amino acid sequence of bovine spleen acid phosphatase. These three proteins possess similar enzymatic properties but whereas the type 5 acid phosphatase is found intracellularly, localized to organelles resembling lysosomes (Lam et al. 1976; Shindelmeiser et al. 1987), uteroferrin appears to be predominantly a secreted protein. However, work carried out by Baumbach et al. (1984) suggests that uteroferrin is in fact a 'hypersecreted' lysosomal enzyme. Their studies showed that uteroferrin synthesized *in vitro* possessed mannose-6-phosphate residues which act as recognition markers by which nascent acid hydrolyases are targeted to lysosomes. However, the mannose-6-phosphate residue was masked and for this reason it appears likely that the

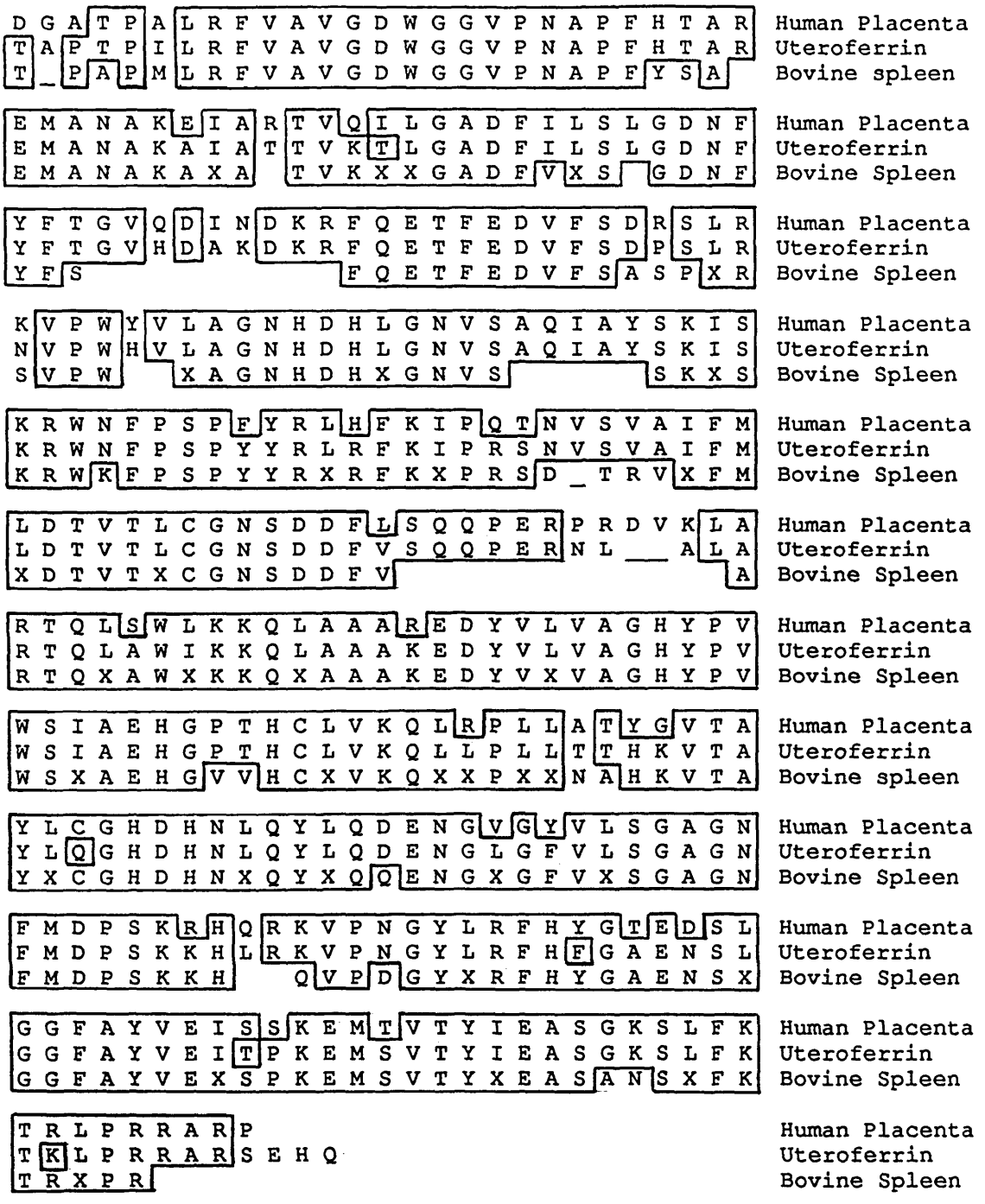


Figure 5.12. Comparison of the deduced amino acid sequence of human tartrate-resistant acid phosphatase with the complete amino acid sequence of porcine uteroferrin (Ketcham et al. 1989) and the partial amino acid sequence of bovine spleen acid phosphatase (Hunt et al. 1987). Blanks are unknown amino acid residues. (X) stands for either leucine or isoleucine. (___) indicates that adjacent residues are connected in the sequence. Sequences that are identical in two or three proteins are boxed.

glycoprotein is secreted by exocytosis rather than retained intracellularly in primary lysosomes.

Using immunocytochemical and isotopic techniques, much evidence has accumulated to indicate that a major function of porcine uteroferrin involves the transplacental transport of iron during pregnancy (Buhi et al. 1982; Ducsay et al. 1982; Renegar et al. 1982). In the pig, placentation is of the diffuse epitheliochorial type (Roberts and Bazer 1985) with several layers separating the maternal and fetal circulation, an arrangement which is believed to require an indirect transfer of iron from mother to the conceptus (Ducsay et al. 1982). Specialised placental structures known as areolae develop in juxtaposition to the openings opposite uterine glands and are believed to be involved in the uptake of secreted proteins. Uteroferrin is the major iron-containing component of porcine uterine secretions (Roberts and Bazer 1980) and can be detected in areolae as well as in the placental venous drainage (Renegar et al. 1982). After [^{59}Fe]-labelled uteroferrin is introduced into the umbilical vein, the radioactivity is rapidly incorporated into the liver, spleen and haemoglobin of the fetus (Buhi et al. 1982). Clearance of the protein seems to occur at two main sites: (1) There is extensive uptake by the macrophages that line the sinusoids of the fetal liver (Saunders et al. 1985) and this process is mediated by receptors that recognize the high-mannose carbohydrate chain of uteroferrin. (2) A second site of clearance is the fetal kidney (Renegar et al. 1982) where uteroferrin-derived peptides enter the urinary filtrate and are excreted in the allantoic sac. The uteroferrin that collects in the allantoic fluid turns over rapidly, and donates its iron to transferrin via a low molecular weight intermediary, such as ascorbate (Buhi et al. 1982). Transferrin mediates transport of the iron to the fetal circulation, where it returns to the liver, the major organ of erythropoiesis in the developing piglet *in utero*.

The striking homology between uteroferrin and human type 5 acid phosphatase described here suggests that the human enzyme may also have a rôle in iron

metabolism. Indeed, type 5 acid phosphatase and uteroferrin have a number of properties which set them apart from other acid phosphatases. First, and of special interest in relation to their proposed function, is that they are both iron-binding proteins. In addition, they have an unusual substrate specificity, possessing a high activity towards phosphoproteins, ATP and aryl phosphates but little action on aliphatic phosphates. A comparison of the predicted amino acid sequence of the human type 5 acid phosphatase with human prostatic (Vihko et al. 1988) and lysosomal acid phosphatases (Pohlmann et al. 1988) has revealed no sequence similarities. Additional computer searches have also failed to show significant sequence similarities between the type 5 acid phosphatase sequence and other lysosomal enzymes. Most lysosomal enzymes are targeted to the lysosome after acquisition of a mannose-6-phosphate residue and it has been suggested that N-acetyl-glucosamine 1-phosphate transferase - one of the enzymes involved in the generation of this residue recognizes a protein domain that is common to all lysosomal enzymes and absent from non-lysosomal glycoproteins (Reitman and Kornfeld 1981; Lang et al. 1984). As yet, a specific lysosomal targetting sequence for high mannose-6-phosphate formation has not been identified, but such a sequence may become evident when more of these proteins have been fully characterized.

Type 5 acid phosphatase, in common with other translocated proteins, appears to possess a hydrophobic amino-terminal signal peptide. Such signal sequences function to sequester the nascent protein within the endoplasmic reticulum and are usually rapidly removed after synthesis (Kornfeld 1986). The hydrophobicity plot depicted in Figure 5.13 shows that the signal sequence (amino acids 1-20) constitutes one of the most hydrophobic regions of type 5 acid phosphatase and that the remaining sequence contains no large uninterrupted region of hydrophobicity that would be expected of a membrane-spanning domain. This is in contrast to lysosomal acid phosphatase where a hydrophobicity profile revealed a hydrophobic sequence close to the carboxy-terminus of sufficient length to span the membrane (Pohlmann et al. 1988). Evidence for the

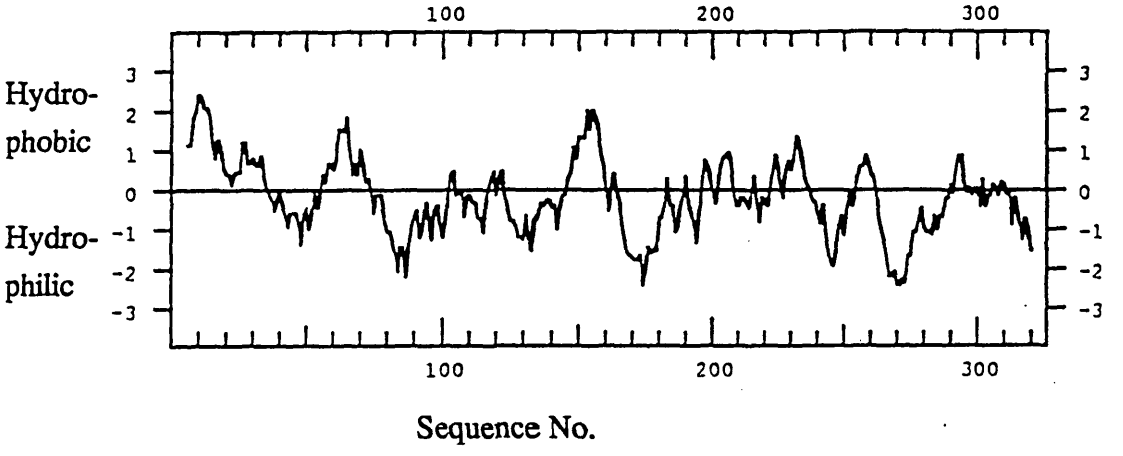


Figure 5.13. A hydrophobicity plot of type 5 acid phosphatase. The deduced amino acid sequence of type 5 acid phosphatase was analysed using a computer program developed by Kyte and Doolittle (1982). This program uses a hydropathy scale in which each amino acid has been assigned a value reflecting its relative hydrophilicity and hydrophobicity. The program continuously determines the average hydropathy of a 'window' of amino acids that is shifted in single amino acid steps through the sequence. Thus, the procedure gives a graphic visualization of the hydrophobic character of the chain from one end to the other, tracking the hydrophilic and hydrophobic regions relative to a universal midline.

association of lysosomal acid phosphatase with membranes comes from the observation that 40% of the lysosomal acid phosphatase activity in human fibroblasts (Lemansky et al. 1985) and in Cos cells transfected with lysosomal acid phosphatase cDNA (Pohlmann et al. 1988) is membrane-associated. *In vitro* studies by Waheed et al. (1988) suggests that lysosomal acid phosphatase is transported as a transmembrane protein to the lysosome where approximately two thirds is proteolytically processed to a soluble form. The mechanism by which this membrane-associated lysosomal acid phosphatase is targeted to the lysosome is believed to be independent of the mannose-6-phosphate receptor pathway of soluble lysosomal enzymes (Waheed et al. 1988, Gottschalk et al. 1989). This also appears to be the case for a number of other lysosomal membrane proteins but the exact mechanism of transfer has not as yet been established (Barriocanal et al. 1986). The existence of a mannose-6-phosphate independent pathway was first suggested by the normal activity of lysosomal acid phosphatase in I cell fibroblasts which are deficient in the enzyme generating the mannose-6-phosphate recognition marker (Gieselmann et al. 1984). However, it should be noted that multiple forms of lysosomal acid phosphatase are found in human fibroblasts and that some of these are soluble and transported to lysosomes in a mannose-6-phosphate-dependent manner. (Lemansky et al. 1985; Waheed et al. 1988). The relationship between these different forms of lysosomal acid phosphatase is unknown (Waheed et al. 1988). The absence of a hydrophobic membrane-spanning domain in the cDNA sequence of type 5 acid phosphatase suggests it is a soluble lysosomal enzyme that is directed to the lysosome in a mannose-6-phosphate receptor-dependent manner.

Previous studies have indicated that iron, which is essential for the enzymatic activity of uteroferrin and spleen acid phosphatase, is present in these proteins as a spin-coupled binuclear iron cluster (Davis and Averill 1982; Antanaitis et al. 1983a). In the enzymatically active pink form of these proteins, the iron is represented by a ferrous-ferric pair, which when analysed by low temperature EPR spectroscopy, generates an

intense EPR signal at g' = 1.74 (Antanaitis and Aisen 1983b). A similarly low g-value has been detected in other specialized iron-containing proteins, e.g., ribonucleotide reductase and hemerythrin (Que and Scarrow 1988) and its presence may be diagnostic of a unique class of metalloproteins. However it must be noted that these proteins, although characterized by a specific EPR signal, show marked divergence in their amino acid sequence that probably reflects their different functions. In contrast, the striking sequence conservation between the purple acid phosphatases and uteroferrin strongly suggests that these proteins may possess a common functional rôle.

There is evidence that histidine and tyrosine residues are involved in the binding of the two iron atoms (Antanaitis and Aisen (1983b). A search for amino acid repeats in human acid phosphatase has revealed a His/Tyr cluster at positions 86-94 and an almost exact mirror image sequence at positions 221-227 (see Figure 5.5). These may represent iron-binding domains. In ribonucleotide reductase, the active centre tyrosine at position 122 is found in conjunction with a highly conserved histidine residue at position 118 (Sjoberg et al 1985) - a configuration that is conserved between porcine uteroferrin, bovine spleen phosphatase and human type 5 acid phosphatase at positions 223 (His) and 227 (Tyr). This region appears to be the most probable candidate for the iron-binding site, but the occurrence of two similar domains in human acid phosphatase raises the possibility that four rather than two iron atoms may be bound per polypeptide, as suggested by analysis of the enzyme isolated from human osteoclasts (Hayman et al. 1989).

Since the sequence of the HP3 cDNA clone was completed, Ketcham et al. (1989) have published a sequence of type 5 acid phosphatase from human placenta. Ketcham et al. used cDNA clones from within the coding regions of uteroferrin to screen a placental *lambda* gt11 library. There are four discrepancies between the sequence reported here and that published by Ketcham and colleagues. Two residues (Lys 160 and Leu 161) were found in the PCR330 clone from human spleen and in the human

placental sequence, HP3, that are not present in Ketcham's sequence, which predicts a protein that is two amino acids shorter. However, because the extra residues were found in cDNA from two independent sources, this sequence is believed to be correct (see Figure 5.14a). In addition, Ala and Arg at positions 26 and 27 are reported here, whereas in Ketcham's sequence, these amino acids are replaced by Gly and Pro, respectively. It seems likely that the Ala and Arg amino acids given here are authentic since their nucleic acid coding sequence contains a *Sma* I site that was used in cloning (see Figure 5.14b). The explanation for these discrepancies is unclear but it is noteworthy that the latter arise in a CpG-rich region which may be subject to sequence compression.

5.3.2 Patterns of tissue expression and chromosomal localization of human type 5 acid phosphatase.

The pattern of tissue expression of specific type 5 acid phosphatase mRNA observed in this study indicates that this isoenzyme is expressed principally in cells of the human mononuclear phagocyte system. The 1.5 kb transcript was found to be abundant in pulmonary alveolar macrophages and osteoclasts from osteoclastoma tissue. Type 5 acid phosphatase RNA was also found within Gaucher's cells - histiocytes containing abnormal accumulation of glycolipid due to a deficiency of glucocerebrosidase (Robinson and Glew 1980a). Gaucher cells are believed to be the counterparts of normal tissue macrophages. Indeed, in a recent cytochemical study of peripheral blood from patients with Gaucher's disease, Troy and colleagues (1985) showed the presence of tartrate-resistant acid phosphatase in peripheral blood monocytes and it was suggested that these circulating cells may represent the precursors of Gaucher's cells in the tissues. The 'hairy cells' of hairy cell leukaemia were also found to contain type 5 acid phosphatase. These cells are believed to be of 'B' cell origin but it is notable that they also possess many surface-markers and functions that are considered to be characteristic of macrophages (Utsinger et al. 1977). The enhanced expression of acid

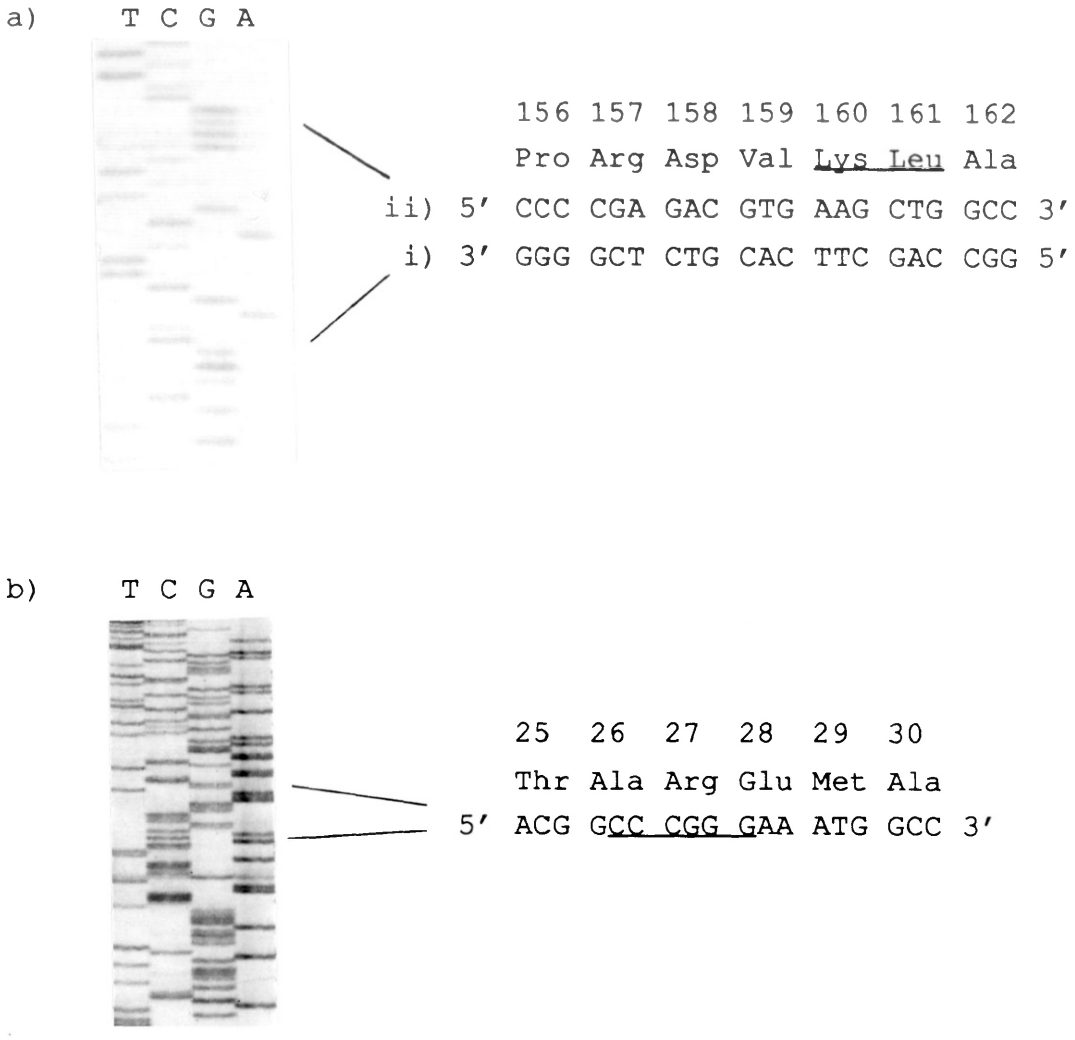


Figure 5.14. Nucleotide sequence of the two regions of HP3 that were found to be different to the sequence of type 5 acid phosphatase published by Ketcham et al. (1989). a) The region encoding two additional amino acids. The sequencing gel and corresponding nucleotide sequence is shown. (i) represents the antisense strand that was sequenced; (ii) represents the complementary sense/coding strand. The two extra amino acids Lys 160 and Leu 161 are underlined. b) The region encoding a *Sma* I restriction site. The sequencing gel and corresponding nucleotide sequence (sense strand) is shown. The *Sma* I site is underlined.

phosphatase in cultured macrophages as compared with that in the mononuclear cell preparation which contained the blood monocytes from which these cells were derived, provides additional evidence for the macrophage-specific nature of the enzyme. This increased expression correlated closely with the data obtained by specific enzymatic immunoassay for type 5 acid phosphatase in these cells.

A number of other studies have been carried out using iso-electric focusing and cytochemical methods to measure type 5 acid phosphatase qualitatively in various human tissues. Using these techniques, Radzun et al. (1983) and Snipes et al. (1986) were similarly able to demonstrate tartrate-resistant acid phosphatase in 'stimulated' monocytes and monocyte-derived macrophages respectively, but not in normal peripheral blood monocytes. Thus, type 5 acid phosphatase appears to be a differentiation marker for transformation of blood monocytes into macrophages. It is noteworthy however, that not all tissue macrophages possess type 5 acid phosphatase activity. Efstratiadis and Moss (1985a) were unable to detect the type 5 isoenzyme in peritoneal macrophages and only trace amounts were found to be present in Kupffer cells. Owing to the difficulty in obtaining pure preparations of the other normal human macrophages (other than the alveolar macrophages and osteoclasts previously discussed), further work on the tissue distribution of acid phosphatase has been limited to the study of biopsy and autopsy specimens. Using such material, Radzun et al. (1983) were unable to demonstrate the type 5 isoenzyme in a variety of tissues. However, these authors limited their investigation to the use of cytochemical staining of acid phosphatase which is now known to be less sensitive than other methods (Drexler et al. 1986a). In fact, further studies carried out using iso-electric focusing techniques have detected type 5 acid phosphatase in tonsils, lymph nodes, bone marrow, liver and spleen from healthy donors (Drexler et al. 1986a). In accordance with these results, we detected acid phosphatase RNA within the liver (albeit at low levels) but we were unable to demonstrate the transcript within the spleen and bone marrow. .

Bone marrow macrophages have been found to express high levels of acid phosphatase under certain pathological conditions (Bianco et al. 1987). It was suggested by these authors that expression of tartrate-resistant acid phosphatase in cells may be a marker of cell activation rather than cell differentiation. However, studies in our laboratory carried out by Dr. Maria Bevilaqua have shown that on treatment of monocytes with γ interferon, a well known activator of these cells, the induction of acid phosphatase activity on culture with serum is retarded. Moreover, acid phosphatase is constitutively expressed by osteoclasts.

Type 5 acid phosphatase, although a known cytochemical marker for the hairy cells of hairy cell leukaemia, is also found within a number of leukaemia cell lines and in certain T cells obtained freshly from patients with leukaemia (Drexler et al. 1986a). The type 5 isoenzyme is not expressed by very immature leukaemic cells but by cells arrested at later stages of differentiation. Moreover, certain myeloid and lymphoid leukaemia cells can be induced *in vitro* by phorbol ester to differentiate to a stage where they express the type 5 isoenzyme (Drexler et al. 1986b). Type 5 acid phosphatase activity might thus correlate with a differentiated status of normal macrophages and malignant cells. It is notable that when chronic lymphatic leukaemia (CLL) cells of B cell origin are treated with phorbol ester, an increase in acid phosphatase activity is accompanied by maturation of B-CLL cells to hairy cells showing a number of macrophage-like phenotypic characteristics (Caligaris-Cappio et al. 1985). It has been suggested that B-CLL cells and hairy cell leukaemia are related disorders and that hairy cells may be an aberrantly activated variant of B-CLL.

Expression of certain other proteins, e.g., the mannose receptor, is tightly regulated in mononuclear cells and similar to the type 5 acid phosphatase, is also stimulated by culture of peripheral blood monocytes in the presence of serum (Lennartz et al. 1989). Although the mechanism by which regulation of macrophage-specific proteins occurs is largely unknown, the use of autologous serum culture of human monocytes should

greatly facilitate study of the control processes involved. Indeed, Ernst et al. (1989) have recently shown that granulocyte and monocyte colony stimulation factor mRNA levels in activated blood monocytes is mediated primarily by stabilization of RNA. Whether the levels of type 5 acid phosphatase RNA are regulated in this manner or by transcriptional mechanisms has yet to be determined.

The physiological function of type 5 acid phosphatase within the cells of the mononuclear phagocyte is largely unknown but the sequence data presented here suggests that this enzyme may be involved in iron metabolism (see previous discussion). The accumulation of acid phosphatase in a number of pathological cells lends additional weight to this argument. Both the Gaucher's and hairy cells are capable of phagocytosing erythrocytes (Hibbs et al. 1970; Nanba et al. 1977). Robinson and Glew (1980b) explored a possible function of the Gaucher's acid phosphatase in this regard and showed the enzyme to be capable of hydrolysing phosphate from erythrocyte membrane proteins. This suggests a possible rôle for type 5 acid phosphatase in the degradation of the phagocytosed erythrocyte. The dephosphorylation of phosphoprotein by the action of a phosphatase may increase the susceptibility of these proteins to subsequent attacks by lysosomal proteases. Furthermore, the Gaucher's cell is known to accumulate large amounts of iron (Hibbs et al. 1970). It is possible that the elevation of the type 5 acid phosphatase is a reflection of abnormal iron metabolism in these cells.

The possibility also exists that the type 5 isoenzyme may serve to modulate the activity of specific phosphoproteins and enzymes through dephosphorylation. However, Robinson and Glew (1981) have shown that such phosphoproteins are generally poor substrates and type 5 acid phosphatase was unable to act on the phosphorylated enzymes of glycogen metabolism. In addition, the lack of amino acid sequence specificity of type 5 acid phosphatase with regard to the phosphopeptide substrate

indicates the enzyme probably serves a catabolic rather than a regulatory rôle as a phosphoprotein phosphatase (Robinson and Glew 1981).

Type 5 acid phosphatase RNA is also abundant within the osteoclast and is in fact a histochemical marker for this multinucleated bone resorbing cell (Minken 1982). The osteoclast has been shown to release type 5 acid phosphatase during bone resorption *in vitro* and it was suggested that this isoenzyme may serve as a biochemical marker of osteoclast function (Minken 1982). Further evidence of an association between acid phosphatase and bone resorption is suggested from the observation that its release, together with other acid hydrolases, is appropriately regulated by hormones that control bone resorption (Chambers et al. 1987). In addition, elevated plasma isoenzyme levels are associated with increased bone turnover during normal physiological growth (Chen et al. 1979) and metabolic bone disease (Stepan et al. 1983). Recent experiments by Zaidi and colleagues (1989) have shown that inhibition of type 5 acid phosphatase by an antibody to uteroferrin results in a marked reduction of bone resorption, suggesting an essential rôle of the osteoclastic enzyme in this process. Thus it appears that type 5 acid phosphatase may well have a different function in various cells of the mononuclear phagocyte system.

This chapter further describes the localization of human type 5 acid phosphatase to chromosome 19p, in collaboration with colleagues at the ICRF. Recently, human type 5 acid phosphatase has been reported to map to chromosome 15 by a newly-described *in situ* hybridization method (Allen et al. 1989). However, we were unable to confirm this localization either by investigation of an extensive panel of somatic cell hybrids (13 of which were discordant for chromosome 15) or by *in situ* hybridization studies(where no visible peak above background was observed for chromosome 15). The reason for this discrepancy is unclear, but the data reported here, using two different methods, independently map the human acid phosphatase gene to human chromosome 19 and further indicates its sub-chromosomal localization to 19p 13.2-

13.3. The identification of a frequent restriction fragment length polymorphism using the *Xba* I restriction enzyme, together with the chromosomal localization, means that this gene may prove to be useful as a marker in studies of human genetic linkage.

Two different lysosomal enzymes (DNase and α–D-mannosidase), as well as two important proteins that are expressed in macrophages (the low density lipoprotein receptor and third component of complement), also map to human chromosome 19p (McKusick (1988), and references therein). Moreover, type 5 acid phosphatase is localized to a region of chromosome 19 involved in translocations in a variety of malignancies. There have been reports of 11;19 translocations involving 19p13 in acute myeloid and lymphoid leukaemias (Kaneko et al. 1986; Hayashi et al. 1988) and the blast crisis of chronic myeloid leukaemia (Morris et al. 1988). These translocations are of particular interest as such rearrangements may give rise to the increased acid phosphatase activity seen in a number of leukaemia cells e.g., in the hairy cells of hairy cell leukaemia. A preliminary investigation into the genomic organization of the acid phosphatase gene in two patients with hairy cell leukaemia was carried out (data not shown) but no rearrangement of this locus was seen compared with normal controls. It should be noted, however, that the DNA was prepared from peripheral blood which contained only about 10 to 20% hairy cells.

HLA studies of families affected by haemochromatosis have mapped the disease gene to the short arm of chromosome 6. Although the type 5 acid phosphatase gene seemed a good candidate for the haemochromatosis gene, its localization to chromosome 19p suggests that this enzyme is unlikely to have a primary causal rôle in the aetiology of the disease. However, there is evidence to suggest that type 5 acid phosphatase may be involved in the retrieval of iron by the cells of the mononuclear phagocyte system. Therefore, the possibility still exists that this isoenzyme is involved in haemochromatosis at a secondary level. The findings reported here open the way for

further examination of the function and regulation of this enzyme in cells of the human monocyte/macrophage series and its possible rôle in human iron metabolism.

SUMMARY AND CONCLUDING REMARKS

Summary and Concluding Remarks

Summary

The gene for hereditary haemochromatosis has been localized to within 1 cM of the HLA-A locus on the short arm of chromosome 6. The work described in this thesis used two different approaches in an attempt to define further the haemochromatosis locus.

1) Pulsed-field gel electrophoresis in combination with a number of probes from the short arm of chromosome 6, was used to search for deletions or rearrangements involving the haemochromatosis locus in the DNA of three pedigrees affected by the disease and six unrelated patients.

•Individuals within each pedigree generally showed the same hybridization patterns for each probe and enzyme combination. Where heterozygosity was observed, it did not segregate with the disease phenotype.

•The size of fragments detected were generally consistent with recent physical maps of the MHC locus and suggest the absence of unmethylated CpG-rich 'HTF islands' 5' to many of the HLA class I genes.

•In the extended study of a number of unrelated patients with haemochromatosis, discrete patterns of banding were identified after hybridization to class I specific probes. These hybridization patterns varied according to HLA marker antigens detected by conventional tissue typing and particular patterns were assigned to certain HLA haplotypes. However, no particular band or pattern of bands could be related directly to the presence or absence of the disease gene.

•Apart from limited variation, the conservation of rare-cutting sites in the highly polymorphic MHC class I region was striking. A similar conservation of the long-range organization of the class II and class III regions of the MHC has been noted.

2) In an alternative approach, a candidate gene, type 5 acid phosphatase, was cloned and then tested for a primary rôle in the aetiology of haemochromatosis by determining its chromosomal localization.

•The human acid phosphatase gene was isolated from a placental cDNA library. The DNA probe used to screen the library was obtained by a novel procedure that utilized the PCR, to amplify DNA sequences using pools of mixed oligonucleotide primers that were designed on the basis of protein sequence, conserved between bovine spleen acid phosphatase and porcine uteroferrin.

•The derived amino acid sequence of human type 5 acid phosphatase showed 85% identity to that of porcine uteroferrin, a protein involved in iron metabolism. A comparison of the predicted amino acid sequence of human type 5 acid phosphatase with human prostatic and lysosomal acid phosphatases revealed no sequence similarities. These observations are consistent with human type 5 acid phosphatase and uteroferrin having a common functional rôle, different from that of the other acid phosphatases.

•A hydrophobicity profile of the human type 5 acid phosphatase sequence showed the presence of a hydrophobic amino-terminal signal peptide but did not reveal an obvious membrane-spanning domain. This is in contrast to lysosomal acid phosphatase which is known to be membrane-associated.

•A search for amino acid repeats in the human acid phosphatase sequence revealed the presence of two histidine/tyrosine clusters that may represent putative iron-binding domains.

•Northern analysis demonstrated the presence of a single RNA species of 1.5 kb within normal and pathological cells of the mononuclear phagocyte system. Further investigation showed increased levels of acid phosphatase RNA expression in cultured macrophages compared with monocytes from which these cells were derived - findings consistent with the macrophage-specific expression of this protein.

•Hybridization of the acid phosphatase cDNA to Southern blots of human genomic DNA digested with *Eco*R I and *Bam*H I restriction enzymes revealed single

fragments consistent with the presence of a single copy gene. In addition, an *Xba* I restriction fragment length polymorphism was shown to be present in two out of ten unrelated individuals and also within a separate pedigree.

•In collaboration with colleagues from the Imperial Cancer Research Fund laboratories, *in situ* hybridization and somatic cell hybrid studies were used to map the type 5 acid phosphatase gene to chromosome 19p 13.2-13.3.

Concluding Remarks

The pulsed-field gel studies suggest that the genetic event responsible for haemochromatosis is not a gross deletion or rearrangement, but that it is in fact more likely to be a relatively discrete structural mutation. The lesion could be a point mutation or even a restricted deletion or rearrangement that is beyond the current resolving power of the PFGE technique. Thus a further advance on the haemochromatosis locus based on its chromosomal location will call for techniques of greater resolution. Such techniques include chromosome walking and jumping which were used successfully in the isolation of the cystic fibrosis gene, but are very labour intensive.

The type 5 acid phosphatase gene was mapped to chromosome 19p13.2. The association of hereditary haemochromatosis with MHC class I antigens has clearly mapped the predisposing gene for this disorder to the short arm of chromosome 6. It thus seems unlikely that type 5 acid phosphatase has a primary causal rôle in hereditary haemochromatosis. However, the evidence previously discussed (see Chapter 5), suggests that this protein may function in human iron metabolism. If this is indeed the case, it is possible that acid phosphatase may be involved in the accumulation of iron seen in hereditary haemochromatosis, albeit at a secondary level. It is clear, therefore, that further work needs to be carried out to define the function of type 5 acid phosphatase. In this regard, questions need to be answered, such as the origin of its

iron and the control of gene expression in cells of the mononuclear phagocyte system. The answers to these questions may well lead to a better understanding of human iron metabolism, and may in turn shed light on the defect that leads to the development of hereditary haemochromatosis.

APPENDICES

APPENDIX 1

Recipes for Buffers and Media

1. Buffers

1. TE	10mM Tris. HCl pH8.0 1mM EDTA pH8.0
2. 1 x TBE	0.089M Tris-borate 0.089M boric acid pH ca 8.5 0.002M EDTA
3. 1M TBE stock	1M Tris-borate 1M boric acid 0.02M EDTA
4. 1 x SSC	0.15M sodium chloride 0.015M sodium citrate
5. 50 x Denhardt's	1% w/v Ficoll 1% w/v polyvinylpyrrolidone 1% w/v bovine serum albumin (fraction 5)

Dispensed into 25 ml aliquots and stored at -20°C.

6. 10 x MOPS	0.2M morpholino propane sulfonic acid 0.2M sodium acetate 0.01M EDTA pH7.0

Sterilized by autoclaving and stored at room temperature.

7. SM buffer — 10mM magnesium chloride
100mM sodium chloride
10mM Tris. HCl pH7.5
0.1% gelatine

Sterilized by autoclaving and stored in 50 ml aliquots at 4°C.

2. Media

1. L broth — 1% w/v bacto-tryptone
0.5% w/v bacto yeast extract
1% w/v sodium chloride pH7.5

+ 1.5% agarose (type II medium EEO) for plates
0.7% agarose " for top agarose

2. YPD broth — 2% w/v bacto-peptone
1% w/v bacto yeast extract
2% w/v glucose

+ 1.5% agarose (type II medium EEO) for plates

Preparation of Salmon Sperm DNA

Salmon sperm DNA was prepared as a stock solution of 10 mg/ml in water. The DNA was denatured by boiling, sonicated and then stored as 1 ml aliquots at -20°C.

Preparation of De-ionized Formamide

50 ml formamide was mixed with 5 g of mixed-bed, ion-exchange resin (Bio-Rad) and stirred for 30 minutes at room temperature. After filtering through Whatman no. 1 filter

paper, the de-ionized formamide was dispensed into 1 ml aliquots and stored at -20°C.

APPENDIX 2

Bacterial Genotypes

1. Y1090

Δ (lac U169), proA+, Δ (lon), ara D139, StrA, SupF, (trpC22 :: Tn10), (pMC9), Restriction : (r+,m+), EcoP1 (-), mcrA (-), mcrB (+).

2. JM101

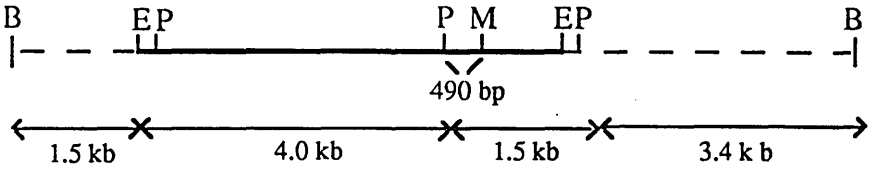
Sup E, thi, Δ (lac - proAB), (F', tra D36, pro AB, lac192ΔM15) Restriction : (rk+, mk+), mcr A+.

JM101 cells were grown on minimal media to select for the episomal proline marker. It is important to maintain the episome as it also codes for the male pilus to which the F1 phage attach before infection.

APPENDIX 3

Preparation of an HLA-A Locus-specific Probe

An HLA-A locus-specific probe was prepared by isolating a 490 bp *Pvu* II-*Msp* I fragment from the 3' untranslated region of a general class I cDNA (Trowsdale et al. 1984), as previously described by Koller et al. (1984). A restriction map was obtained for the class I cDNA clone (in pBR328), as shown below. (The cDNA is designated by a solid line and the restriction endonuclease-cleavage sites indicated are B, *Bam*H I; E, *Eco*R I; P, *Pvu* II; M, *Msp* I).



The 1.5 kb *Pvu* II fragment was subcloned into pEMBL which was then digested with restriction endonucleases *Msp* I and *Pvu* II. The DNA digest was subjected to electrophoresis in a 4% acrylamide gel. The 490 bp *Msp* I-*Pvu* II fragment was then isolated from the acrylamide gel according to Maxam and Gilbert (1980).

APPENDIX 4

Abbreviations and Units

1. Abbreviations

(d)ATP	(deoxy) Adenosine triphosphate
BSA	Bovine serum albumin
CLL	Chronic lymphatic leukaemia
(d)CTP	(deoxy) Cytidine triphosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EPR	Electron paramagnetic resonance
FCS	Foetal calf serum
FIGE	Field inversion gel electrophoresis
(d)GTP	(deoxy) Guanosine triphosphate
HLA	Human leucocyte antigen
HTF	*Hpa* II tiny fragment
ICRF	Institute of Cancer Research Fund
IPTG	Isopropyl - β - D - thiogalactopyranoside
ITP	Inosine triphosphate
MES	N - morpholino ethane sulfonic acid
MHC	Major histocompatability complex
MOPAC	Mixed oligonucleotide primed amplification of cDNA
MPS	Mononuclear phagocytic system
MRC	Medical Research Council

NIH	National Institute of Health
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
Poly A	Poly adenylic acid
PPP	Platelet-poor plasma
PRPDS	Platelet-rich, plasma-derived serum
RF	Replicative form
RFLP	Restriction fragment length polymorphism
RPMI 1640	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TEMED	N, N, N', N', tetra-methyl-ethylene-diamine
TRIS	Tris (hydroxy methyl amino methane)
d(TTP)	Thymidine triphosphate
uv	Ultraviolet

2. Units

Concentration

M molar

mM millimolar

μM micromolar

Volume

L	litre
ml	millilitre
µl	microlitre

Length

cm	centimetre
bp	base pair
kb	kilobase
mer	oligonucleotide length (b)

Weight

Da	Daltons
g	gram
mg	milligram
µg	microgram
ng	nanogram

others

OD	optical density
cpm	counts per minute
w/w	weight/weight
w/v	weight/volume
V	volts

APPENDIX 5

Publications Associated with the Work Presented in this Thesis

1. Cox, T.M., Lord, D.K. (1989) Hereditary Haemochromatosis. *Eur. J. Haematol.* **42**, 113-125.

2. Lord, D.K., Cross, N.C.P., Bevilacqua, M.A., Rider, S.H., Gorman, P.A., Groves, V.A., Moss, D.W., Sheer, D., Cox, T.M. (1990) Type 5 acid phosphatase: sequence, expression and chromosomal localization of a differentiation-associated protein of the human macrophage. *Eur. J. Biochem.* **189**, 287-293.

3. Lord, D.K., Dunham, I., Campbell, R.D., Bomford, A., Strachan, T., Cox, T.M. Molecular analysis of the human MHC class I region in hereditary haemochromatosis - a study by pulsed-field gel electrophoresis. *Hum. Genet.* (in press as at June 1990).

4. Bevilacqua, M.A., Lord, D.K., Cross, N.C.P., Whitaker, K.B., Moss, D.W., Cox, T.M. Regulation and expression of type 5 (tartrate-resistant) acid phosphatase in human mononuclear phagocytes (submitted June 1990 and under consideration)

5. Dokal, I.S., Lord, D.K., Bydder, G., Cox, T.M. Hereditary haemochromatosis: HLA analysis and magnetic resonance imaging in a unique pedigree. (in preparation as at June 1990)

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