Genetic Variation at the α- and β - Fibrinogen Loci and its Association with Plasma Fibrinogen Levels; Ethnic Differences and Environmental Interactions

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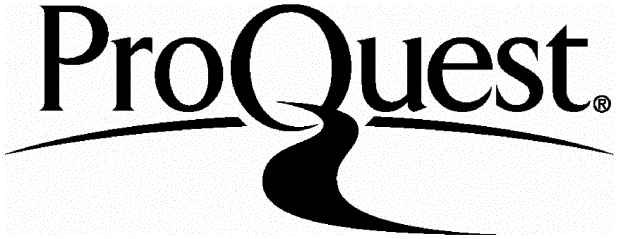

Abstract

Three previously described polymorphisms of the α- and β-fibrinogen genes detectable with the restriction enzymes TaqI, AvaII and Bcl I were mapped and appropriate regions of the gene sequenced so that detection of all three polymorphisms was possible using the polymerase chain reaction technique. The allele frequencies of these polymorphisms together with two others in the 5'-flanking region of the β-fibrinogen (detectable with the enzymes HaeIII and Hind III) were determined in five population samples in order to investigate their relationship with each other and to estimate their contribution to variance in plasma fibrinogen level.

The HaeIII and HindIII polymorphisms and the AvaII polymorphism (intron 1 of the β-fibrinogen gene) were found to be in complete linkage disequilibrium. The Bcl I polymorphism (3' end of the β-fibrinogen gene) was in strong linkage disequilibrium with these three polymorphisms, while the TaqI polymorphism (3' untranslated region of the α-fibrinogen gene) showed no such association. However, in a sample of Afrocaribbean individuals not only was the frequency of the HaeIII, HindIII and AvaII polymorphisms significantly different from that in Caucasians, but the linkage disequilibrium was not complete between the HaeIII and HindIII polymorphisms although it remained so between the HaeIII and the AvaII polymorphisms. The HaeIII and HindIII polymorphisms, in both Caucasians and Afrocaribbeans, were due to a G to A substitution and a C to T substitution at -455bp and -148bp respectively from the start of transcription of the β-fibrinogen. gene. The AvaII polymorphism was due to a T to G substitution at

+1689bp in Caucasians. The base changes resulting in the TaqI and the Bcl I polymorphisms were not determined but the TaqI polymorphism was found to lie within an Alu repeat sequence at the 3' untranslated region of the α-fibrinogen gene.

In two population samples of healthy Caucasian men, there was a significant association between the A^{-455} allele of the G/A^{-455} polymorphism and higher fibrinogen levels. An extended genotype was determined in one of the samples and this showed that in non-smokers the G/A^{-455} β-fibrinogen polymorphism and the TaqI α-fibrinogen polymorphism together explained a larger proportion of the variance in plasma fibrinogen level than any one polymorphism alone. No interaction was demonstrated between smoking and genotype in either sample. However, there was a significant interaction between age, genotype and smoking in the larger sample compatible with the hypothesis that older men who smoke and possess the A^{-455} allele, who would be predicted to have amongst some of the highest plasma fibrinogen levels, are not healthy.

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Abbreviations

ACTH	Adrenocorticotrophic hormone
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
CAD	Coronary artery disease
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
C/EBP	CAT enhancer binding protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
cpm	Counts per minute
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytosinetriphosphate
dGTP	Deoxyguanosinetriphosphate
dTTP	Deoxythymidinetriphosphate
ddNTP	DideoxyA/C/G/TTP
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
FVII	Factor VII
FVIIc	Factor VII coagulant activity
FVIII	Factor VIII

FVIIIa	Activated factor VIII
FIX	Factor IX
FIXa	Activated factor IX
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated FXI
FXII	Factor XII
FXIIa	Activated factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
HMWK	High molecular weight kininogen
HNF-1	Hepatic nuclear factor-1
IHD	Ischaemic heart disease
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-6RE	Interleukin -6 responsive element
kDa	Kilo Daltons
kb	Kilo base pairs
LDL	Low density lipoprotein
mRNA	Messenger ribonucleic acid
NF-IL6	Nuclear transcription factor-interleukin-6
OD	Optical density
PCR	Polymerase chain reaction
PDB	Phage dilution buffer

PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS page	SDS polyacrylamide gel electrophoresis
SSC	Standard saline citrate solution
SSPE	Standard saline phosphate EDTA solution
Taq	Taq polymerase thermostable DNA polymerase from thermus aquaticus
TE	Tris-EDTA
TGF-β	Transforming growth factor β
Tm	Melting temperature
TPT	Thrombosis Prevention Trial
w/v	Weight by volume

1. Introduction

Cardiovascular disease now accounts for up to 50% of the total mortality in Western countries such as the United Kingdom (Prentice, 1981; Balarajan,1991). It accounted for 49% of deaths among men between the ages of 20-69 years in England and Wales during 1979-1983 and 37% of deaths among women at these ages (Balarajan, 1991). Ischaemic heart disease forms the largest component of cardiovascular disease and during 1979-1983 caused the deaths of 73% of men and 54% of women who died from circulatory diseases between the ages of 20-69 years (Balarajan,1991). It is therefore of great relevance to identify aetiological factors in the development of cardiovascular disease so that modification of these factors by drugs or life-style can improve these mortality figures.

In the early 1950's, Morris showed that the rapid increase in ischaemic heart disease (IHD) that had occurred since the 1920's could not be explained solely in terms of atheroma (Morris, 1951; Morris and Crawford, 1958). Morris conducted a retrospective study analysing coronary artery reports on around 6000 patients aged 30-69 years who had a post mortem at the London Hospital during the period 1908-1949. He showed that despite a decrease in 'advanced' atheroma, that is lesions with calcification, ulceration, haemorrhage, stenosis or occlusion, from an age-standardized prevalence of 30.4% in the period 1908-1913 to a prevalence of 16.0% in the period 1944-49, there was a seven-fold increase in the number of ischaemic heart disease cases (Morris, 1951). He later showed that the

incidence of IHD was inversely related to the level of occupational activity (Morris et al, 1953) but from a national study, that there was no relationship between the pathological manifestations of arterial disease, as measured by the extent of atheroma, and the level of occupational activity (Morris and Crawford, 1958). From this data, some other process or processes had to be involved in the development of IHD other than atheroma, and thrombosis was a logical candidate.

1.1 Role of thrombosis in ischaemic heart disease

There has been some reluctance to study the haemostatic system and its relation to IHD in the past, and this might in part stem from the controversy as to how important thrombosis is in the clinical manifestations of IHD, namely sudden coronary death, myocardial infarction and angina. The importance placed on atherogenesis in the aetiology of IHD despite Morris's findings, and the predominance of the hypothesis that dietary fat and blood lipid levels are the most important determinants of atherogenesis may also have contributed to this reluctance. With improved imaging techniques such as angioscopy and angiography (Fuster et al,1990; Davies, verbal communication, British Society for Haematology,1994), by autopsy angiography and histology (Davies and Thomas,1984) and indirectly from the success of thrombolytic treatment (Simoons et al,1986; GISSI,1986) it is now generally accepted that thrombosis plays a crucial role in IHD. Case-comparison studies and prevalence studies can yield useful information but it can be impossible to distinguish between cause and

effect. Also, in the case of sudden coronary death, little information as to causation would be gained with these types of study. Over the past 15 years several large prospective epidemiological studies have been conducted where the measurement of interest is made before the clinical event (Wilhelmsen et al, 1984; Stone and Thorp, 1985; Meade et al, 1986; Kannel et al, 1987). These have provided powerful evidence to support the hypothesis that thrombosis is an important aetiological factor in IHD.

1.2 Epidemiological evidence for the role of fibrinogen in the pathogenesis of ischaemic heart disease

1.2.1 Epidemiological screening to define risk factors

The traditional use of epidemiology is in studying the contrasts between groups and the knowledge that these contrasts provide on the extent, distribution and causes of disease. A hypothesis for the pathogenesis of a disease can be put forward and other data such as that gained from pathological studies or intervention studies used to strengthen or refute the hypothesis. However, although a variable may be identified as being of pathogenetic significance in a population, the association is not always of practical value to the individual. This is particularly so when the variables being measured are continuous and there is no clear value at which an individual will develop the disease or not. In addition, not all those in the high risk group as defined by the variable will develop the disease

just as some who are in the low risk group will. It is therefore important to distinguish between the biological and screening uses of epidemiology. This is not to say that an individual who is found to be in a high risk group should not be advised or treated, but that population screening should not be carried out simply because of an association established in a study of pathogenesis. When epidemiological screening is carried out, the repeatability and the sensitivity and specificity of the test variable is of great importance. For some of the haemostatic variables, for instance fibrinogen and factor VII coagulant activity (FVIIc), this presents a problem since there is a wide within-individual variation as well as laboratory variation (Meade et al,1986; Thompson et al,1987; Machin and Mackie, 1993). Laboratory variation can be minimized by standardizing the methods and the operators but this still leaves within-individual variation which has been shown to be as high as 28% for fibrinogen (Thompson et al,1987) compared to 12% for cholesterol (Thompson et al,1987). This will tend to lead to an underestimate of any association in a population study as well as making the interpretation of a single measurement in an individual even more difficult.

1.2.2 Haemostasis and ischaemic heart disease

Despite these difficulties, the importance of haemostasis has been clearly demonstrated in relation to the development of IHD (Wilhelmsen et al, 1984; Stone and Thorp, 1985; Meade et al, 1986; Kannel et al, 1987). In the study by Wilhelmsen and colleagues (1984), 792 men aged 54 years were followed for

13.5 years for development of cardiovascular disease (CVD). Base-line measurement of blood pressure, serum cholesterol, smoking and plasma fibrinogen level were found to be significant risk factors for myocardial infarction by univariate analysis and blood pressure and fibrinogen were risk factors for stroke. When multivariate analysis was used, the relationship between fibrinogen and myocardial infarction was weaker and only that between fibrinogen and stroke remained significant. Interestingly, there also seemed to be an interaction between blood pressure and fibrinogen levels in association with stroke. In a study by Stone and Thorp, the incidence of IHD was examined in 297 men aged between 40 and 69 years. In this study, the relationship between fibrinogen levels and IHD was stronger than those of cholesterol, blood pressure or smoking (Stone and Thorp,1985). An interaction was also shown between fibrinogen and blood pressure and the incidence of IHD. During the tenth biennial examination of the Framingham study (Kannel et al,1987) 1315 men and women free of cardiovascular disease had fibrinogen levels measured and were followed for 12 years for evidence of CVD. For both men and women, base-line fibrinogen levels were significantly associated with IHD, and the incidence of cardiovascular events correlated positively with the age-dependent increase in plasma fibrinogen levels in both men and women up to the age of 60 years. However, although the association held for men, in women over 70 years, the cardiovascular risk showed no relation to fibrinogen. Fibrinogen level was also related to the development of stroke in men but not women. By univariate analysis, fibrinogen was comparable with the major risk factors such as blood pressure, haematocrit, obesity, cigarette

smoking and diabetes. In a multivariate analysis, fibrinogen was still significantly associated with IHD in both men and women. In the Northwick Park Heart Study (Meade et el,1986), 1511 white men between the ages of 40-64 years at the age of recruitment had measurements of plasma fibrinogen and FVIIc taken at base-line and were then followed for a minimum of 5 years. High levels of FVIIc and fibrinogen were associated with an increased risk of IHD, especially within 5 years of recruitment. Further follow-up of individuals from this study (mean 16.1 years from recruitment) shows that the association of high plasma fibrinogen with ischaemic heart disease is maintained in the long term (Meade et al,1993). These associations seemed to be stronger than those for cholesterol with elevations of one standard deviation in FVIIc, plasma fibrinogen and serum cholesterol being associated with increases in the risk of an episode of IHD within 5 years of 62%, 84% and 43% respectively. In a multiple regression model, plasma fibrinogen and FVIIc still showed a significant association with IHD but not so serum cholesterol. The risk of IHD with high fibrinogen levels or FVIIc levels decreased with age, perhaps because other, unidentified favourable characteristics were also present which abbrogated the effect of high fibrinogen levels. No interaction was shown between blood pressure and plasma fibrinogen levels as in the two other studies described (Wilhelmsen et al,1984; Stone and Thorp, 1985) but most of those diagnosed as hypertensive before recruitment (7%) had been treated, so the relationship between fibrinogen, blood pressure and CVD may have been altered.

1.3 Epidemiological characteristics of plasma fibrinogen

The general epidemiological characteristics of plasma fibrinogen concentration, also support the hypothesis that it is important in the aetiology of IHD. Plasma fibrinogen level has a positive correlation with nearly all other cardiovascular risk factors: age, hypertension, hyperlipoproteinaemia, cigarette smoking, diabetes, body mass index, oral contraceptives, blood group and social class (Meade and North, 1977; Meade et al,1979; Meade et al,1987; Lancet,1981; Balleisen et al, 1985). It is also lowered to a moderate extent by alcohol intake and exercise (Meade et al,1977; Meade et al,1979; Kozararevic et al,1980; Meade et al,1987; Carmassi et al,1992) which is negatively correlated with the incidence of IHD (Kozararevic et al,1980). Despite these associations, fibrinogen has emerged as a statistically significant independent risk factor for both CVD and IHD (reviewed by Ernst 1991). Increased fibrinogen concentration may be a common mechanism by which several risk major factors promote coronary artery disease. For instance, when both a univariate and multivariate analysis was done on the relationship between smoking, age and fibrinogen with IHD, the effect of smoking was not significant once fibrinogen levels had been taken into account (Meade, 1987). It seems likely therefore that a substantial part of the relationship between smoking and IHD is mediated through the rise in plasma fibrinogen level (Meade et al, 1987). However, comparing fibrinogen levels between white and black ethnic groups, the latter having a lower incidence of IHD, in general no difference in plasma fibrinogen level has been found (Meade et al,1977; Meade et al,1978).

The level was raised in rural Gambians who experience little or no IHD, but this is likely to be due to other factors such as parasitic infection.

For high fibrinogen to cause IHD, it is likely that there is interaction with some other characteristic(s) for instance blood pressure, which was shown in two other studies (Wilhelmsen et al, 1984; Stone and Thorp, 1985), but the precise nature of these interactions is unknown.

1.4 Pathological evidence for the role of fibrinogen in the pathogenesis of ischaemic heart disease

1.4.1 Atherosclerosis and the structure of the atherosclerotic plaque in coronary artery disease

It is now generally accepted that there are two main elements in the aetiology of ischaemic heart disease - atherogenesis and thrombogenesis, and there is evidence that coagulation factors are of importance in both these processes. Atherogenesis in its early stages can be explained as a protective mechanism associated with inflammation and repair. However, should these responses become excessive or prolonged, due to continued injury from whatever cause, then they can both destroy sufficient tissue and induce such a chronic, inflammatory and proliferative response that they cause a pathological lesion, the atherosclerotic plaque. The 'response to injury' hypothesis as a cause of

atherosclerosis has been modified over the past few years in the light of increasing observation and knowledge of the clinico-pathological consequences of the disease (Ross,1986; Ross,1993). Before this unifying hypothesis, there were two main hypotheses, not mutually exclusive, as to the pathogenesis of atherosclerosis; the 'encrustation' hypothesis and the 'lipid' hypothesis. Duguid, while examining recanalized thrombosed coronary arteries, noticed that the thrombosis extended along the vessel in the form of a thick inner lining which had organized and had the appearance of an intimal overgrowth. He concluded that mural thrombi had become incorporated into the atherosclerotic plaque (Duguid,1946). A further study on mural thrombi in the aorta confirmed his findings but he also described a 'variety of fine, fibrinous encrustations' often not visible macroscopically which he also concluded became organized and were a source of intimal thickening (Duguid,1948). In both studies he reasoned that the organized thrombus underwent fatty change producing an appearance identical to atherosclerosis. The lipid hypothesis, put forward by Virchow, stressed the importance of infiltration of blood lipid into the arterial wall. It was argued that this formed complexes with acid mucopolysaccharides and that there was an imbalance between deposition and removal which resulted in accumulation of the lipid (reviewed by Fuster et al,1992).

The earliest recognisable lesion of atherosclerosis is the 'fatty-streak' which is an aggregation of lipid-rich macrophages and T lymphocytes within the intima. Stary showed that these lesions can be found in the coronary arteries of 45% of infants

in the first 8 months of life (Stary 1989). There is then a decrease in the number of lesions but a further, steady increase from puberty, so that 65% of 12-14 year olds had lesions which included foam cells, lipid droplets in smooth muscle cells and extracellular lipid. 8% of children in this age group had preatheromatous lesions and there was a steady increase to 34% of 29 year olds who possessed these lesions (Stary, 1989). A recent report investigating atherosclerosis in youth has shown more extensive fatty streaks and raised lesions in both coronary arteries and aorta in those between 15-35 years with a prediabetic or early diabetic state as indicated by elevated glycohaemoglobin levels, and with obesity (McGill et al,1995). Injury caused to the endothelium, especially at branch points in the arterial tree (Sadoshima,1979; Stary,1989), result in a degree of dysfunction of the endothelium which leads to increased trapping of lipoprotein in the artery and the appearance of adhesive proteins on the surfaces of the endothelial cells. Monocytes and T lymphocytes attach to these proteins and migrate between the endothelial cells in response to cytokines and growth factors released by the altered endothelium, the adherent white cells and possibly underlying smooth muscle cells (Ross,1993). As the monocytes and lymphocytes reach deeper beneath the endothelial surface, the monocytes become macrophages and accumulate lipid thus becoming foam cells and, together with the lymphocytes, form the 'fatty-streak'. If injury, in its broadest sense, persists, then more advanced lesions can form and lead to the atherosclerotic plaque. Thus it can be seen that platelet-endothelial interactions are unlikely to be the initiating factors for the development of a plaque (Davies,1990; Ross,1993) but, as described below, they

have an important role in the advancement of plaques and the formation of thrombi.

The fundamental lesion of atherosclerosis is the intimal raised plaque which leads ultimately to both chronic stenosis of coronary arteries and episodic coronary thrombosis. The progression of atherosclerosis is through two main pathways, primary growth of the fatty streak and thrombosis, both major and minor. Primary growth of the fatty streak occurs in response to continued injury such as that resulting from exposure to oxidized low density lipoprotein or due to cigarette smoking or hypertension, by smooth muscle proliferation and lipid accumulation which form alternating layers and result in an intermediate lesion, the fibrofatty plaque (Ross,1993). As the lesions accumulate more cells and the macrophages scavange the lipid, some of the lipid-laden macrophages may migrate back into the blood stream by pushing apart the endothelial cells (Ross,1993). Exposure of the underlying lipid-filled macrophages, particulary at sites such as arterial branches and bifurcations where blood flow is turbulent, may produce thrombogenic areas that lead to the formation of platelet mural thrombi. These mural thrombi can then become incorporated into the plaque and lead to further growth of the lesion. In addition, such thrombi can release cytokines and growth factors. Platelet derived growth factor (PDGF) may be one of the principal growth regulatory hormones responsible for the migration of smooth muscle cells into the intima and for proliferation of existing collections of smooth muscle cells. There is evidence that one of the sources of PDGF is the macrophage (Ross et al,1990)

and thus macrophages provide not only a site for platelet interactions but also a potent chemotactic and growth-stimulatory molecule to the intimal smooth muscle cells. Smooth muscle cells form new connective tissue matrix as well as proliferate and therefore play an important role in the progression of the plaque. Major thrombosis can also occur, often in association with plaque fissuring (Fuster et al, 1990; Davies, 1990), and this can lead to an acute clinical event.

The advanced plaque is made up of a lipid core and a collagenous cap separating the lipid from the lumen of the vessel. In the plaque is tissue factor, crystalline cholesterol and fragmented collagen with variable amounts of elastin and proteoglycans (Davies,1990). The lipid core which is predominantly cholesterol, is either intracellular in foam cells, or extracellular as a pool of lipid. The margins of the lipid core also contain monocytes/macrophages concentrated at the shoulder area of the plaque. The normal cap consists of interwoven collagen strands with lacunae in which lie the smooth muscle cells, making it a strong structure. The smooth muscle cells produce and maintain the collagenous matrix which is important both for the integrity of the plaque and for that structural integrity to be maintained (Davies,1990; Davies, verbal communication,1994). The plaques can be either concentric in the artery which will produce a fixed degree of obstruction, or eccentric, where a crescent of normal vessel wall is retained allowing changes in the tone of medial smooth muscle which in turn will vary the degree of stenosis. From the study of necropsy specimens of men with stable angina, more than 50% of patients had at least one high-grade (>50% occlusion), eccentric and potentially

variable stenotic lesion (Hangartner et al,1986). Examination of high-grade stenoses shows that 48% were caused by fibrous, concentric plaques, which is higher than that found at lesser degrees of stenosis (Hangartner et al, 1986). This suggests that fibrous lesions may represent a progression of an earlier lipid-rich lesion that has undergone an episode of thrombosis and organisation (Hangartner et al,1986).

1.4.2 Relationship of the atherosclerotic plaque to thrombosis

Most advanced plaques do not cause stenosis or symptoms, but those that do, do so either by primary growth due to smooth muscle proliferation and hence collagen production and lipid accumulation or secondary to thrombosis. This latter is the major factor precipitating acute regional myocardial infarction, unstable angina and sudden coronary death. This is corroborated by autopsy angiography and histology (Davies and Thomas 1984), angiography in life and angioscopy (Fuster et al,1990) and indirectly from the success of thrombolytic treatment (GISSI,1986; Simoons,1986). There are two mechanisms of thrombosis. The first is endothelial denudation described above which results in superficial tissue injury and a thrombus which adheres to the plaque. Small thrombi can become incorporated into the plaque and thus make it bigger. This may eventually lead to obstruction. The second mechanism is by plaque fissuring or disruption with tearing of the cap. This produces deep intimal injury and exposure of collagen, lipid and smooth muscle cells which leads to the activation of platelets and the

coagulation cascade. Thrombus is formed inside the plaque which may then protrude or project into the lumen but not necessarily occlude it (Fuster et al,1990; Davies et al,1993). This is relatively more important than the superficial thrombi and can be seen at angiography. When a protruding, non-occlusive thrombus is formed, large clumps of platelets are found downstream with very little stainable fibrin. Distal embolisation of fragments can give transient ischaemia which might result in a transient ischaemic attack if from a carotid artery or chest pain if from a coronary artery. Plaque disruption may be occlusive with thrombus downstream. However, there may be a massive intraluminal thrombus with a minor plaque event, since disruption is a local stimulus for arterial thrombosis. There are various factors which influence whether an intraluminal thrombus forms: the amount of collagen and lipid exposed, the local blood flow , the systemic lytic potential and the systemic thrombotic potential. For example, angioplasty disrupts plaques and might therefore be expected to lead to increased thrombosis. However, angioplasty also leads to increased blood flow and the lytic and thrombotic potential is optimized pharmacologically. From angiographical data, 75% of the intimal lesions underlying major thrombi are secondary to plaque rupture and 25% superficial (Davies,1990). Not all plaques are unstable and plaque fissuring does not necessarily lead to occlusive thrombus, the thrombus can also be incorporated into the plaque and increase its size. Studies have shown that plaques with an increased extracellular lipid content appear more prone to rupture (Fuster et al,1990; Davies et al,1993), particularly when the lipid pool is localized eccentrically within the intima (Fuster et al,1990). In the study by Davies, 155

aortic plaques from 13 men under the age of 69 years who had died within 6 hours of the onset of symptoms of ischaemic heart disease were examined at necropsy. In 91.1% of the plaques undergoing thrombosis, lipid pools occupied more than 40% of the cross sectional area of the plaque. Only 10.9% of the intact plaques had lipid pools of this size. In addition, those undergoing thrombosis contained a smaller volume of smooth muscle cells and a larger volume of monocytes/macrophages in the plaque cap (Davies et al, 1993). It is possible that the cap is weakened by this infiltration of macrophages and is a contributory factor to plaque fissuring (Davies et al,1993). Studies on non-ischaemic sudden death patients have shown that in 69 individuals where there was no history of hypertension or diabetes mellitus (controls), 91.3% of all plaques present were stable whereas in 60 individuals with the above conditions only 78.3% of the plaques were stable. In the controls 8.7% of the plaques had fissured but none had associated thrombus. In the hypertensive or diabetic individuals 16.7% of the plaques had fissured and 5% had associated thrombus (Davies,1990). Cap tears reflect the interplay between the mechanical force exerted on the cap and its mechanical strength. Maximum stress is placed on the cap in systole and from the above studies it seems that increased lipid in the plaque makes the plaque less able to withstand that stress, possibly partly because of the increased size of the plaque. The tendency to fissuring is also related to its strength. Cap strength is controlled by the presence or absence of focal areas of collagenous change or loss and replacement of these areas by clusters of macrophages weakens cap strength. The reduction in smooth muscle cell results in decreased collagen

production and increased numbers of monocytes in the cap which are active destroyers of collagenous structure and are probably driven by an increased lipid content. If any of these causes can be modified, this could lead to reduced risk of an ischaemic cardiac event by increasing plaque stability (Davies,1990; Fuster et al,1990; Davies, verbal communication,1994).

1.5 The Role of Fibrinogen

1.5.1 Fibrinogen and platelets

The common pathway for agonists of platelet aggregation is the saturable binding of fibrinogen to the stimulated platelet (Marguerie et al,1986). Studies on patients with afibrinogenaemia or Glanzmann's thrombasthenia have shown that fibrinogen is essential for normal platelet aggregation (Nurden,1987). Patients with fibrinogen deficiency have a bleeding diathesis and investigation of their platelets shows poor aggregation which is correctable by the addition of fibrinogen. Patients with Glanzmann's thrombasthaenia have platelets which lack the GPIIb/IIIa receptor, the receptor which binds fibrinogen to the platelet, and they too have poorly aggregating platelets and a bleeding diathesis.

In vivo aggregation of platelets has been shown in patients with unstable angina or sudden cardiac death (Davies et al, 1986; Fuster and Cheseboro, 1986). Meade et al, 1985, showed that sensitivity of platelets to ADP was correlated with

plasma levels of fibrinogen. Increases in plasma fibrinogen promote platelet aggregation in whole blood under low-shear conditions (Lowe,1987a; Meade et al,1985) probably by cross-linking of platelets by fibrinogen via the GPIIb/IIIa receptor complex.

1.5.2 Fibrinogen and blood rheology

There is increasing evidence that abnormalities of variables important in the determination of whole blood viscosity are present in disease states and are of pathological significance (Lowe GDO, 1987a). Whole blood viscosity is determined by plasma viscosity, haematocrit and red cell deformability at high shear rates. Changes in blood viscosity can be compensated for in the normal circulation but not in the compromised, low-flow circulation such as occurs in stenotic vessels. In these circumstances, a systemic increase in plasma viscosity, haematocrit, red cell aggregation and the number of circulating rigid red or white cells can perpetuate low-flow states and ischaemia.

Fibrinogen is by far the most active red cell aggregating protein and is a major determinant of blood flow resistance, as well as contributing at least 22% of the plasma viscosity due to high its molecular weight and asymmetry.

Haemodynamic factors are probably important for localising atherosclerotic lesions. For instance, at bifurcations, blood flows at low velocity and low shear

rates, promoting increases in red cell aggregation and blood viscosity, especially in the presence of increased fibrinogen (Lowe,1987b) and this is where early atherosclerotic plaques have been observed (Stary, 1989). Deposition of fibrinogen, fibrin and breakdown products in developing arterial lesions has also been demonstrated (Smith et al,1990; Smith and Staples 1981: Smith and Walker, 1986) and fibrin is a major component of many atherosclerotic plaques (Smith and Staples, 1981).

Thus in coronary artery stenosis, the decreased perfusion pressure can be insufficient to overcome resistance of even blood with normal rheological characteristics and vasodilatation is limited. This decrease in pressure leads to an increase in blood viscosity and a positive feedback loop of increased blood resistance and a further reduction of blood flow is set up. In addition, high shear stresses of stenotic plaques can lead to endothelial damage and increased risk of thrombosis. Increases in plasma fibrinogen will contribute to this loop and it has been shown that the extent of the occlusive arterial disease detected at angiography is correlated with fibrinogen levels (Broadhurst et al, 1990: Lowe et al, 1980; Handa 1989).

1.5.3 Fibrinogen and the vessel wall

Woolf and coworkers demonstrated fibrin in atherosclerotic lesions but showed that there were two distinct ditribution patterns - one a diffuse pattrn with no

evidence of platelet involvement and the second localised fibrolipid plaques in a proportion of which platelet antigens were present (Woolf and Carstairs, 1967). Later Woolf and coworkers went on to show using pig aorta that platelet antigen demonstrated in fibrous plaques declined over months and therefore concluded that even if platelet antigen could not be detected it does not necessarily mean that it was not present earlier (Woolf and Carstairs, 1969). From these experiments, he concluded that fibrinogen was deposited within the vessel wall by two mechanisms: infiltration and incorporation of thrombi. Smith and Staples agree that haemostatic proteins can infiltrate the intima of arteries (Smith and Staples, 1981) and that increased levels of these proteins in the plasma will be reflected by increased levels of these substances in the intima (Smith et al, 1979). They showed, using immunoelectropheresis that prothrombin, fibrinogen and fibrin were present in varying amounts in healthy intima, gelatinous thickenings and the lipid-rich plaques of atherosclerotic lesions and in the mural thrombi. There was increased fibrinogen in the mural thrombi and gelatinous thickenings and markedly increased fibrin in mural thrombi and lipid-rich centres in association with increased prothrombin levels (Smith et al, 1981; Smith et al, 1986). The conclusions drawn were that fibrinogen was converted to fibrin within the lesions which then bound low density lipoprotein (LDL) with further sequestration of fibrinogen and clotting factors forming a self-amplifying system.

Further studies on similar lesions examining the products of fibrinolysis by SDS page and immunoblots showed that continuous formation of cross-linked fibrin and

fibrinolysis within the intima was likely and that the fragments generated may have atherogenic properties (Smith et al, 1990). Smith showed that arterial thrombi, fibrin emboli and fibrin deposits were rapidly invaded by smooth muscle cells but it was not clear whether the fibrin was acting as a scaffold or actually stimulating proliferation. Fibrinogen and fibrin degradation products have been shown to be chemotactic for leucocytes (Smith and Walker, 1986), the most significant being the monocyte/macrophages and smooth muscle cells. Fibrin degradation products have also been shown to stimulate mitosis and collagen synthesis (Thompson et al,1990).

Kadish and coworkers (1979) showed that endothelial cells in contact with fibrin become disorganized and provide a nidus for further fibrin deposition and platelet aggregation. In vitro culture of endothelium with clot showed a separation of the endothelial cells, this migratory property not being shared by epithelial cells or fibroblasts. Zwaginga and coworkers (1990) showed that endothelium from atrium was thrombogenic and contained tissue factor under unstimulated conditions. Platelet aggregation and fibrin deposition were found in vitro after perfusion with whole blood and that this could be blocked using tissue factor antibody. This therefore has important implications if the endothelial cell layer is damaged to expose deeper layers of the vessel wall.

In summary, fibrinogen is important in plaque initiation and also in plaque growth and plays a major role in enhancing arterial obstruction by thrombosis.

Thrombosis itself is brought about by both platelets and fibrinogen and is of importance in plaque growth and the progression that can be detected angiographically. It is also of vital importance in the initiation of acute clinical events.

1.6 The coagulation cascade

Haemostasis in man requires a balance between procoagulant factors and anti-cogulant factors so that the blood remains fluid, enabling it to reach all parts of the vascular tree, but able to clot to prevent leakage of blood out of the system should there be a breech in the vasculature. The coagulation cascade is a series of enzymatic reactions accelerated by co-factors which convert inactive precursors to their activated forms and culminate in the production of thrombin which in turn cleaves fibrinogen to fibrin. Clotting can occur with only a small initial stimulus because the coagulation cascade enables amplification of the system so that large amounts of thrombin are generated. The exact process of initiation of coagulation in vivo has not been fully elucidated but two potential initiators have been well studied in vitro; the contact phase which intiates the intrinsic pathway and tissue factor which intiates the extrinsic pathway.

1.6.1 The contact phase

Surface contact activation initiates the intrinsic pathway and occurs when factors XII, XI (FXI) and kallikrein and the cofactor high molecular weight kininogen (HMWK) bind to a negatively charged surface and interact to produce activated factor XI (FXIa). This factor is then released and activates factor IX (FIX). Activated factor IX (FIXa) binds to the phospholipid surface of an activated platelet in complex with factor X (FX) and activated factor VIII (FVIIIa) as cofactor. This 'tenase' complex activates FX to form activated X (FXa) which then forms part of the prothrombin activator complex also on the phospholipid surface of the platelet along with activated factor V (FVa) as cofactor and prothrombin (factor II). Thrombin is then formed which cleaves four peptides from fibrinogen, 2 fibrinopeptides A from the Aα chains of fibrinogen and 2 fibrinopeptides B from the Bß chains, to form fibrin. Fibrin is released as a monomer and spontaneously polymerizes to form a meshwork, the basis of the clot. Factor XIII is also cleaved by thrombin to its active form (FXIIIa) and stabilizes the meshwork by forming covalent cross-links between the α- and γ-chains of fibrin.

1.6.2 Tissue factor dependent pathway

Tissue factor initiates the extrinsic pathway of coagulation and is an apoprotein present in the surface membranes of many cell types but is especially abundant in vessel wall cells as well as in brain and other highly vascular tissues such as

placenta. It is fully active but normally separated from flowing blood by the endothelium. Leakage of blood into tissue allows tissue factor to promote the assembly of factor VII (FVII) and FX. There appears to be reciprocal activation of these two clotting factors and the FXa so formed then enters the prothrombinase complex as outlined above. Tissue factor also activates FIX and the FIXa so generated will enter the 'tenase' complex already described. In this way, thrombin is generated and thus fibrin from fibrinogen.

1.6.3 Platelets

Platelets are essential for effective haemostasis due to their primary role in forming a haemostatic plug, the provision of a phospholipid surface and their receptors for coagulation proteins. Platelets release pro-coagulants locally and form part of the fibrin clot. Platelet membrane receptors are glycoproteins and the glycoprotein IIb/IIIa receptor reacts chiefly with fibrinogen and fibronectin and the glycoprotein I receptor with von Willebrand factor. Binding of these proteins is essential for the adherence of platelets and release of procoagulants from the platelets.

1.7 Fibrinogen - structure

Human fibrinogen is a 340 kDa protein and is made up of two identical subunits joined together by disulphide bonds (for review see Doolittle,1994). Each subunit

consists of three polypeptide chains Aα, Bß and γ (Henschen et al,1986). The Aα chain has 610 amino acid residues, the Bß chain 461 and the γ chain 411 residues. About 10% of γ chains in plasma have an extra 20 residues at their C-terminals, due to alternative processing and polyadenylation of the γ-fibrinogen gene transcript. The three pairs of chains are grouped together at their N terminals with fibrinopeptides A and B protruding. The six chains then bifurcate into two bundles of three strands each. These are gathered together at two nodes, the disulphide rings, where the sequence of each of the three chains is Cys-Pro-X-X-Cys (Doolittle et al,1978). The intervening sequence of each chain has features strongly suggesting α-helical conformation and are almost certainly the regions of the coiled coils proposed originally by Doolittle (Doolittle et al,1978). The terminal domains are composed mainly of the C-terminal two thirds of the ß- and γ-chains. A short section of the α-chain proceeds through the terminal domain before emerging as a highly polar 'tail' which is susceptible to proteolytic attack,especially by plasmin. The three chains are similar in sequence but the individual sequences confer specific properties to each of the chains. For example fibrinopeptides A and B are released from the N-terminals of the Aα- and Bß-chains respectively after cleavage by thrombin; cross-linking of fibrin chains by factor XIIIa involves specific residues on the α and γ chains only.

1.8 Evolution of the fibrinogen gene cluster

Each of the three different polypeptide chains of human fibrinogen, Aα, Bß and γ, is encoded by a separate mRNA transcribed form three, distinct, single copy genes (Imam et al,1983; Kant and Crabtree,1983; Kant et al,1985) which lie within a 50kb segment of DNA in the distal third of the long arm of chromosome 4, bands q23 to q32 (Kant et al,1985; Olaisen et al, 1982; Humphries et al,1984). The nucloetide sequence for each of the cDNA's has been determined (Crabtree and Kant,1981; Chung et al, 1983a; Chung et al,1983b; Imam et al,1983, Kant et al, 1983; Rixon et al,1983; Rixon et al,1985) and comparison of these sequences shows marked homology with each other. Overall, the ß- and γ-gene sequences resemble each other more than either resembles the α-chain, consistent with the proposal that the duplication of the ß- and γ-genes are more recent than the α-gene as discussed below (Fu et al,1992; Crabtree et al,1985). Comparison of the cDNA with other vertebrates again shows marked homolgy. There is a high identity between human, rat and bovine fibrinogen ß-gene cDNA (Eastman and Gilula,1989; Chung et al,1981), human and rat α-gene cDNA (Crabtree et al,1985) and human and rat γ-chain cDNA (Rixon et al,1985). Moderate homology is seen between human and lamprey ß-gene cDNA (Eastman and Gilula,1989;) and lamprey γ-chain cDNA (Pan and Doolittle,1992). The genomic sequences of human and rat also show homology (Kant et al,1985; Crabtree et al,1985) particularly the first 133bp of the 3' untranslated region of the ß-fibrinogen gene (Eastman and Gilula,1989). This may indicate that, although non-coding, this

region is of functional importance. It has already been mentioned that there is alternative processing and polyadenylation of the γ-fibrinogen gene transcript. Alternative splicing and use of polyadenylation sites is also found in the rat γ-fibrinogen gene (Haidaris and Courtney,1992). A study by Mirashi and coworkers (1990) has shown that one epitope of fibrinogen concerning a fibrin polmerization site and two epitopes responsible for tPA binding to fibrin are conserved in several mammalian fibrinogens and they conclude that the sites have not been modified for at least 80 million years. Further analysis of the fibrinogen genes from both rat and human has revealed that the α- and γ-genes lie in the opposite transcriptional orientation to the ß-gene (Kant and Crabtree,1983; Kant et al,1985). This suggests that the present fibrinogen genes evolved from a common ancestral gene through a series of duplications and inversions (Kant et al,1985; Crabtree et al,1985). Since fibrinogen contains three chains in all vertebrates, including the lamprey (one of the most primitive extant vertebrates) this duplication must have occured before their development, that is at least 450 million years ago. Further analysis of amino acid sequence data shows that the estimated time is around that when plants and animals diverged, around 1 billion years ago (Doolittle,1983). However, there is evidence that the domain structure of the fibrinogen molecule and the exon/intron structures of its genes evolved independently during the later stage of evolution (Medved,1990; Crabtree et al,1985).

1.9 Regulation of fibrinogen synthesis - the acute phase response

The response to inflammation in humans occurs in three phases (for review see Fey and Fuller,1987); immediate local responses such as vasodilatation and release of lysosomal enzymes; secondary responses such as neutrophil chemotaxis and thirdly systemic reactions including pain, fever and a rise in the plasma concentration of certain proteins collectively known as the acute phase reactants. A common characteristic of these proteins is their involvement in defence mechanisms against tissue damage, infection or bleeding. Fibrinogen is one such protein. It is important that there is regulation of the response so that it gradually becomes quiescent and homeostasis is restored, otherwise significant tissue damage can occur.

Interleukin-6 (IL-6) is the major regulator of the acute phase response (Castell et al,1989) and monocytes, macrophages, fibroblasts and endothelial cells are probably the major sources of IL-6 in the inflammatory state. The liver is the major target organ in vivo, although other cell types in transformed cell lines have shown some response in vitro (Baumann et al,1986). Hepatocytes have a specific IL6 receptor on their surface which comprises two proteins, one of which binds IL6 and through interaction with the second, a trans-membrane tyrosine kinase (Kishimoto et al,1992), stimulates the phosphorylation of specific cytoplasmic proteins. This initiates a cellular cascade of events which results amongst other things in the rapid modification of a nuclear transcription factor NF-IL6, which

significantly enhances the DNA-binding ability of this protein (Akira et al,1992). NF-IL6 is a 'leucine-zipper' containing protein which has homology to the transcription factor CAT enhancer binding protein (C/EBP). The transcription of a number of liver specific genes are controlled by C/EBP binding, due to the presence of a sequence element (consensus TGTGGAAA) in the promoter region of both positive and negative acute phase genes; such an element is found in both the promoters of the negative acute phase proteins, albumin and apoAI. It appears that NF-IL6 competes for C/EBP binding in these genes and this has the effect of suppressing the transcription of negative acute phase proteins. By contrast, positive acute phase proteins have related sequence elements which are recognised only by NF-IL6 and binding results in strong transcription; such elements have been identified in the fibrinogen gene promoter amongst others. IL-6 induces a liver-specific nuclear protein to bind to the promoter region of the acute phase genes (Poli and Cortese,1989). Dalmon and coworkers (1993) have shown that in the ß-fibrinogen gene promoter, there are three subdomains in the region of the IL-6 responsive element (IL-6RE) and that all three are needed for full response to IL-6. Apart from the IL-6RE itself, the other two domains are the hepatic nuclear factor 1 (HNF1) binding site, which is both the major determinant of and essential for ß-fibrinogen gene tissue specific expression, and a second site which binds several distinct nuclear proteins from the C/EBP family and plays an important role in the constitutive expression of the gene. These three domains lie in the region -70bp to -160bp from the start of transcription (Dalmon et al,1993).

In addition to IL-6, glucocorticoids are important mediators of the acute phase response and experiments examining their effects are of interest. Baumann and coworkers (1990) showed in HepG2 cells that dexamethasone, a powerful synthetic glucocorticoid, increased the transcriptional activity of several acute phase proteins including fibrinogen and α_1-acid glycoprotein. The response for fibrinogen was specific to the HepG2 cells, whereas that for the α_1-acid glycoprotein also occured in mouse L-cells. Experiments using rat hepatocytes and the FAZA rat hepatoma cell line showed that IL-6 increased mRNA production of the fibrinogen genes resulting in a 1.5-3 fold rise in fibrinogen protein levels (Otto et al,1987). Dexamethasone alone increased the fibrinogen protein levels to the same degree but did not increase mRNA levels. The dexamethasone effect was blocked in the FAZA cells by cycloheximide showing that protein synthesis was required for maximum transcription to occur. Using IL-6 and dexamethasone together, the rise in fibrinogen was 15-20 fold, the dexamethasone enhancing the IL-6 effect on mRNA production. Using HepG2 cells, Rose-John and colleagues (1990) showed that dexamethasone induced a time- and dose-dependent upregulation of IL-6 receptor levels which caused an earlier and increased response to IL-6 as shown by increased γ-fibrinogen mRNA. They postulated that expression of the IL-6 receptor might be the rate-limiting step in the acute phase response. Mazzorana and colleagues (1991) showed that the ß-fibrinogen mRNA increased 5 fold in response to addition of glucocorticoids to cultured adult human hepatocytes. Huber and colleagues (1990) demonstrated a domain between

-2900bp and -1500bp from the start of transcription which confers dexamethasone inducibility.

The role of the cytokine transforming growth factor ß (TGFß) in the acute phase has been examined and appears to be an important modulator in inflammation and tissue repair (Mackiewicz et al,1990). In Hep3B cells and HepG2 cells, TGFß has been shown to decrease the production of fibrinogen protein and in HepB3 cells, this was shown to be due to a decrease in α-gene mRNA production (Mackiewicz et al,1990). TGFß appears to inhibit the induction of fibrinogen by IL-6 due to decreased stability of mRNA (Mackiewicz et al,1990). The effect of TGFß on fibrinogen production was confirmed by Hassan and colleagues (1992) in Hep3B cells and in addition they showed that the antagonism to IL-6 induction of fibrinogen occurs late (12-48 hours) not early (6 hours). This early induction by IL-6 and late inhibition by TGFß provides a mechanism by which to regulate fibrinogen protein production during the acute phase.

Interleukin-1 (IL-1), another cytokine, has been shown to decrease fibrinogen production in adult human hepatocytes (Castell et al,1989) and in HepG2 cells (Baumann et al,1987) but no such effect was seen in Hep3B2 cells (Darlington et al,1986). However, IL-1 has also been shown to induce the expression of IL-6 in a variety of cell types and possibly provides a mechanism for regulating the acute phase response in vivo (Ray et al,1988; Zhang et al,1988).

Both IL-1 and IL-6 have been found to stimulate a mouse pituitary tumour cell line to release adrenocorticotrophic hormone (ACTH) which in turn stimulates glucocorticoid production (Woloski et al,1985). Dexamethasone was shown to inhibit the production of IL-6 by cultured monocytes (Woloski et al,1985). These findings led Woloski and coworkers to propose that tissue injury caused monocytes to release IL-1 and IL-6 which then stimulated ACTH and thus glucocorticoid production. These factors together elicit acute phase protein production in the liver while the glucocorticoids also inhibit IL-1 and IL-6 production thus removing the stimulation to the pituitary to release ACTH and consequently, causing a reduction in glucocorticoid levels. This provides a model for the initiation, amplification and subsequent limitation of the acute phase response.

The regulation of constitutive fibrinogen expression in liver is complex; α- and ß-gene expression is mostly dependent on HNF-1 (Courtois et al,1988) while γ-gene expression is regulated by three ubiquitous nuclear proteins, Sp1, major late transcription factor and CAAT-binding factor (Morgan et al,1988). During the acute phase of inflammation, a coordinate accumulation of α, ß and γ mRNA is observed (Crabtree and Kant,1982) and this effect can be mimicked by recombinant IL-6 (Geiger et al,1988). It has been shown using pulse-chase experiments and later by transfection of the Bß chain cDNA into HepG2 cells that synthesis of the Bß chain is the rate limiting step in the formation of fibrinogen (Yu et al,1983; Yu et al,1986; Roy et al,1990) and it is therefore reasonable to assume that changes in

the rate of transcription of the ß fibrinogen gene will alter the rate of production of the protein.

1.10 Smoking and Coronary Artery Disease

Smoking contributes to coronary artery disease (CAD) in several ways, both acutely and chronically with the balance between these different effects varying between individuals (for review see Oliver 1989). Acute effects tend to be direct and include endothelial cell damage, demonstrated by a rise in von Willebrand Factor (Blann,1992), and a consequent increase in arterial wall permeability to fibrinogen (Allen et al,1988). Smoking also increases the adrenergic state which results, amongst other things, in an increased heart rate and blood pressure as well as increased plasma free fatty acids.

The chronic effects most importantly include a rise in plasma fibrinogen level but also increases in platelet aggregation, white blood cell count and haematocrit (Lowe,1987a; for review Lowe 1993). All these factors will increase viscosity which is an important factor in IHD, especially at bifurcations in blood vessels and other areas of low shear rate where early atherosclerotic plaques have been observed (Stary,1989).

It is postulated that the rise in plasma fibrinogen is due to chronic, low grade stimulation of the acute phase response. Cigarette smoke chronically stimulates

lung macrophages, causing them to produce IL-6, which in turn results in chronic stimulation of fibrinogen synthesis through binding of positive transcription factors (Ito et al,1989; Poli and Cortesel,1989) to the IL-6 responsive elements in the ß-fibrinogen gene (Huber et al,1990).

Smoking is chronically associated with a number of potentially atherogenic changes in plasma lipid levels (Richmond et al,1987), particularly the lowering of the concentration of high density lipoprotein cholesterol, a negative risk factor for CAD. Smokers also have lower levels of linoleic acid in their adipose tissue (Logan et al,1978), and low linoleic acid is strongly associated with CAD in case-control studies (for review see Oliver,1989). Linoleic acid is the principal polyunsaturated fatty acid consumed in the Western diet and it cannot be synthesized in the body. Smokers and others living in high incidence areas for CAD consume significantly lower amounts of linoleic acid, for smokers this may be because smoking affects taste, a suggestion which is supported by the observation that smokers also add more salt to their food (for review see Oliver,1989). Linoleic acid has potential anti-thombotic effects by reducing platelet aggregation as well as by reducing plasma levels of LDL.

Thus, smoking contributes to CAD because of direct cell-mediated acute effects on endothelial integrity and vascular physiology, because of its more chronic effects in promoting thrombosis and increasing blood viscosity, both through

cellular effects and through changes in transcription of genes in the liver, and because of alterations in the plasma lipid balance to a more atherogenic profile.

1.11 The significance of polymorphisms in the genetics of haemostatic problems

Genetic polymorphisms contribute to the understanding and management of haemostatic problems in varying ways. Polymorphisms in or near a gene of interest, such as the factor VIII gene in haemophilia A, are used to track an abnormal gene through a family in order to predict with greater certainty whether family members will be affected by the disease or are carriers of the particular trait. The polymorphism itself is not the cause of the disease state but is a marker to identify on which of the alleles the causal mutation lies. More recently, genetic polymorphisms have been used in population studies to see if genotype can be used to predict levels of a particular protein. The main difference in these circumstances is that the levels of the protein are within the normal range and the genetic variation detected by the polymorphism is, by definition, common (ie the frequency of the rare allele is >1%). Instead of a few people having a major defect, a large number of people have a small change in protein levels. The overall impact of this change in a population can be significant, as has been shown by the increased incidence of IHD, in those with a plasma fibrinogen level raised one standard deviation above the mean (Meade et al,1986). As has been described earlier in this chapter, such a rise in plasma fibrinogen levels resulted in an 84%

increased risk of an episode of IHD within the following five years. Genetic-environment interactions can be studied using this approach. The polymorphisms used in these associations may or may not be functional themselves, however, they help to pinpoint the functional changes. This is important for several reasons. Firstly in the understanding of normal control and function of a gene and secondly in the future because of the possibility of directed therapeutic strategies with genetic specificity. If gene-interaction is shown, advice on avoidance of certain lifestyles can be targetted more effectively.

The extent to which genetic factors are involved in determining plasma fibrinogen levels is of interest since individual and environmental factors account for only around 20-28% of the population variance in fibrinogen (Meade et al,1976; Thompson et al,1987). The level of fibrinogen observed in the plasma of an individual at any particular time is determined by interaction between a number of specific environmental factors experienced by the individual and their genetic make-up. Many environmental factors affect plasma fibrinogen levels as described previously, but of these, smoking is the single, target environmental factor determining fibrinogen levels in individuals in the general population. It has been suggested that a large part of the relationship between smoking and IHD is mediated through the rise of fibrinogen levels (Meade et al,1987). In one small study of fourteen healthy individuals, the degree of within-individual variation for fibrinogen was estimated to be at least twice that for cholesterol (Thompson et al,1987), and this is confirmed by other larger studies (Meade and North,1977);

thus a single measure of an individual's plasma fibrinogen level will result in a significant underestimate of the true relationship between fibrinogen and, for example, subsequent risk of disease.

There is less information on the relative contribution of genetic variation to the determination of plasma fibrinogen levels in the general population. One study using path analysis in families of healthy individuals and smokers estimated a heritability of 0.5 (Hamsten et al,1987) and a recent study by Bara and coworkers (1994) investigating plasma fibrinogen levels in young adults with a paternal history of premature myocardial infarction support the hypothesis that fibrinogen is a transmissible risk factor of coronary artery disease in males. Livshits and coworkers (1995) using segregation analysis found that total genetic effects explain about 80% of phenotypic variance in plasma fibrinogen levels. However, two studies in twins reported a low heritability of 0.3 (Berg and Keirulf,1989; Reed et al,1994). It is likely that variation at the gene locus coding for the fibrinogen protein may contribute to the genetic component determining plasma fibrinogen levels. Since synthesis of the Bß chain is the rate-limiting step in the production of mature fibrinogen (Yu et al,1983; Yu et al,1986; Roy et al,1990), this has prompted investigation of genetic variation in this region, especially the ß fibrinogen promoter, by exploiting DNA polymorphisms. A report by Humpries and coworkers (1987) showed an association between such variation and differences in plasma levels of fibrinogen in healthy individuals but not all studies have shown this association (Berg and Keirulf,1989; Connor et al,1992). The same association

has been shown in some but not all studies of patients with peripheral arterial disease (Monsalve et al,1988; Fowkes et al,1992). In the study by Fowkes and coworkers (1992) ß-fibrinogen genotype was an independent predictor of risk of peripheral arterial disease (Fowkes et al,1992), but this was not observed in the ECTIM study of myocardial infarct survivors and controls from Belfast and France (Scarabin et al,1993). Determination of the size of the effect of genetic variation at the fibrinogen gene locus on plasma fibrinogen levels and interaction with environmental factors are the subjects of this thesis.

Approaches

The studies in this thesis explore the relationship between genetic variation at the fibrinogen gene locus and plasma fibrinogen levels. The approach used has been to choose a candidate protein, fibrinogen, and investigate genetic polymorphisms within or near functional regions of that gene locus. Synthesis of the Bß-chain has been shown to be the rate-limiting step in the production of mature fibrinogen (Yu et al,1983; Yu et al,1986; Roy et al,1990) and therefore the polymorphisms first studied were in the promoter region of the ß-fibrinogen gene of healthy men, smokers and non-smokers. The position of other, known polymorphisms were then identified and an extended genotype determined. In this way, both gene-environment interactions and interactions between the polymorphisms themselves were studied.

Aims

The overall aim of the project is to determine the contribution of genetic variation at the fibrinogen gene locus to the differences in plasma level of fibrinogen in healthy male smokers and non-smokers and to determine whether there was any significant interaction between the polymorphisms themselves or between smoking and genetic variation.

Experiments were designed to:

i) determine the extent of association of the ß-fibrinogen gene G/A^{-455} polymorphism with plasma fibrinogen levels in different population samples of healthy male smokers and non-smokers and to look for evidence of gene-environment interaction

ii) determine the position and sequence changes of other known polymorphisms of the ß-fibrinogen gene (C/T^{-148}; T/G^{+1689}; BclI) and α-fibrinogen gene (TaqI) to enable large numbers of individuals to have their extended genotype determined and examine the relationship between the haplotypes and level of plasma fibrinogen and for gene-environment interaction

iii) examine the linkage disequilibrium between the G/A^{-455}, C/T^{-148}, T/G^{+1689}, BclI and TaqI polymorphisms in population samples from different ethnic backgrounds and the association with plasma fibrinogen levels.

iv) determine whether the sequence changes of the G/A^{-455} and C/T^{-148} polymorphisms were the same in individuals of different ethnic background.

I set up a population based study with detailed smoking history to:

i) investigate the influence of smoking and genetic variation both independently and together on plasma fibrinogen levels.

ii) determine the importance of passive smoking on plasma fibrinogen levels.

2 Materials, Methods and Subjects

2.1 Materials

Chemicals were obtained from BDH, Gibco-BRL or Sigma unless otherwise stated.

Enzymes and biochemicals were obtained from Pharmacia, Sigma, BRL, BCL, Promega or Anglian biotechnology unless otherwise stated.

Radiochemicals, Hybond-N and X-ray film were obtained from Amersham.

2.2 Methods

2.2.1 Human genomic DNA extraction from whole blood

a Triton X-100 lysis method

10ml frozen whole blood was thawed at 4^0C and the cells lysed with 80ml cold sucrose lysis solution. The nuclei were pelleted in three aliquots by centrifugation at 1000 rpm at 4^0C for 10 minutes and then each pellet resuspended in 4.5ml 0.075M NaCl/0.024M EDTA (pH 8.0) at 4^0C. 250µl 10% SDS were added with 100µl proteinase K (10mg/ml) and 150µl ddH_2O and the suspension incubated at 56^0C for 3 hours. Phenol, phenol/chloroform, chloroform (with isoamylalcohol 24:1) extraction was then performed and 500µl of 3M NaOAc (pH 5.0) added to

the supernatant and the DNA precipitated with 11ml ethanol. The DNA was dissolved in 1ml TE buffer (10mM EDTA, 25mM Tris-HCl, pH 8.0) and rotated at 4^0C until dissolved. Concentration and purity were checked by scanning spectrophometrically from 240-300nm. DNA produces a peak at optical density (OD) 256nm and the height of this peak was compared with the OD reading at 280nm, corresponding to any protein contamination. The ratio of these two peaks was calculated and was between 1.6-2.0. If the ratio was less than this, the DNA extraction steps were repeated. The DNA was aliquoted and stored at 4^0C and -20^0C.

Sucrose Lysis Solution: 0.32M sucrose

10mM Tris pH7.5

5mM Magnesium chloride

1% Triton X-100

b Rapid method for small scale extraction (Talmud et al (1991))

100µl frozen, thawed whole blood was mixed with 400µl freshly prepared 0.17M NH_4Cl and incubated at room temperature for 20 minutes. After spinning in a microfuge, the pellet was washed three times in 800µl cold 0.9% NaCl. After the final wash, the pellet was resuspended in 200µl 0.05M NaOH then boiled for 10 minutes and neutralized with 25µl 1M Tris(pH 8.0). The DNA preparation was stored at -20^0C.

2.2.2 Southern blotting

a Enzyme digestion of genomic DNA

Digestion with two restriction enzymes was usually required for Southern blotting. The first digest was carried out using 5μg genomic DNA in a 20μl total reaction volume with one tenth volume of 10x reaction buffer and one tenth volume of restriction enzyme and incubated for at least four hours. The first enzyme used was that requiring the buffer with the lowest salt concentration. One tenth volume was then run on an agarose gel to check that digestion was complete and if not, further enzyme was added with buffer and ddH_2O and the incubation continued. The second digestion reaction volume did not exceed 50μl and reaction buffer of the appropriate salt concentration and restriction enzyme each were added at one tenth the additional volume followed by incubation for a further four hours minimum. The digested DNA was then run on 1.5% agarose gels in 1xTAE buffer containing EtBr at 35 volts overnight to separate the fragments.

b Agarose gel electrophoresis

Agarose gels were made with 1-2% agarose in 1xTAE containing 1μg/ml EtBr. The solution was boiled and allowed to cool before being poured into a gel former with a comb(s) to form wells for loading DNA samples. After the gel had set, the

comb(s) was removed and the gel in the gel former was placed in a horizontal electrophoresis tank containing 1xTAE. Before loading, the samples were mixed with 10% volume 10x loading buffer and the gel then run at a suitable voltage to separate the DNA fragments. The resulting bands were then visualized using U.V. transillumination.

10x TAE buffer:	0.4M Tris
	0.01M EDTA
	pH 8.0 with glacial acetic acid
10x loading buffer:	0.25% bromophenol blue (w/v)
	25% Ficoll 400 (w/v)
	0.1M EDTA

c Endlabelling DNA oligonucleotide probes

The oligonucleotides were diluted with H_2O to give a concentration of 20ng/μl. 3μl [γ-^{32}P] ATP, 5μl 10xT_4 kinase buffer and 1μl T_4 kinase were added to 40ng of oligonucleotide probe and made up to 50μl with H_2O. The reaction mixture was incubated at 37^{0}C for 30 minutes, diluted with an equal volume of H_2O and poured into a Sephadex G25 column. When the probe had passed through, the column was spun for 3 minutes at 1000rpm to obtain the last of the probe. The

radioactivity was counted. Generally 10^6cpm were used per ml of hybridisation solution.

d Southern blotting - hybridisation

Genomic DNA was double digested as described with either the restriction enzymes BclI or AvaII and then with HindIII, SacI or BglII. Each of the double digests was run on an agarose gel as described along with samples digested with just BclI or AvaII. The gel was washed for 50 minutes in denaturing solution and then rinsed twice in tap water. The DNA was transferred from the gel to the membrane (Hybond N, Amersham) by sandwiching a piece of Hybond N between the gel, which was placed on an inverted perspex gel former covered with two layers of 3MM Whatman filter paper dipping into 20x SSC and two layers of 3MM Whatman filter paper soaked in 3x SSC placed on top, taking care to avoid air bubbles between the layers. A stack of paper towels was placed on top with a weight of approximately 0.5kg and left overnight. The position of the gel slots was marked with a black water-resistant pen. The filter was removed and washed in 3x SSC for 10 minutes, dried for 10 minutes in an oven at 80^0C and then fixed by U.V. light on a transilluminator for exactly 3 minutes. The remaining gel was viewed by U.V. light to ensure that the DNA had transferred successfully. The filter was cut to size, wetted with 3x SSC, rolled up and placed in a 50ml falcon tube with 15ml hybridization buffer. The filter was prehybridized at 65^0C for 3 hours in a rotating oven. The hybridization buffer was

then replaced with 10ml of the same buffer containing $1x10^6$cpm/ml of labelled probe. The filter was hybridized for 48 hours after which it was removed and washed for 30 minutes in 3x SSC/0.1% SDS at 65^0C and then washed in 1x SSC/0.1% SDS for 15 minutes at 65^0C. This second wash was repeated if required. The filter was monitored with a Geiger counter at each stage to prevent over- or underwashing. The filter was then mounted on card, wrapped in cling film and autoradiographed at -70^0C for 7 and 14 days.

Denaturing Solution: 0.5M NaOH

1.5M NaCl

20x SSC: 3M NaCl

0.3M sodium citrate

Hybridization buffer: 6x SSC

5x Denhardt's solution

0.5% SDS

0.002% salmon sperm

2.2.3 PCR

a Double stranded

The polymerase chain reaction (PCR) was carried out under standard conditions (Saiki et al 1988; Mullis and Faloona 1987). 200-500ng of genomic DNA were used as a template with 200ng of each primer in a 40μl reaction volume. The PCR contained 200μM each of dTTP, dGTP, dCTP and dATP, 10mM Tris-Hcl (pH8.3), 1.5mM $MgCl_2$, 50mM KCl, 10% dimethyl sulphoxide or 5% W-1 and 0.5-1.0 units BRL Taq polymerase. Samples were overlaid with paraffin oil and typically underwent one cycle of 95^0C followed by 29 cycles of 95^0C, 55^0C and 72^0C for one minute each on a Cambio Intelligent Heating Block using the plate temperature setting. Primers were obtained from The Advanced Biotechnology Centre,Charing Cross and Westminster Medical School or Severn Biotech Ltd, Kidderminster, England. Precise details are shown in table 2.1.

b Enzyme digestion of PCR product

Restriction enzyme digests were set up in 20μl volumes. 10μl of PCR product were mixed with 2μl of 10x restriction enzyme and 2μl of the appropriate buffer and incubated at the temperature appropriate for the enzyme for at least two hours. For genotyping samples from PCR the whole volume was electrophoresed on a 1.5-2% agarose gel in 1x TAE buffer containing EtBr.

c Single stranded

Standard conditions applied as in 2.2.3.a. 1μg restriction enzyme (HindIII) digested genomic DNA was used as template with 500ng of a single primer in a 50μl reaction volume. The PCR contained 200μM each of dTTP, dGTP, dCTP and dATP, 10mM Tris-Cl (pH8.3), 1.5mM $MgCl_2$, 50mM KCl, 5% W-1 and 1 unit BRL Taq polymerase. Samples were overlaid with paraffin oil and underwent one cycle of 95^0C for 5 minutes followed by a further 50 cycles of 95^0C, 55^0C and 72^0C each for one minute in a Cambio Intelligent Heating Block at plate temperature setting.Primers were obtained from The Advanced Biotechnology Centre,Charing Cross and Westminster Medical School or Severn Biotech Ltd, Kidderminster, England.

Table 2.1 Polymerase chain reaction conditions and primer sequences.

Gene	PCR purpose	Primer sequence (5'-3')	Reaction Conditions•				
			Denaturing	Annealing	Extension	Cycle No	Fragment Size (bp)
ß-fibrinogen	Detection of C/T^{-148} G/A^{-455}	AAGAATTTGGGAATGCAATCTCT GCTACCT CTCCTCATTGTCGTTGACACCTT GGGAC	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (1 min)	30	1,301
ß-fibrinogen	Detection of T/G^{+1689}	*TGGTTAATCTGGTTAAGTCTGG *GTCAGTAGCTATACATCCTTTG	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (1 min)	30	710
ß-fibrinogen	Detection of BclI polymorphism	*ACCTGGTTTCTCTGCCACAAG *AATAGTTCTCATACCACAGTGT	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (1 min)	30	2500
α-fibrinogen	Single stranded-PCR 3'end of α-fibrinogen gene	CAATATCAAGCTATCCGG	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (2 min)	50	900 (predicted)
α-fibrinogen	Sequencing 3'end of α-fibrinogen gene	CCCGGATCCCCCCCCCCC CAATATCAAGCTATCCGG	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (1 min)	29	900 (predicted)
α-fibrinogen	Detection of TaqI polymorphism	AGCCGTGCCTATCTTTG *TGTCTCAGGTACATTTAGC	95^{0}C (1 min)	55^{0}C (1 min)	72^{0}C (1 min)	40	900

• Reaction mixtures were heated initially to 95^{0}C for 5 minutes to denature DNA

* Oligonucleotides designed from results chapter 3

2.2.4 Detection of allelic phase of polymorphisms by oligomelting

a Preparation of DNA

This method was used to identify the phase of the G/A^{-455} and C/T^{-148} polymorphisms. Amplification of the ß fibrinogen promoter was performed using PCR, digested with HaeIII and run on an agarose gel. The DNA was blotted onto Hybond-N (Amersham) and then probed (as outlined in 2.2.2.d) with each of 2 radiolabelled oligonucleotides: 5'-3' coding strand AAATAAGC*TTTGCTGG and 5'-3' non-coding strand CCAGCAAAA*CTTATTTA corresponding to the C^{-148} and the T^{-148} alleles respectively.

b Oligonucleotide Hybridisation (Tm 44^0C)

The blots were prehybridised in hybridisation solution for 15 minutes at 39^0C and solution removed. Further hybridisation solution was added with 10^6cpm of probe/ml solution and rotated gently at 39^0C for 70 minutes. The blots were then washed at 42.5-43.5^{0}C for 3 minutes, the membranes dried and autoradiographed overnight.

SSPE: 3.6M Ncl

0.2M sodium phosphate pH7.7

0.02M EDTA

Hybridisation Solution:	5xSSPE
	5x Denhardt's Solution
	0.5% SDS
Washing Solution:	5x SSC
	0.1% SDS

2.2.5 Microbiological Techniques

a Lambda packaging

i Bacterial strains

Cells for growing up phage λ were LE 392(recA-ve).

ii Packaging

This procedure was used to package the Nαß clone. To prepare the host bacteria, LE 392 cells (recA-ve) were streaked out and incubated overnight at 37^0C. A single colony in 10ml L broth with 100μl 1M $MgSO_4$ and 100μl 20% maltose were shaken at 37^0C until $O.D._{600}$nm ~ 1.0. The culture was then spun for 10 minutes at

room temperature at 2000rpm (Sorvall SA-600 rotor) and the cells resuspended initially in half volume 10mM $MgSO_4$ and diluted to $O.D._{600}nm$ ~ 0.5 with 10mM $MgSO_4$ prior to storage at 4^0C. Gigapack II Gold Packaging Extract (Stratagene, California) was used according to the manufacturer's instructions to package the Nαß clone. Titrations were performed by making 1 in 10 to 1 in 10,000 dilutions with phage dilution buffer of the packaged clone. 10µl of each dilution were then added to 200µl bacteria in suspension prepared as above and incubated at 37^0C for 20 minutes. 3ml melted top agar was added to each culture and poured onto dry, pre-warmed LB plates, incubated overnight at 37^0C and plaques counted.

L Broth (per litre):
- 10g tryptone
- 5g yeast extract
- 10g NaCl

Top agar (per 100ml):
- L broth
- 0.7g agarose

LB Plates (per litre):
- 10g NaCl
- 5g yeast extract
- 10g tryptone
- 20g agar

Phage Dilution Buffer:

0.1M NaCl

10mM $MgSO_4$

0.05M Tris-HCl, Ph 7.5

0.01% gelatine

b Isolation of bacteriophage DNA

i Preparation of phage λ lysate

Phage λ plate lysate was prepared by overlying overnight culture plates of phage λ containing LE 392(recA-ve) cells with phage dilution buffer and leaving at room temperature for 72 hours. The phage dilution buffer was pooled and the plates rinsed with further PDB which was also pooled. 5% chloroform by volume was added to the lysate and spun for 5 minutes at room temperature at 2,000 rpm. (Sorvall SA-600 rotor). Chloroform 0.3% volume was added to the supernatant and stored at 4^0C.

ii PEG precipitation of phage particles

14ml of plate lysate was centrifuged at 8000g (Sorvall SA-600 rotor) for 10minutes at 4^0C. RNAse A and DNAse I were added to the supernatant to a final concentration of 1μg/ml each and incubated for 30 minutes at 37^0C. An equal

volume of ice-cold 20%PEG/2M NaCl/SM was added and incubated for 1 hour at 0^0C. After centrifugation at 10,000g for 20 minutes at 4^0C the pellet was recovered and respun for 1 minute at 5,000g to remove all traces of PEG. After resuspension of the bacteriophage particle in 0.5ml phage dilution buffer, 5µl 10% SDS, 5µl 0.5mM EDTA (pH 8.0) and proteinase K to a final concentration of 50µg/ml were added and incubated at 68^0C for 30 minutes. Standard phenol, phenol chloroform, chloroform (with isoamylalcohol 24:1) extraction was performed and an equal volume of isopropanol added to the supernatant and kept at -70^0C for 20 minutes. After thawing and centrifuging (Eppendorf microfuge), the pellet was washed in 70% ethanol, dried and resuspended in 50µl TE (pH 8.0). Concentration and purity was checked spectrophometrically at wavelengths 240-300nM as described in 2.2.1.a.

iii Amplification of DNA packaged in phage λ

Single colonies of LE 392 (recA-ve) cells were prepared. Approximately 1ng of packaged DNA (Nαß clone) in 10µl of phage dilution buffer was added to the prepared plating cells and incubated at 37^0C for 20 minutes. 3ml of melted top agar was added to each culture and poured onto dry, prewarmed LB plates and incubated overnight. Lysates were prepared (see below) and the DNA stored at 4^0C.

c Competent cells - Calcium chloride method (XL1-blue)

A single colony from fresh overnight culture of XL1-blue cells was placed into 20ml L broth with 250μg tetracycline and shaken at 37^0C until $O.D._{550}$nm ~ 0.3. The culture was then spun at 3000 rpm for 7 minutes and the cells resuspended in 25ml 50mM $CaCl_2$, chilled on ice and left for 20 minutes. The cells were respun as before, resuspended in 5ml 50mM $CaCl_2$ and left at 4^0C for 18 hours. The cells were then aliquotted, snap frozen in liquid nitrogen and stored at -70^0C.

d Subcloning

i Ligation

The vector used was pUC13 predigested with HindIII and treated with bovine alkaline phosphatase. The clone Nαß was digested with HindIII and a phenol/chloroform extraction performed on a 1μg aliquot, ethanol precipitated and resuspended in 10μl water. Ligations were set up in 10μl total volumes with molar ratios of 2:1,4:1 and 7:1 insert:vector, 1μl 10x ligase buffer and 1U T4 DNA ligase (Boehringer). The ligation reactions were incubated at 12^0C overnight and frozen after 20 hours at -20^0C. A background control was also set up containing prepared vector but no insert.

10x ligase buffer:

10mM ATP

600mM Tris.HCl pH 7.5

50mM $MgCl_2$

10mM dithiothreitol

ii Transformation

Competent E.Coli XL-1 blue cells were used. 17.4μl ß mercaptoethanol and 1ml of sterile water were cooled on ice. 10μl of this solution were placed into each of seven cooled Eppendorf tubes on ice. The competent cells were thawed from -70^{0}C to room temperature and 100μl added to each tube. The samples were left on ice for 3 minutes. 5μl of each of the ligation reactions, a background control of linearized vector and transformation controls of two concentrations of uncut pUC13 were added and left on ice for a further 40 minutes. The samples were then heat shocked at 42^{0}C for 3 minutes and placed on ice for 10 minutes. 500μl SOB broth enriched with 10mM $MgSO_4$ and 10mM $MgCl_2$ were added and incubated at 37^{0}C for 30 minutes to allow expression of the antibiotic resistance genes. The broth was then plated out onto L-agar plates (300μl/plate) with 50μg/ml ampicillin added to select for transformed cells. The plates were incubated at 37^{0}C overnight. Cells alone were plated out to check the efficacy of the ampicillin.

Single colonies were picked from appropriate plates and grown overnight in 5ml LB broth containing 250μg ampicillin. Plasmid DNA was extracted from 1.5ml of each culture using the miniprep method (see below). 5μl plasmid DNA from each colony were then digested with HindIII in a total volume of 20μl and 5μl run on an agarose gel alongside linearized pUC13 vector (2.2.2.3.e). The different banding patterns produced were compared with the predicted fragment lengths

if certain fragments of the Nαß clone had been successfully inserted into the vector. Further identification of an insert of the same length as the vector was achieved by a second digestion with XbaI. The transformed plasmids were then amplified (2.2.2.e.iv) and the inserts sequenced to enable amplification of the same region of the gene locus in genomic DNA by PCR (2.2.3).

e Isolation of recombinant plasmid DNA

i Bacterial strains

Competent cells for transformation with recombinant plasmids were from the E.Coli strain XL-1 Blue.

ii Miniprep

DNA was extracted in small quantities using the alkali lysis method (miniprep) from 1.5ml fresh overnight culture. The cells were pelleted and resuspended in 100μl TE. The bacterial cell membranes, chromosomes and proteins were removed by lysis and precipitation with 0.2M NaOH, 1% SDS followed by 3M potassium/5M acetate solution. The remaining reannealed plasmid DNA was ethanol precipitated and the DNA pellet washed with 70% ethanol, dried under a vacuum and resuspended in 20μl TE. It was then treated with 1μl of 10mg/ml DNAse free pancreatic RNAse at 37^0C for 30 minutes. Standard phenol, phenol

chloroform, chloroform (with isoamylalcohol 24:1) extraction was performed followed by ethanol precipitation before storage at -20^{0}C.

iii Enzyme digestion of plasmid DNA

Restriction enzyme digests were set up in 20-30μl volumes. 5μl of plasmid DNA were mixed with 2μl of 10x restriction enzyme and incubated at the temperature appropriate for the enzyme for 1-2 hours. For plasmid digests one tenth of the volume was electrophoresed on a 1.5-2% agarose gel in 1x TAE buffer containing EtBr to check that digestion was complete. In the event of incomplete digestion, more enzyme was added and the incubation continued. The volume of enzyme did not exceed one tenth of the total sample volume.

iv Amplification of recombinant plasmid DNA in bacteria

Transformed XL-1 blue cells were streaked out and incubated at 37^{0}C overnight. Single colonies were incubated in 5ml LB broth and 5μl ampicillin (50mg/ml) overnight. DNA was extracted using the miniprep method.

2.2.6 DNA preparation

a DNA purification using Geneclean

This procedure was used in the purification of PCR products prior to direct sequencing and prior to ligation. The geneclean kit (BIO 101 INC., California, USA) was used according to manufacturer's instructions.

b Alkali denaturation of double stranded DNA

This procedure was used when preparing DNA from recombinant plasmids for direct sequencing. 0.2mM EDTA and 0.2M NaOH were added to 20μl from DNA from appropriate minipreps and incubated at 37^0C for 30 minutes. The DNA was ethanol precipitated and washed and dissolved in 30μl H_20. The DNA was stored at -20^0C.

c G-tailing single stranded DNA

2μl or 10μl of genecleaned product from single-stranded PCR (2.2.4.b) were added to 5μl of 10mM dGTP, 10μl x5 tailing buffer and 1μl (15U) terminal deoxynucleotidyl transferase (BRL). Distilled water was added to a total volume of 50μl. The cocktail was incubated at 37^0C for 30 minutes and then placed immediately on ice.

x5 Tailing Buffer (supplied): 0.5M potassium cacodylate (pH 7.2)

10mM $CoCl_2$

1mM DTT

2.2.7 DNA sequencing

Several regions of the fibrinogen gene locus were sequenced; part of the ß fibrinogen gene promoter in Afrocaribbeans and parts of the region between the α and ß fibrinogen genes (table 2). DNA for sequencing was obtained either from recombinant plasmids or from PCR product.

a Recombinant plasmid

Sequencing was carried out using a modification of the dideoxy method (Sanger et al, 1977) after subcloning regions of DNA under investigation into the plasmid pUC 13. The Sequenase version 2.0 kit (United States Biochemical) was used which provides a modified T7 DNA polymerase for single stranded DNA synthesis. Annealing was carried out in a total volume of 10μl using the primer and template in a molar ratio of approximately 25:1. 1μl primer and 2μl 5x Sequenase buffer were added to 7μl of alkali denatured DNA (2.2.1.e) and warmed to 65^0C for 2 minutes. The cocktail was allowed to cool slowly to less than 30^0C when annealing was complete and placed on ice. The annealing reaction was used

within 4 hours. A "labelling mixture" was made for 6 reactions: 6μl dithiothreitol, 12μl labelling mix prediluted 1 in 5 with water, 3μl (10μCi/μl)[α-^{35}S] dATP and 12μl Sequenase (T7 DNA polymerase) prediluted 1 in 8 in ice-cold Sequenase buffer. 5.5 μl labelling mix was added to the annealed template-primer, mixed thoroughly and incubated for 2-5 minutes at room temperature. Once incubation was complete, 3.5μl of the labelling incubation was added to separate microtitre wells, prewarmed to 37^{0}C, containing either 2.5μl ddATP, 2.5μl ddCTP, 2.5μl ddGTP or 2.5μl ddTTP termination mixes. The plate was then spun to 1,000 rpm to mix and incubated at 37^{0}C in a water bath for 3-5 minutes. 4μl stop solution was added to each termination reaction and spun again. The plate was placed immediately on ice until ready to load sequencing gel or frozen at -20^{0}C to store. The termination reactions were heated to 75-80^{0}C for 2 minutes prior to loading onto a 4mm, 6% polyacrylamide urea gel and electrophoresed as described below. The gel was autoradiographed at room temperature for between 18 hours and 2 weeks depending on the strength of the signal, using Hyperfilm ßmax (Amersham UK).

5x Sequenase buffer:	200mM Tris.HCl pH 7.5
	100 mM $MgCl_2$
	250 mM NaCl
5x labelling mix:	7.5μM dCTP
	7.5μM dGTP
	7.5μM dTTP

dd A,C,G, or T termination mix:	80μM dATP, 80μM dCTP, 80 μM dGTP, 80μM dTTP, 50mM NaCl, 8μM ddATP/ddCTP/ddGTP or ddTTP
Enzyme dilution buffer:	10mM Tris.HCl 5mM DTT 0.5mg/ml BSA
Stop solution:	95% Formamide 20mM EDTA 0.05% Bromophenol blue 0.05% Xylene cyanol FF

b DNA from PCR product

PCR product was prepared for sequencing using Geneclean and then sequenced directly. An annealing mixture was made for 5 reactions and contained 0.75μl primer (=0.75μg), 10μl 5x Sequenase buffer, 1.5μl 10% NP-40 and 12.75μl TE (pH 7.6-8.0). 5μl of mixture was added to 2μl of each purified template (primer/template ratio >20 but <200), boiled for 3 minutes, spun briefly and then snap-cooled on dry ice. A labelling mix was then made containing 2.5μl (10μCi/μl) [α-^{35}S] dATP, 5μl 0.1M dithiothreitol, 0.75μl Sequenase (T7 DNA polymerase),

2.25μl 10% NP-40 and 12μl TE (pH 7.6-8.0) and 4μl added to each annealed primer. These were spun briefly and 2μl of each annealing reaction were added to separate microtitre plate wells containing either 2.5μl ddATP, ddCTP, ddGTP or ddTTP termination mixes to give 4 termination reactions for each primer. The plate was spun to 1,000rpm, incubated at 37^0C for 5 minutes and briefly spun again. 2μl chase solution was added to each termination reaction and incubated at 37^0#C for a further 5 minutes. After a brief spin, 4μl stop solution was added and the plate placed immediately on ice. The procedure for loading the gel described in 2.2.3.a was then followed.

5x Sequenase buffer: as 2.2.3.a

Chase Solution:
- 0.25mM dATP
- 0.25mM dCTP
- 0.25mM dGTP
- 0.25mM dTTP
- 0.5% NP-40

ddATP termination mix:
- 80μM dCTP
- 80μM dGTP
- 80μM dTTP
- 80nM ddATP
- TE (pH 7.6-8.0)

ddCTP/GTP or TTP termination mixes:	80μM dCTP
	80μM dGTP
	80μM dTTP
	80μM ddCTP/ddGTP or ddTTP
	TE (pH 7.6-8.0)
Stop solution:	as in 2.2.3.a

c Denaturing polyacrylamide gel electrophoresis for DNA sequencing

0.4mm spacers were placed between two clean plates of glass, one of which had been siliconised using Repelcote (BDH) which were then taped together to prevent leakage of the acrylamide solution. 75ml of a 6% acrylamide solution was made using 19:1 acrylamide:bisacrylamide solution, 42% urea and 1xTBE. The solution was placed in a water bath at 37^0 C to dissolve the urea after which it was polymerized with the addition of 75μl of a cross-linking agent, TEMED (NN N'N'-tetramethylethylenediamine) and 75μl of freshly made 25% ammonium persulphate solution. The solution was then poured into the space between the glass plates, ensuring no bubbles were formed, and two 0.4mm sharks tooth combs were inserted flat side down at the top of the glass plates and clipped in place to form a horizontal surface at the top of the gel. Once the gel was set, the clips and tape were removed and the gel placed in a vertical electrophoresis tank.

The combs were carefully removed and reinserted tooth side down between the glass plates so that the teeth indented the gel by a few millimetres thus forming small wells for samples. The gel was pre-run in 1xTBE for 20-30 minutes, until warm, and the wells were flushed out with 1xTBE to remove any urea that may have leached out of the gel. Samples for loading were prepared by adding 50% volume formamide dye, boiling for 3 minutes, placing immediately on ice and then loading without delay. The gel was run at a constant power of 60W (maximum current 55mA, maximum voltage 2000V) until the required separation was achieved. At the end of the gel run, the gel was transferred to 3MM filter paper (Whatman) and dried under vacuum at 80^0C (at least 90 minutes). The isotope used for the sequencing was ^{35}S and therefore autoradiography was carried out overnight at room temperature.

10x TBE: 89mM Tris-borate
2mM EDTA
pH 8.3

Formamide dye: 100ml deionized formamide
0.1% xylene cyanol
0.1% bromophenol blue
20mM EDTA

Table 2 Source of DNA and primers used in sequencing reactions

Source of DNA	L.H./Forward Primers	R.H./Reverse Primers
Nß1 clone αß intergenic PCR product	GGATCTGGGAATTCCCCG	ACAAAAATTAGCCAGGCGTGA CCGA(A/G/T)TCACATCCCCAC AGAGTGGTAGTTGCCAGG
Nαß clone subcloned 0.7kb fragment	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGAC
Nαß clone subcloned 1.0kb fragment	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGAC
Nαß clone subcloned 2.8kb fragment	GTAAAACGACGGCCAGT CTTCCTGCTTAACATTAGCTCC	CAGGAAACAGCTATGAC
ß fibrinogen promoter PCR product containing C/T^{-148} and G/A^{-455} polymorphisms	[GGG]TTTAATAGCCCCTTTTGAA [GGG]TTTAATGGCCCCTTTTGAA CCTGATTGCAACACACAAGTGA ACAGAC	CCCAGCAAAGCTTATTTACTTG TCATAC CCCAGCAAAACTTATTTACTTG TCATAC

Measurement of plasma fibrinogen concentration

Plasma fibrinogen concentrations for the Thrombosis Prevention Trial (TPT) studies were measured under the direction of Yvonne Stirling at the laboratory for the MRC Epidemiology and Medical care Unit at Northwick Park Hospital, Harrow, Middlesex. Each of the men in the studies was informed of the purpose of the study and a blood sample was taken, 4.5 ml into 0.5 ml of 3.8% sodium citrate and sent as plasma to Northwick Park Hospital and assayed for fibrinogen concentration by the Clauss method (Clauss, 1957). Thrombin reagent for the test came from Leo laboratories and reference plasma from Immuno (Vienna). Samples were diluted 1 in 10 with physiological saline and 0.2 ml added to 0.1 ml of thrombin (50u/ml) and time taken to clot ascertained. This was achieved by running samples in duplicate on a KC-10 coagulometer against a concentration curve prepared from the reference material. The coefficient of variation of the test was 4% for the period of sample testing. All assays were performed in mixed batches with the routine TPT samples.

At least three molecular forms of fibrinogen have been identified in plasma, as a high molecular weight (HMW) form, with two intact carboxyl ends of the Aα-chain and as two low molecular weight forms, one with one degraded Aα-chain carboxyl end (LMW) and one with both Aα-chain carboxyl ends degraded (LMW'). In normal plasma, 70% of fibrinogen is HMW, 26% is LMW and only 4% is LMW' (Review de Maat, 1995). Clotting rate based assays such as the Clauss method (Clauss,1957) yield a functional fibrinogen level and not an absolute concentration of fibrinogen. Alteration in the distribution of the three fibrinogen forms (HMW, LMW and LMW') in plasma may affect the Clauss assay since the LMW and LMW' forms have a prolonged thrombin clotting time. Also the presence of fibrin and fibrinogen degradation products (FDP's) and heparin in plasma will alter the clotting rate.

The subjects in the TPT studies were selected as a sample of healthy men free of symptoms of past or present ischaemic heart disease. It is therefore assumed

that the distribution of the three fibrinogen forms will conform to average and that factors such as increased FDP's or heparin will not be present in the plasma.

When assessing the contribution of environmental or genetic factors to variance in plasma fibrinogen level, it is important to minimise variation from other sources such as the laboratory. The Clauss method has been shown to be the most accurate haemostatic assay for the measurement of fibrinogen (Thompson SG et al,1989) and 4% coefficient of variation in the measurements for fibrinogen for this study was achieved. Within individual variation can account for up to 27% of sample variance in standardised assays (Thompson et al,1987) and this was more difficult to control. To standardise the timing of the samples, those used for measurement of plasma fibrinogen were taken at the subject's point of entry into the TPT study for all men. Since within individual variation and laboratory variation cannot be eliminated altogether, estimates for the contribution of genetic and environmental factors to variation in plasma fibrinogen levels will almost certainly be lower than their true contribution.

References

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2.2.8 Measurement of serum cotinine

Serum cotinine levels were performed by M sian from the Vascular Research Unit at charing Cross Hospital, London. Cotinine was extracted from serum using a modification of the method described by Feyerabend and Russell,1980 and by a gas chromatographic method, developed at Charing Cross Hospital (M Sian, personal communication), using a capillary megabore column. a capillary GC, model 3500, Varian Associates, Walton-on-Thames, UK fitted with a Thermionic Specific Detector was used and an autosampler, model 8035 (Varian Associates, UK).

2.3. Subjects

2.3.1 The TPT studies

Individuals participating in this study were selected from general practices in the Thrombosis Prevention Trial (TPT) (Meade et al 1988). Sample size was estimated based on mean differences in plasma fibrinogen levels between smokers and non-smokers already available from those previously screened for the TPT study and on the allele frequencies of restriction fragment length polymorphisms of the fibrinogen gene cluster investigated in a previous study (Humphries et al, 1987). It was estimated that to detect a difference in plasma fibrinogen levels between smokers and non-smokers with a significance level of

5% and a power of 80%, a sample of 130 cases in either smoking group was required. Up to 600 in each group would be necessary to detect a difference in frequency of the rarest genotype. The two groups studied recruited 302 and 810 participants.

Men with a history of ischaemic heart disease and those who had contraindications to warfarin or aspirin treatment were excluded from the trial. This rendered about a third of those registered with the chosen practices ineligible. The remainder were invited to be screened for TPT.

a TPT 1

In this sample, 302 individuals were selected from four general practices. The men were classified by smoking status as current smokers or non-smokers. Ex-smokers were included in the latter group as risk assessment in TPT is based on current smoking habit. A sample of current smokers was then selected at random across the distribution of plasma fibrinogen levels available from the screening and were matched for age and body mass index (BMI) with a sample group of non-smokers.

b TPT 2

The 810 individuals participating in this study were selected from a further 4 general practices. The men were classified in this study as current, ex- or never smokers. The ex-smokers and the never smokers were combined to form the not current smokers group. A passive smoker was defined as someone who i) considered they were working in a smoky atmosphere ii) was living with someone who smoked iii) had someone in their immediate workplace who smoked. A detailed smoking history was obtained at screening using a questionnaire which the participants completed unsupervised on site (appendix 7). The questionnaire included questions on duration of smoking, quantitative and qualitative data on cigarettes/cigars smoked and how many years since cessation of smoking where appropriate. Numbers of cigarettes (cigs+) included total numbers of cigarettes plus cigars plus cheroots if any smoked. If only pipe or other material was smoked, data was coded as missing; the non-smokers were coded as 0. Questions on passive smoking were also asked. Age and body mass index (BMI) were obtianed and serum cotinine levels measured in all subjects. All the current smokers were selected and were matched for age and BMI with a sample group of ex- and non-smokers.

2.3.2 Ethnic study

Individuals were recruited for a study on ethnic differences in insulin and plasma C-peptide. (Criukshank 1991). Ethnic group was determined by grandparental origin with at least three grandparents being from one ethnic group.

2.4 Statistical methods

The data were analysed using the statistical package SPSS (SPSS,1990).

2.4.1 Chapter 5

2.4.1.a TPT 1

Observed numbers of each genotype were compared with those expected if the sample were in Hardy-Weinberg equilibrium using χ^2 analysis. Allele frequencies in different groups were compared by gene counting and χ^2 analysis. Polymorphism information content (PIC) values were calculated according to the method of Botstein et al, 1980. Standard disequilibrium statistics were calculated as described by Chakravati and colleagues, 1984.

2.4.1.b Ethnic study

Allele frequencies were calculated as in 2.4.1.a and frequencies compared between ethnic groups using χ^2 analysis.

2.4.2 Chapter 7

2.4.2.a TPT 1

The distribution of fibrinogen was found to be skewed to the right. $Logarithmic_{10}$ transformation normalised the distribution and transformed values were used in the analyses. Where appropriate, data is presented as the geometric mean with 95% confidence intervals. Age, body mass index (BMI) and plasma fibrinogen level were compared between genotype groups and smokers and non-smokers using one-way analysis of variance and Student's unpaired t-test. Combined genotypes were devised by counting the number of alleles in any given genotype combination that were associated with higher fibrinogen levels. Although some associations did not reach significance in this study, the trend seen was similar to that in a previous study (Humphries et al,1987) and the designated 'fibrinogen-raising' alleles determined from this. Thus for an extended genotype involving two different polymorphisms with two alleles each, there are five categories having 0,1,2,3 or 4 'fibrinogen-raising' alleles. For the extended genotype involving all three polymorphisms there are seven possible

categories. A quadratic term was calculated for each of the genotype groups and tested with a linear term in a multiple regression analysis. There was no evidence of non-linearity in any of the genotype groups except for the combined HaeIII/TaqI in non-smokers. The genotypes were therefore entered into any regression analysis as n categories except for the HaeIII/TaqI combination when dummy variables were used. In order to evaluate the relative contribution to the variance in plasma fibrinogen level of the genotypes and their combinations, multiple regression analysis was used, first adjusting for age and BMI which are known to affect plasma fibrinogen level and then entering the various genotypes or their combinations to see if any further significant effect was seen. the analysis was performed separately in smokers and non-smokers. Interaction terms were calculated between smoking and age, smoking and BMI and smoking and genotype and entered into the multiple regression model.

2.4.2.b Ethnic study

Fibrinogen levels, adjusted for age, BMI and sex, were compared between different genotype groups in Afrocaribbeans using one way analysis of variance and Student's unpaired t-test.

2.4.2.c TPT 2

Allele frequencies were calculated and compared as in 2.4.2.a. Fibrinogen was normally distributed and was not therefore $\log_{10}$ transformed. Plasma cotinine levels were $\log_{10}$ transformed to normalise the data. Variables were compared using one-way analysis of variance and Student's unpaired t-test as in 2.4.2.a. The correlation coefficients between fibrinogen, smoking and cotinine level were calculated using the Pearson product moment coefficient of correlation. Interaction between allele frequency, age and smoking was assessed using multiple logistic regression and was carried out by Dr Rob Elton, Department of Medical Statistics, Edinburgh University, Edinburgh. Where appropriate, data is presented as the geometric mean with 95% confidence intervals.

3. Characterisation of the AvaII, BclI and TaqI polymorphisms of the ß-fibrinogen gene: results

A study by Humphries et al, 1987, showed that genetic variation at the fibrinogen gene locus is implicated in determining an individual's basal fibrinogen level. In that study, three polymorphisms were investigated: the first detectable with a ß-fibrinogen cDNA probe after digestion of genomic DNA with the restriction enzyme AvaII, the second detectable using a ß-fibrinogen cDNA probe after digestion of genomic DNA with the restriction enzyme BclI and the third detectable with an α-fibrinogen cDNA probe after digestion of genomic DNA with the restriction enzyme TaqI. Since these polymorphic sites had not been mapped or sequenced and therefore were detectable only by Southern blotting, conducting studies on large numbers of individuals would be very time consuming and technically more difficult than using PCR analysis. In addition, sequencing of these polymorphisms to identify the base changes involved might provide information as to the possible mechanisms contributing to the determination of an individual's fibrinogen level.

In order to study these polymorphisms more easily in different population samples, the polymorphisms of the ß-fibrinogen gene, detected with the restriction enzymes AvaII and BclI, were mapped by Southern blotting. By studying the known sequence (AvaII) or by sequencing the appropriate region of the fibrinogen gene cluster (BclI) oligonucleotide primers suitable for amplification of regions

containing the polymorphisms using the polymerase chain reaction (PCR) were identified.

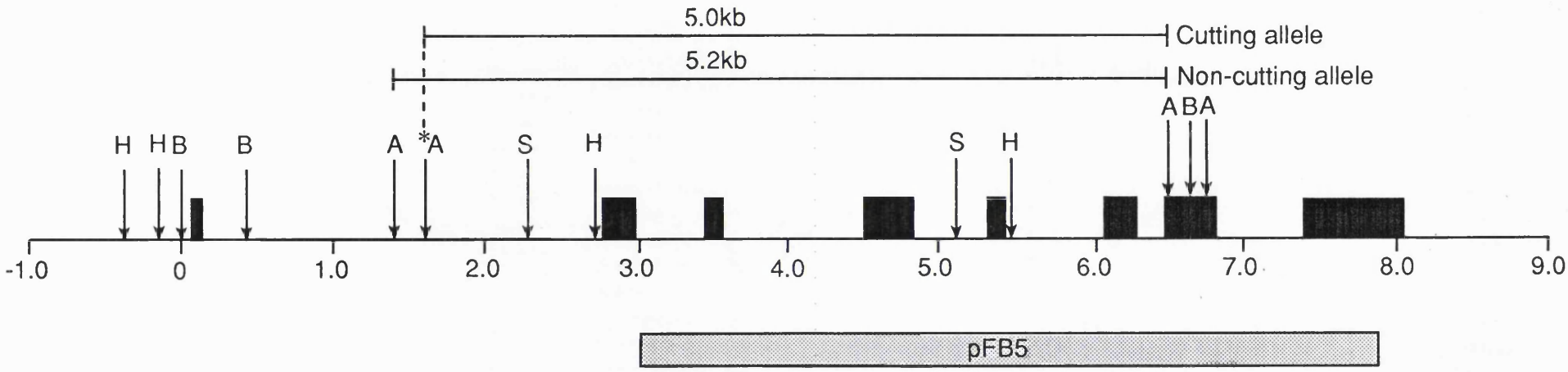
3.1. Characterisation of the AvaII polymorphism

3.1.1. Mapping of the AvaII polymorphism by Southern blotting

The ß-fibrinogen cDNA probe used for the mapping experiments was a 700bp Pst1 fragment from pFB5 (Humphries et al, 1987), which spans the region of genomic DNA 2936-7862bp (excluding introns) from the start of transcription and includes the 3' end of exon 2 to the 5' end of exon 8. Preparation of the probe was performed by Dr Anne Lane.

Southern blots were performed on genomic DNA digested with several different restriction enzyme combinations, from individuals homozygous for either the cutting or non-cutting site of the AvaII polymorphism, determined by previous Southern blot analysis. The restriction enzymes chosen in addition to AvaII were SacI, HindIII and Bgl II because the cutting sites for these enzymes were known from previous maps (Kant et al, 1985; Chung et al, 1990; Courtois et al, personal communication) (figure 3.1). Genomic DNA was digested with AvaII alone and double digested with AvaII/HindIII, AvaII/SacI and AvaII/Bgl II. Results of the Southern blots are shown in table 3.1 and figure 3.2.

Fig. 3.1 Restriction map (not to scale) of the β fribrinogen gene with predicted Ava II polymorphic site and fragment lengths seen on Southern blot



H = Hind III
B = Bgl II
A = Ava II
S = Sac I
= Exons
* = predicted Ava II polymorphic site
= β fibrinogen cDNA probe

1.0kb

Table 3.1

Mapping of the AvaII polymorphism by Southern blotting; predicted and actual fragment length after AvaII restriction digest of genomic DNA and hybridization with a ß-fibrinogen probe

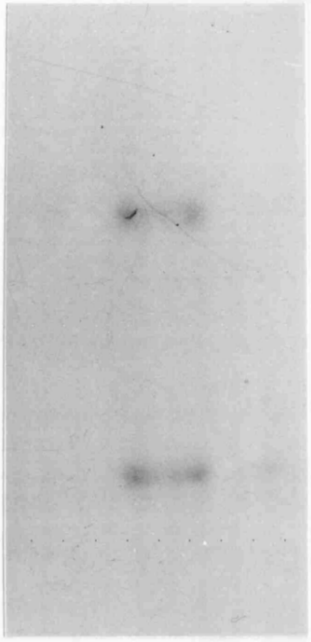
Restriction enzyme digest	Fragment length with ß fibrinogen probe (kb) (predicted[#]) **actual**	
	AvaII Genotype	
	11	22
AvaII	(5.1) **5.0**	(5.1) **5.2**
	(1.6) **2.0**	(1.6) **2.0**
AvaII/SacI	(2.9) **2.8**	*
	(1.6) **2.0**	*
	(1.4) **1.35**	*
AvaII/HindIII	(2.8) **2.7**	(2.8) **2.7**
	(1.6) **2.0**	(1.6) **2.0**
	(1.1) **1.0**	(1.1) **1.0**
AvaII/Bgl II	(5.1) **5.2**	(5.1) **5.2**
	(1.6) **1.6**	(1.6) **1.6**

[#] from restriction map, figure 3.1 and previous southern blot results (Humphries et al,1987) * No result from Southern blot

The only difference in the Southern blot results between the individual who was homozygous for the cutting allele and the individual homozygous for the non-cutting allele was in the AvaII digest alone. There was a 200bp difference in length

of the largest fragment resulting from the AvaII digest which spans the two constant AvaII sites found at +1441bp and +6552bp from the start of transcription in the published sequence (Chung et al, 1990). The polymorphic site was predicted to lie approximately 200 bp downstream from the most 5' constant site (+1441bp), which is the only position compatible with both the known restriction map and the Southern blot results (figure 3.1). Examination of this stretch of sequence revealed three possible polymorphic AvaII sites in the appropriate position, differing from the recognition sequence by a single nucleotide (figure 3.3).

Fig. 3.2 Auotradiograph of a Southern blot showing the hybridisation pattern obtained from an Ava II digest of 5μg of genomic DNA from individuals homozygous for either the non-cutting allele (A-A-) or the cutting allele (A+A+) using the pFB5 cDNA probe



The Southern blot shown in the autoradiograph has faded before photography and initially demonstrated two bands at 5.2kb and 2.0kb in the A-A- individual and two bands at 5.0kb and 2.0kb in the A+A+ individual as shown in the cartoon below.

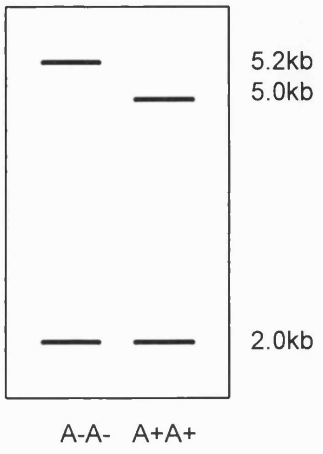


Figure 3.3 Sequence of intron 1 of the ß-fibrinogen gene from +1039 to +1758 from the start of transcription (Chung et al,1990). The oligonucleotide primers used for subsequent PCR are underlined, with the possible AvaII sites (5 nucleotides in length) also underlined. The constant AvaII site is in bold, with the polymorphic AvaII site, identified by examining the fragment lengths after PCR and AvaII digestion, in bold and underlined.

ATATGGTTAA TCTGGTTAAC TCTGGTTAAT CATATTAGGT AAGAAAAATG TAAAGAATGT
GTAAGACGAA ATTTTTGTAA AGTACTCTGC AAAGCACTTT CACATTTCTG CTTATCAACT
AAACCTCACA GAGATAGTTT AATAGTTTAG GCTTTAAAAT GGATTTTGAT TATTCAACAA
GTGGCCTTCA TAATTTCTTT AAGTGTTTTT CTTTAAGTAT ATACTTTCTT TAAATATTTT
TTAAAATTTC CTTTTCTCTA GTAAAGCCAG ACCATCCATG CTACCTCTCT AGTGGCACTC
TGAAATAAAA AGAAAATAGT TTTCTCTGTT ATAATTGTAT TTGTAATAAG CAGATGAATC
ACATTTCTTA AAATTTGTTT TAGAGAGGGT AAGCTCTGAC TAG**GACC**ATG ACTTCAATGT
GAAATATGTA TATATCCTCC GAATCTTTAC ATATTAAGAA TGTATATAGT CAACTGGTTA
AACAGGAAAA TCTGGAACAG CCTGGCTGGG TTTTAATCTT AGCACCATCC TACTAAATGT
TAAATAATAT TATAATCTAA TGAATAAATG ACAATGCAAT TCAAATAGA GTTCATCTGA
TGACTTCTAG ACTACAAAAT TGCAAGAGAG CTCAGTTGTT GCTCAGTT**GT TCC**AAATCAT
GTCGTTTGTT AATTTGTAAT TAAGCTCCAA AGGATGTATA GCTACTGACA AAAAAAAAA

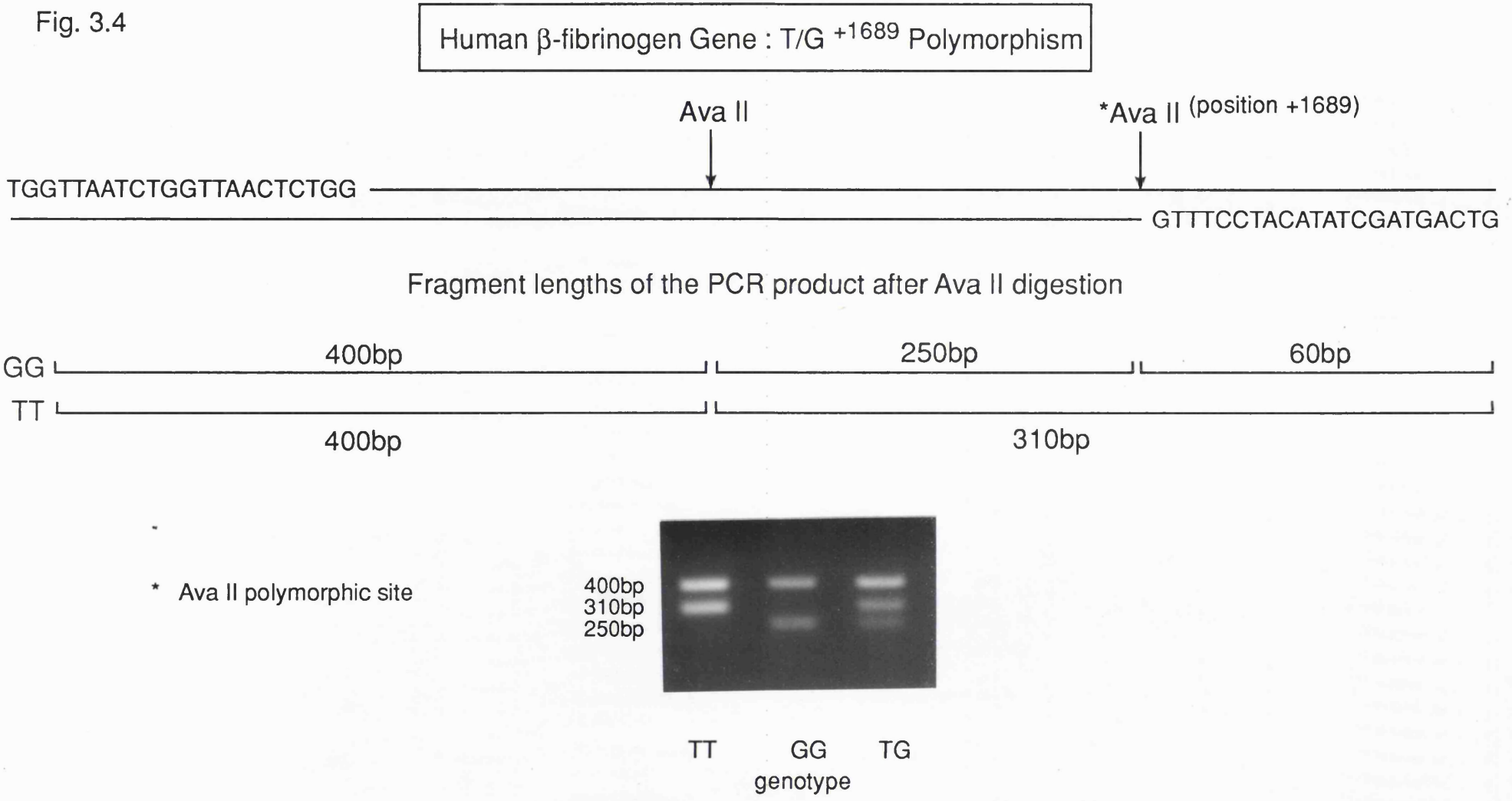
3.1.2. Identification of oligonucleotide primers necessary for PCR

Oligonucleotide primers were identified flanking the three predicted possible sites of the AvaII polymorphism in intron 1 of the ß-fibrinogen gene by examination of the published sequence (Chung et al, 1990) (figure 3.3).

3.1.3. Amplification of the AvaII polymorphic site

The two oligonucleotide primers identified above were used to amplify the intervening region of DNA which was then digested with AvaII. This resulted in two or three fragments dependent on the presence or absence of the polymorphic AvaII site and whether the individual was homozygous or heterozygous for a particular allele. The undigested PCR product was 710bp and on digestion of DNA from an individual homozygous for the non-cutting allele, two fragments resulted of 400bp and 310bp in length. Digestion of DNA from an individual homozygous for the cutting allele revealed two fragments 400bp and 250bp in length. The smallest fragment of 60bp was not visible on the gel. Digestion of DNA in those heterozygous for the polymorphism revealed all three fragments (figure 3.4).

Fig. 3.4



Oligonucleotide primers for PCR amplification of the T/G $^{+1689}$ polymorphic site in intron I of the β - fibrinogen gene and the sizes of Ava II digestion products for the two alleles

3.1.4. Identification of the base change causing the AvaII polymorphism

Of the possible polymorphic sites identified in figure 3.3, the most 3' site which lies at +1689bp from the start of transcription was compatible with the results of the PCR amplification and digestion, with either a T or a G at this position (figure 3.3). The polymorphism is referred to as the T/G^{+1689} from now on in this thesis.

3.2. Characterisation of the BclI polymorphism

3.2.1. Mapping of the BclI polymorphism by Southern blotting

The ß-fibrinogen cDNA probe used for the mapping experiments was a 700bp Pst1 fragment from pFB5 (Humphries et al, 1987), as described in 3.1.1.

Southern blots were performed on genomic DNA digested with several different restriction enzyme combinations, from individuals homozygous for either the cutting or non-cutting site of the BclI polymorphism, determined by previous Southern blot analysis. The restriction enzymes chosen in addition to BclI were SacI, HindIII and Bgl II because the cutting sites for these enzymes were known from previous maps (Kant et al, 1985; Chung et al, 1990; Courtois et al, personal communication) (figure 3.5). Genomic DNA was digested with BclI alone and double digested with BclI/HindIII, BclI/SacI and BclI/Bgl II. Results of the Southern blots are shown in table 3.2. and figure 3.6.

Table 3.2

Mapping of BclI polymorphism by Southern blotting; predicted and actual fragment length after BclI digestion of genomic DNA and hybridization with α ß-fibrinogen probe

Restriction enzyme digest	Fragment length with ß fibrinogen probe (kb) (Predicted[#]) **actual**	
	BclI genotype	
	11	22
BclI	(6.6) **7.0**	(6.6) **7.0**
	(5.3) **5.4**	(4.2) **4.2**
BclI/SacI	(5.3) *	(4.2) *
	(2.9) **2.8**	(2.9) *
	2.0	*
	(0.7) **1.35**	(0.7) *
BclI/HindIII	(5.3) *	(4.2) *
	(2.8) **2.7**	(2.8) **2.7**
	2.0	**2.0**
	(0.3) **1.0**	(0.3) **1.0**
BclI/Bgl II	(5.3) **5.4**	(5.3) *
	(4.4) *	(3.3) *
	1.7	*
	(0.9) **0.8**	(0.9) *

[#] Predicted from previous Southern blot results (Humphries et al, 1987) and Restriction map (figure 3.5.) * No Southern blot result

Fig. 3.5 Restriction map (not to scale) of the β fribinogen gene with two predicted Bcl I polymorphic sites compatible with the southern blot results

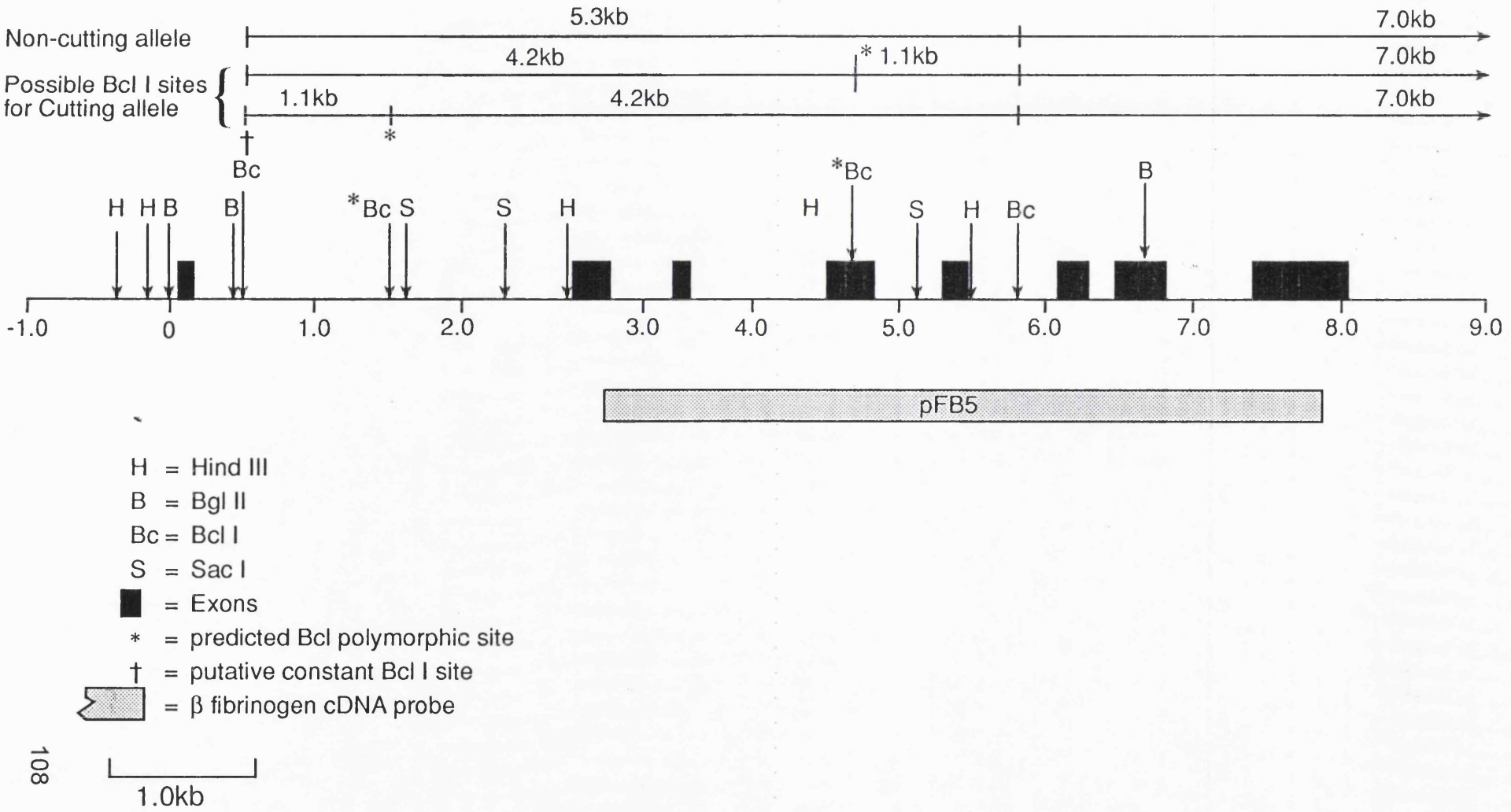
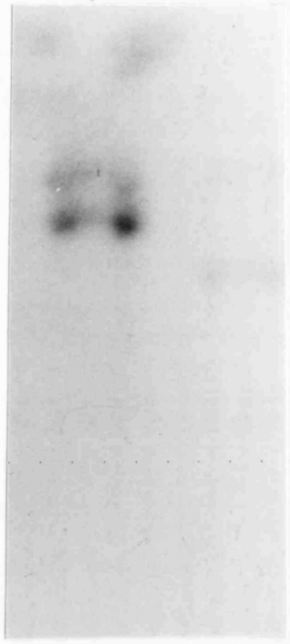
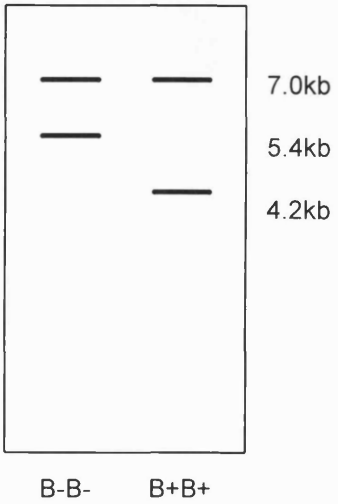


Fig. 3.6 Autoradiograph of a Southern blot analysis showing the hybridisation pattern obtained from a Bcl I digest of 5µg of genomic DNA from individuals homozygous for either the non-cutting allele (B-B-) or the cutting allele (B+B+) using the pBF5 CDNA probe

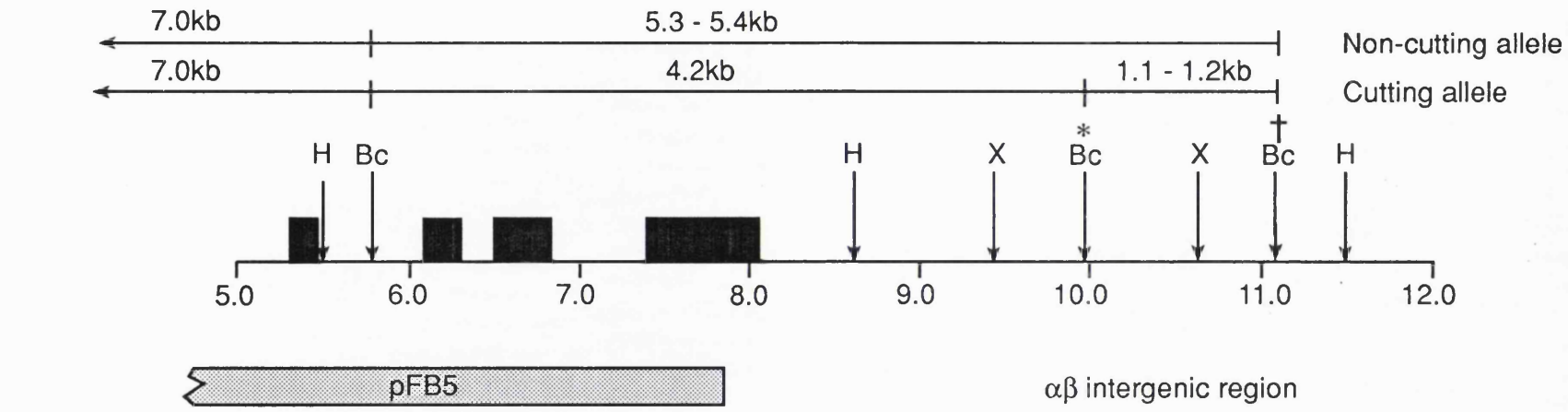


The Southern blot shown in the autoradiograph has faded before photography and initially demonstrated two bands at 7.0kb and 5.4kb in the B-B- individual and two bands at 7.0kb and 4.2kb in the B+B+ individual as shown in the cartoon below.



A constant BclI site lies in the 5th intron of the ß-fibrinogen gene (+5792). The other constant sites must therefore be 6.6-7.0kb in one direction and 5.3-5.4kb in the other, with the polymorphic site lying 4.2 or 1.1-1.2kb in the latter direction (table 3.2). If the BclI polymorphic site were mapped to the 5' end of the ß-fibrinogen gene there should be a constant site at approximately +400-500bp and a potential polymorphic site either at approximately +1590bp (5792-4200) or between +4600-4700bp (5792-1100) as shown in figure 3.5. On searching the known sequence (Chung et al, 1990), not only are such sites not present but if the latter potential polymorphic site is correct, the probe used in the Southern blotting experiments should pick up both the 4.2kb and the 1.1-1.2kb fragment, which it did not (figure 3.5 and table 3.2). The BclI polymorphic site was therefore mapped to the 3' end of the ß-fibrinogen gene and predicted to lie within the 2.8kb fragment of the HindIII restriction map of the intergenic sequence (Humphries et al,1984; Kant et al,1985; Chung et al,1990; Courtois (personal communication) and my own observations)(figure 3.7). The results of the double digests were compatible with this hypothesis, although not all the fragment sizes identified could be explained by the present restriction map.

Fig. 3.7 Restriction map (not to scale) of 3' end of the β fribinogen gene with two predicted Bcl I polymorphic sites compatible with the Southern blot results



H = Hind III
Bc = Bcl I
X = Xba I
■ = Exons
* = predicted Bcl I polymorphic site
† = putative constant Bcl I site
▭ = β fibrinogen cDNA probe

1.0kb

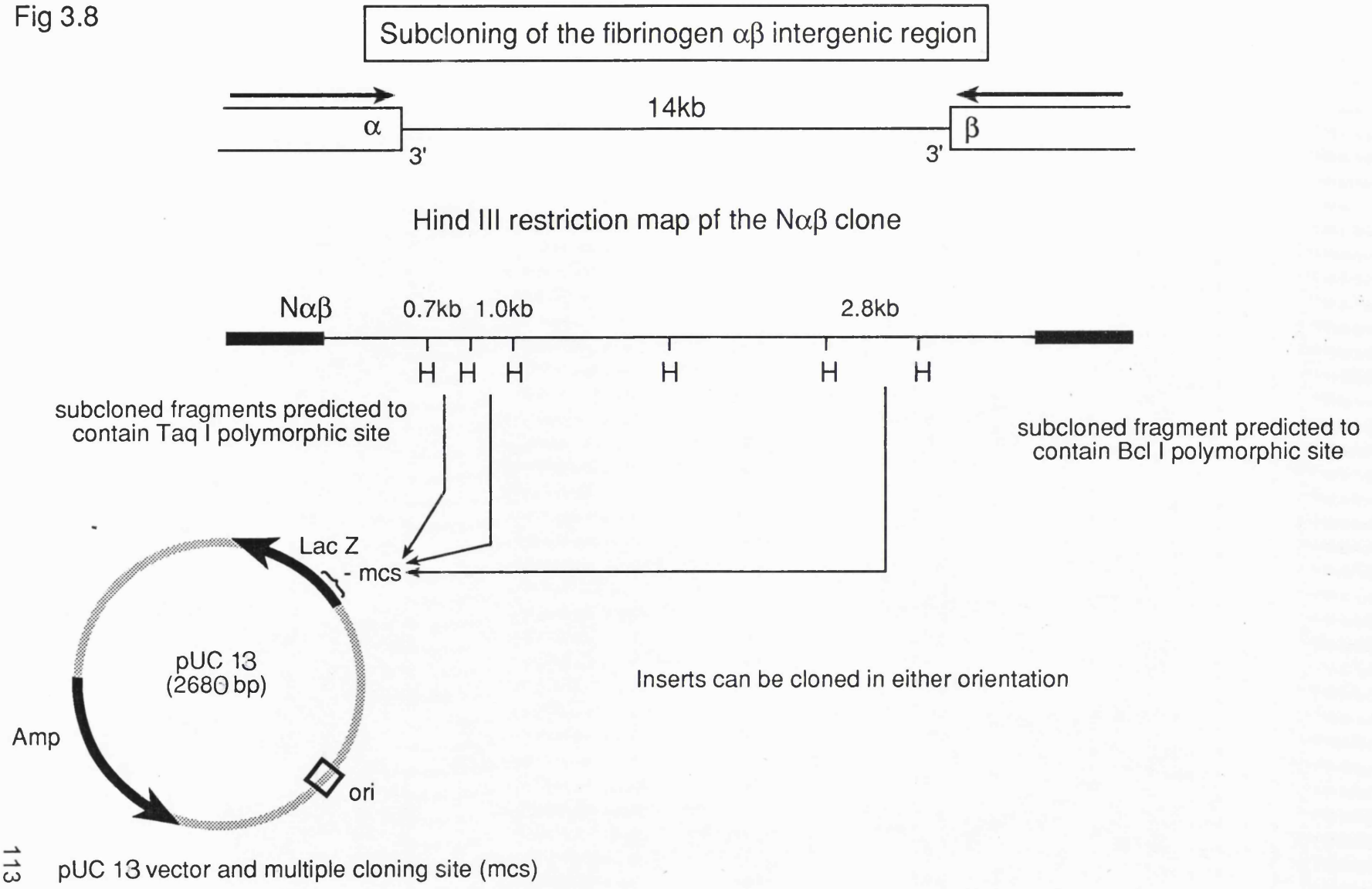
3.2.2. Identification of oligonucleotide primers necessary for PCR

3.2.2.a Subcloning of the fibrinogen αß intergenic region

The polymorphic BclI site was mapped to the 3' end of the ß-fibrinogen gene (3.2.1) but the nucleotide sequence of the region between the α- and ß- fibrinogen genes was unavailable. In order to obtain sufficient sequence to design PCR primers, a bacteriophage λ clone, Nαß, (kind gift of Dr Giles Courtois, INSERM U217, Grenoble, France) containing the 3' ends of the α- and ß-fibrinogen genes and the intergenic region was packaged, amplified and the phage DNA extracted by PEG precipitation (2.2.4.a,b). The clone was then digested with HindIII and subcloned into pUC13 (figure 3.8).

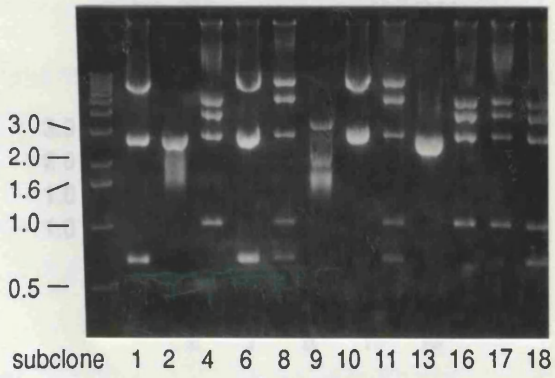
The transformed XL-1 blue cells were plated out and 18 colonies were picked, grown up and miniprepped (2.2.4.d). After digestion of the subcloned DNA with HindIII, two subclones (subclones 2 and 13) had a banding pattern of a major 2.8kb band which could represent the expected 2.8kb fragment (figure 3.7) running with the pUC13 vector which is approximately 2.7kb in length (figures 3.8 and 3.9). Within the multiple cloning site of the pUC18 vector is a unique XbaI site (figure 3.10c). From the restriction map of the intergenic region (Kant et al, 1985) there are two XbaI sites within the 2.8kb HindIII restriction fragment (figure 3.7) approximately 1.0kb apart and therefore the subclones were digested with XbaI. Three bands resulted in subclone 13, the two smaller bands being 1.0kb and 0.9kb in length (figure 10a), compatible with the hypothesis that subclone 13 contained the 2.8kb fragment (figure 3.10b).

Fig 3.8



pUC 13 vector and multiple cloning site (mcs)

Fig. 3.9 Hind III digested pUC18 vector with inserts from the fibrinogen gene αβ intergenic region



The pUC 18 vector is seen as a 2.7 kb band

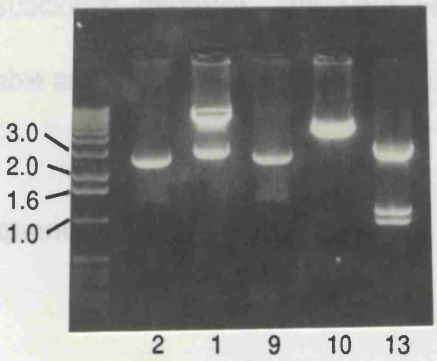
subclones 1 and 6 show 0.7kb insert

subclones 4,16 and 17 show 1.0kb insert

subclones 8,11 and 18 show both a 0.7kb and a 1.0kb insert

subclones 2 and 13 show a possible insert of ~ 2.7 kb

Fig. 3.10a Hind III /Xba I double digest of several subclones of the αβ intergenic region



Double digestion of subclone 13 results in two new bands approximately 0.9kb and 1.0kb in length. Subclone 1 'loses' the 0.7 kb band after digestion.

Fig.3.10b 2.8kb Hind III restriction fragment from the fibrinogen αβ intergenic region showing the two Xba I sites from a restriction map of the region (Kant et al, 1985)

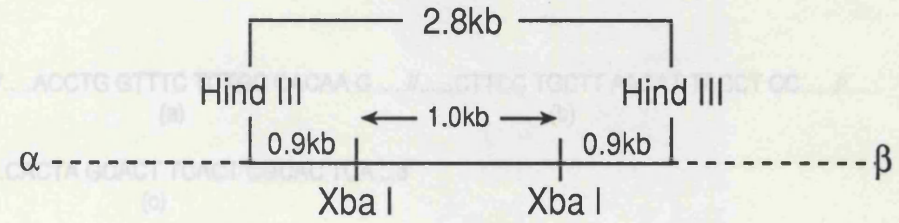
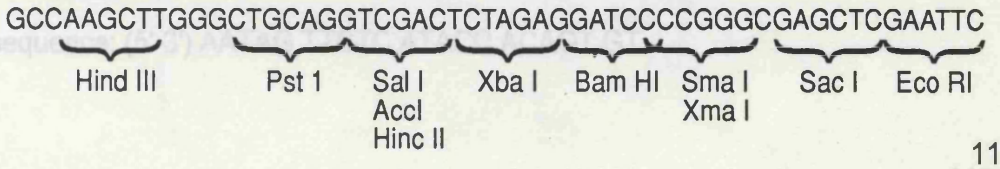


Fig 3.10c Multiple cloning site at pUC 13 vector showing unique Xba I restriction site 24bp from unique Hind III site.



A double digest with HindIII/BclI was performed on the 2.8kb insert but the banding pattern was too complex to be certain that the polymorphic BclI site was present in the subcloned fragment. The DNA sequence of the insert was determined to enable amplification by PCR and subsequent digestion with BclI.

3.2.2.b Direct sequencing of the 2.8kb insert

The 2.8kb insert (subclone 13) was partially sequenced, shown at appendices 1 and 2, using universal forward and reverse primers initially. Three new oligonucleotide primers were designed from the forward sequence of the 2.8kb fragment to allow both PCR amplification (a) and further sequencing (a,b,c)(figure 3.11).

Figure 3.11 Oligonucleotides designed from sequencing of the 2.8kb insert. Oligonucleotide (a) was used for PCR amplification to detect the BclI polymorphism as well as further sequencing of the 2.8kb fragment.

5'.....ACCTG GTTTC TCTGC CACAA G...../...../.....CTTCC TGCTT AACAT TAGCT CC...../...../.....
(a) (b)

...CACTA GCACT TCACT CGCAC TCA....3'
(c)

../// represents a break in sequence

Autoradiographs of part of the sequencing gel identifying primers (a) and (b) are shown in figures 3.12 and 3.13. An oligonucleotide primer (d) for PCR amplification was designed from the reverse sequence (figure 3.12) and had the sequence: (5'-3') AATAG TTCTC ATACC ACAGT GT.

Fig 3.12. Autoradiograph of direct sequencing gel of 2.8 kb insert (subclone 13) with both forward and reverse universal primers

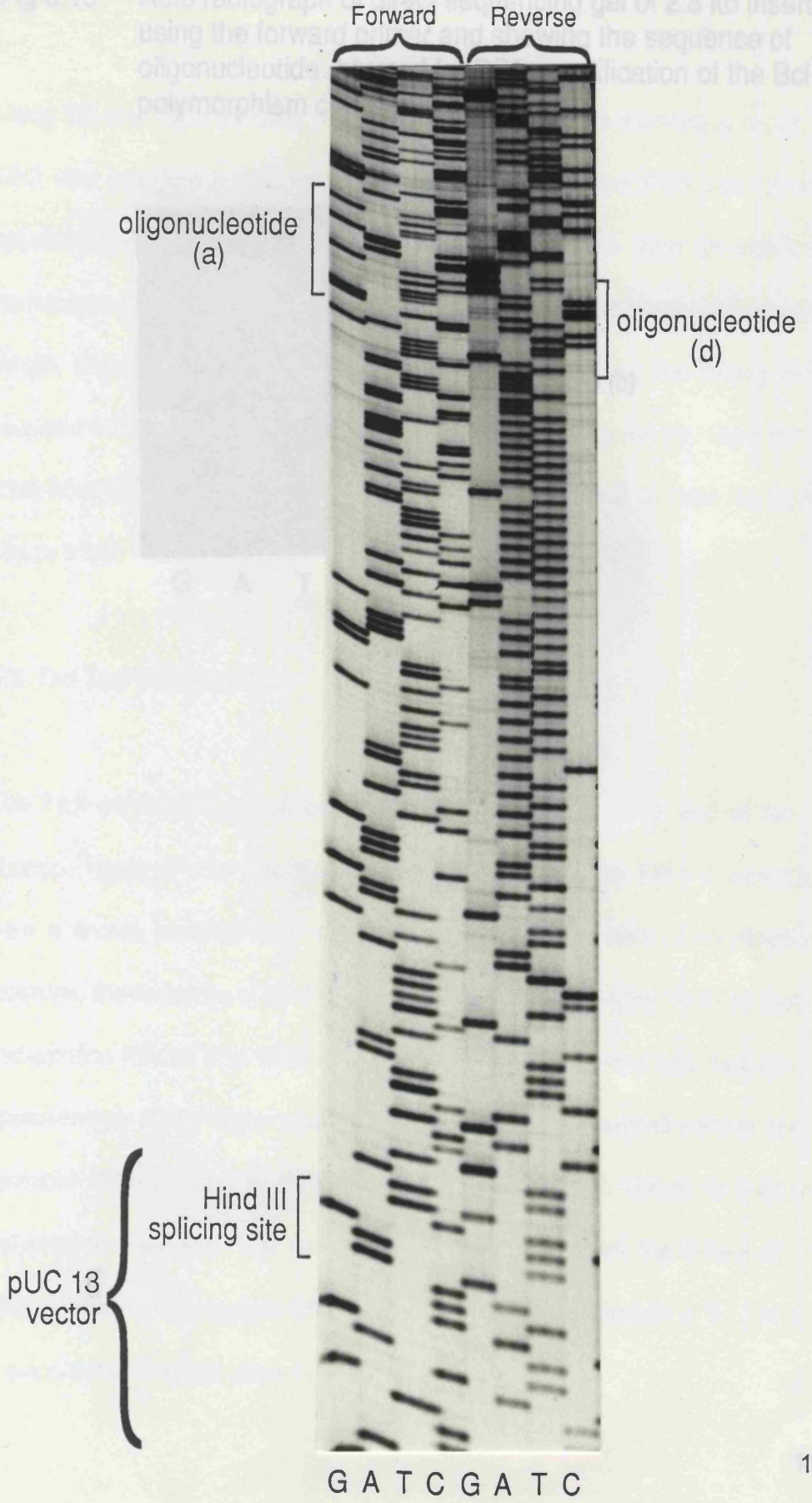
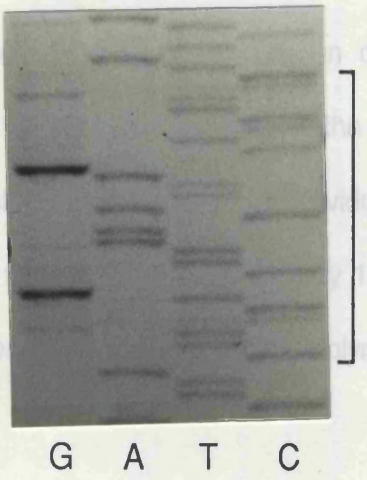


Fig 3.13 Auto radiograph of direct sequencing gel of 2.8 kb insert using the forward primer and showing the sequence of oligonucleotide (b) used for PCR amplification of the Bcl I polymorphism containing fragment



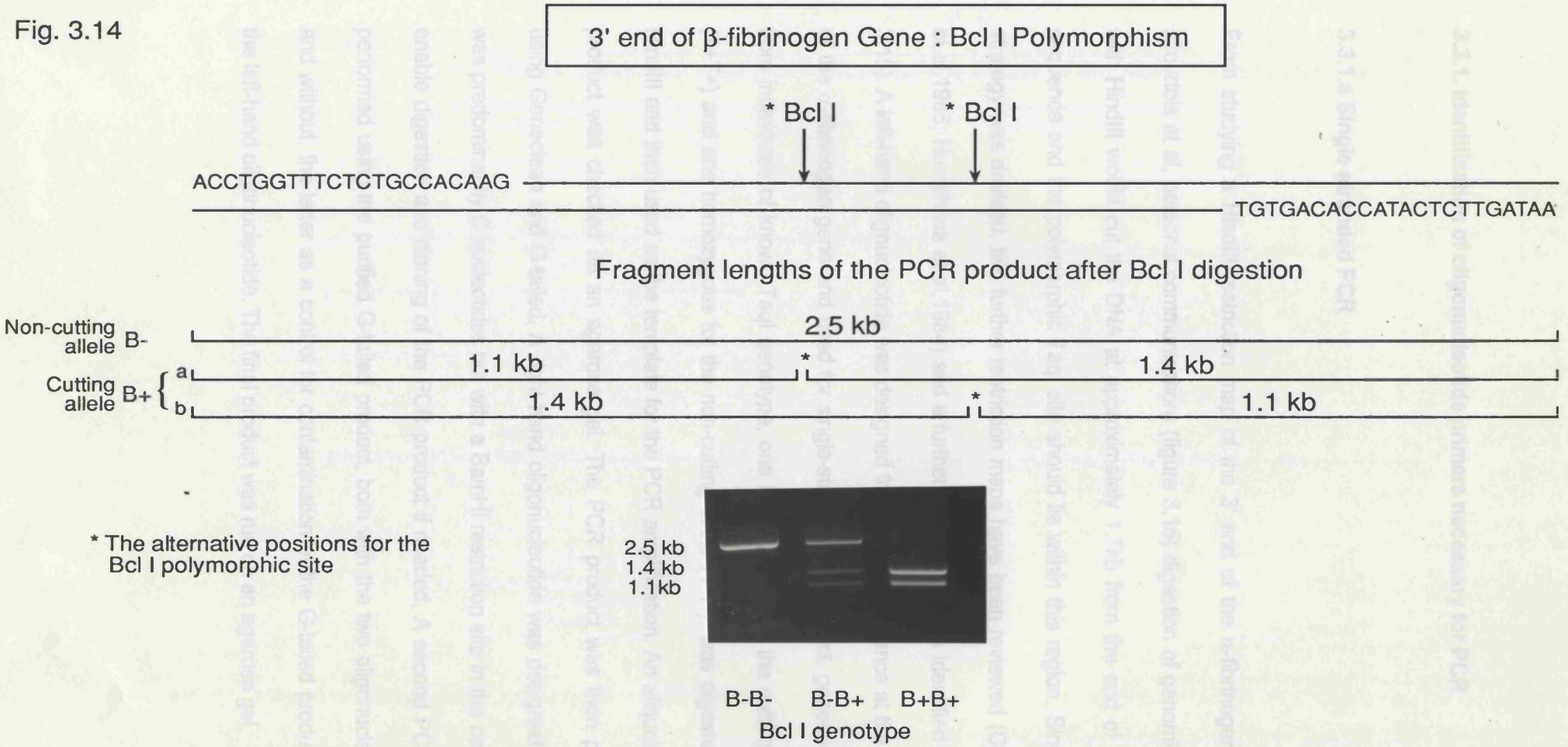
3.2.3 Amplification of the BclI polymorphic site

Using the two oligonucleotide primers identified above, the intervening region of DNA was amplified and digested with BclI. The undigested PCR product was approximately 2.5kb in length, and on digestion of DNA from an individual homozygous for the non-cutting allele, the PCR product remained unchanged in length. Digestion of DNA from an individual homozygous for the cutting allele revealed two fragments of approximately 1.4kb and 1.1kb in length. Digestion of DNA from those heterozygous for the polymorphism revealed all three fragments (figure 3.14).

3.3. The TaqI polymorphism

The TaqI polymorphism has been previously mapped to the 3' end of the α-fibrinogen gene (Humphries et al, 1984) and predicted to lie 1.6kb downstream from a known constant TaqI site at +4833bp from the start of transcription. However, the sequence of the 3' region is known only to +5942bp from the start of transcription (Chung et al 1990) and the polymorphic TaqI site would therefore lie approximately 500bp further downstream. A strategy was devised utilising single stranded PCR in order to try to amplify the region which should contain the polymorphic TaqI site. This was unsuccessful and therefore the fibrinogen αß intergenic region was subcloned from the phage λ clone described in 3.2.2.a and the appropriate regions sequenced.

Fig. 3.14



Oligonucleotide primers for PCR amplification of the BclI polymorphic site at the 3' end of the β fibrinogen gene and the sizes of the BclI digestion products for the two alleles

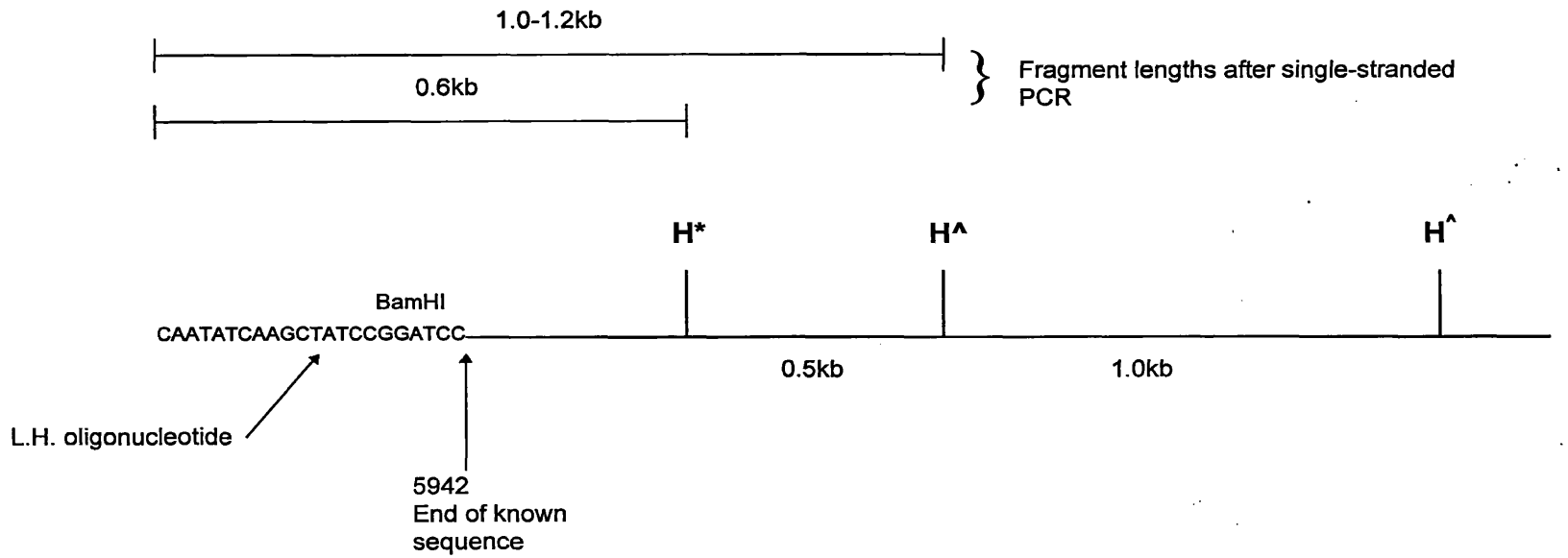
3.3.1. Identification of oligonucleotide primers necessary for PCR

3.3.1.a Single stranded PCR

From studying a HindIII restriction map of the 3' end of the α-fibrinogen gene (Courtois et al, personal communication) (figure 3.15) digestion of genomic DNA with HindIII would cut the DNA at approximately 1.7kb from the end of known sequence and the polymorphic TaqI site should lie within this region. Since this strategy was devised, two further restriction maps have been reviewed (Courtois et al,1985; Humphries et al,1984) and a further two HindIII sites identified (figure 3.15). A left-hand olignucleotide was designed from known sequence at the 3' end of the α-fibrinogen gene and used for single-stranded PCR. First, genomic DNA from individuals of known TaqI genotype, one homozygous for the cutting allele (T+T+) and one homozygous for the non-cutting allele (T-T-), was digested with HindIII and then used as the template for the PCR amplification. An aliquot of the product was checked on an agarose gel. The PCR product was then purified using Geneclean and G-tailed. A right-hand oligonucleotide was designed which was predominantly C nucleotides but with a BamHI restriction site in the centre to enable digestion and cloning of the PCR product if needed. A second PCR was performed using the purified G-tailed product, both with the two oligonucleotides and without, the latter as a control for contamination of the G-tailed product with the left-hand oligonucleotide. The final product was run on an agarose gel.

Figure 3.15

HindIII restriction map (not to scale)of the 3' end of the alpha fibrinogen gene



H* HindIII restriction site; Humphries et al,1984

H^ HindIII restriction sites; Courtois , personal communication

H*+H^ HindIII restriction sites; Kant et al, 1985

After the first round of PCR, designed to produce single stranded DNA of approximately 0.9kb in length, two distinct main bands were seen for each HindIII digested DNA sample when run on the agarose gel (figure 3.16a). The fragment sizes were approximately 600bp and 1.0kb for the individual of genotype T-T- and approximately 600bp and 1.2kb for the individual of genotype T+T+. However, on several repeat PCR's, the two different samples produced bands of the same size, as each other, 700bp and 1.4kb, but different from the first PCR (figure 3.16b).

Single stranded PCR of 3' end of α fibrinogen gene

Fig. 3.16a

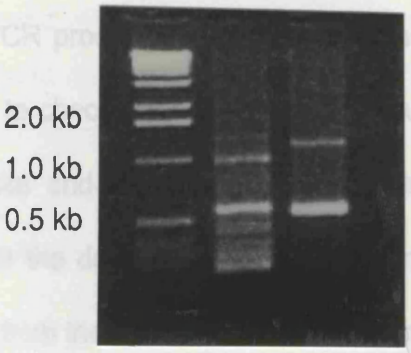
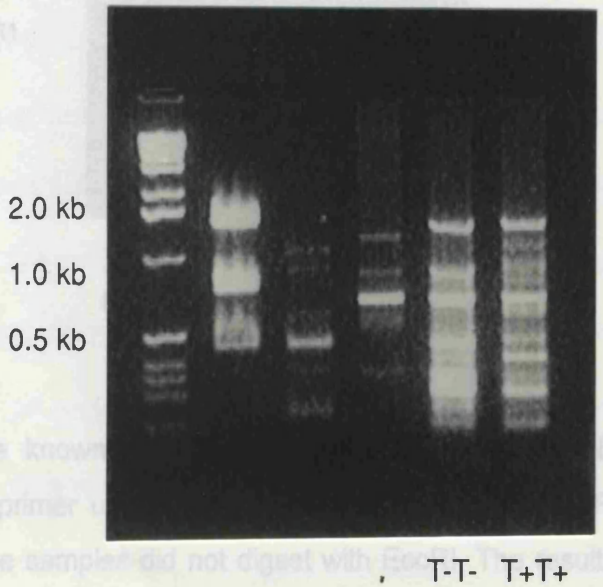


Fig. 3.16b



After the second round of PCR, using the two oligonucleotides, the same bands were observed after electrophoresis as with single-stranded PCR, but they were more intense, indicating that further amplification had taken place. No bands were seen in the control lane. EcoRI and TaqI restriction digests were performed on both first round PCR product (single-stranded) and second round PCR product (double-stranded) to check for the presence of double-stranded DNA and also a constant EcoRI site and the polymorphic TaqI site. Both the putative single-stranded DNA and the double-stranded DNA from the T-T- allele cut with TaqI whereas the DNA from the T+T+ allele did not (figure 3.17).

Figure 3.17

TaqI digested 'single-stranded' PCR of the 3' end of the α-fibrinogen gene

ds ss ds

E U T E U T E U T

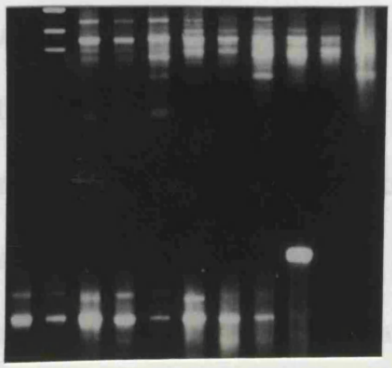
ds = double stranded DNA

ss = single stranded DNA

E = Digested with EcoR1

U = uncut

T = digested with TaqI



T-T-

T+T+

E U T E U T E U T

ds ss ds

On reviewing the known sequence (Chung et al,1990), the EcoRI site lies upstream of the primer used and was therefore not in the PCR product, thus explaining why the samples did not digest with EcoRI. The results of the single-stranded PCR can be explained if there are at least three HindIII sites 6-700bp

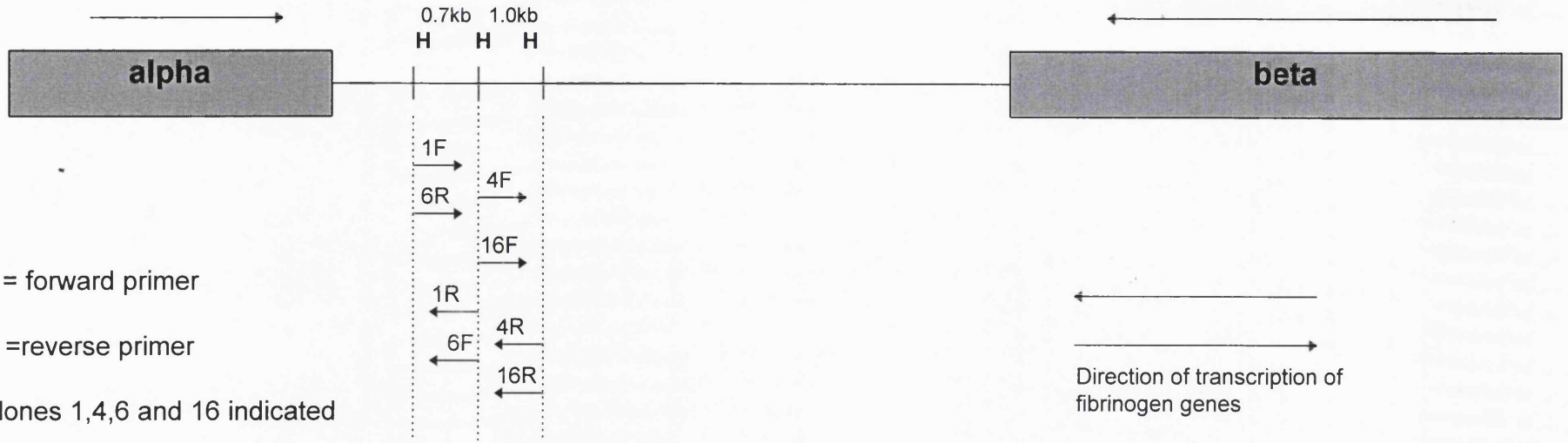
and 1.0-1.4kb apart at the 3' end of the α-fibrinogen gene and incomplete digestion of genomic DNA with HindIII (figure 3.15). This is compatible with Kant's HindIIII restriction map of the fibrinogen gene cluster (Kant et al, 1985). For the production of double-stranded DNA using a single oligonucleotide there would have to be an identical or almost identical sequence to this oligonucleotide in the complementary strand of DNA where the HindIII restriction sites are found. No explanation was found why TaqI digested the known non-cutting allele and not the known cutting allele. In order to investigate these findings further, the Nαß clone which contains the fibrinogen αß intergenic sequence was subcloned and appropriate inserts sequenced.

3.3.1.b Subcloning of the fibrinogen αß intergenic region

The same bacteriophage λ clone, Nαß, was used and prepared as described in 3.2.2.a, up to the miniprep extraction of DNA and HindIII digestion of the inserts which had been subcloned. Inserts of predicted length, 0.7kb and 1.0kb (Kant et al, 1985 and own observations), were identified in several of the clones. Some of the clones contained both inserts, which would support the findings of Kant et al, that the three most 5' HindIII sites in the intergenic region lie within 1.7kb of each other. The individual inserts were double digested with HindIII/TaqI and although the banding patterns were complex, they indicated that there were TaqI cutting sites in both the 0.7kb insert and the 1.0kb inserts (not shown). Therefore the DNA sequence of selected 0.7kb and 1.0kb inserts was determined to enable amplification by PCR and subsequent digestion with TaqI. The orientation of the subcloned fragments is shown in figure 3.18.

Figure 3.18

Intergenic region (not to scale) of the α and β fibrinogen genes and orientation of subcloned fragments



3.3.1.c Direct sequencing of the 0.7kb and 1.0kb inserts

Two each of the 0.7kb and 1.0kb inserts were partially sequenced initially using universal forward and reverse primers. New oligonucleotide primers were designed to allow further sequencing and/or PCR amplification of genomic DNA.

Sequence of 1.0kb insert (subclones 4 and 16)

The sequence of the 1.0kb insert using the forward primer is shown in figure 3.19 and the autoradiograph from the sequencing gel of the Alu consensus sequence with the TaqI site within it is shown in figure 3.20. The partial sequence of the 1.0kb insert using the reverse primer is shown at appendix 3 and figure 3.21.

Figure 3.19 Sequence of 1.0kb insert (subclones 4 and 16) forward primer The sequence of the pUC18 vector is underlined with the oligonucleotide subsequently used for further sequencing shown in bold. Where it was impossible to identify whether a base was present or not, or repeated, these single bases are shown in bold. The TaqI site is shown in bold and underlined.

C GGGGA TCCTC TAGAG TCGAC CTGCA GCCCA AGCTT CTAGT TCCTC

TTTAT TTATG AACCA A**GAGA AACAG CTAAC TCAGG GATTG** TTTTT

AAACT GACTA CAGAT TCCCA CCATG GGGCA AAAAT ATGGG TGACA

TGACA CATCA CCAGC TGCAG TAGCT CAGCT CACAT TTCTG TTTAA

GCATT ACACA CATAA TATCC TGTAT GCTGA TCATT TAAAC TTACT

GACGC TTGTA ATCCC AGCAC TTTGG GAGGC CGAGG GGGGT GAATC
TGTA ATCCC AGCAC TTTGG GAGGT CGAGG CGGGC AGATC

ATGAG GTCAG GAAAT TGAGA CCATC CTGGA CAACA TGGTG
AAGAG GTCAG GAGAT CGAGA CCATC CTGGC CAACA TGGTG

AAA**TC GA**AAC AGTGT CTCTA CTAAA A**A**TAC A
A----------AAC CCCAT CTCCA CTAAA AATGC A.....

The second underlined portion = consensus sequence homologous to the Alu repeat with occasional nucleotide differences. Alu repeat consensus sequence is shown in italics underneath the intergenic sequence.

Sequence of 0.7kb insert, subclones 1 and 6

The 0.7kb insert was partially sequenced with both forward and reverse universal primers. The sequence identified using the forward primer in subclone 1 and the reverse primer in subclone 6, from which the right-hand oligonucleotide for the PCR was designed, is shown at appendix 4 and the oligonucleotide sequence is shown below.

5'-3' TGTCT CAGGT ACATT TAGC

Fig 3.20 Autoradiograph of sequencing gel of 1kb insert (subclone 16) forward primer, showing Taq I site within part of Alu consensus sequence

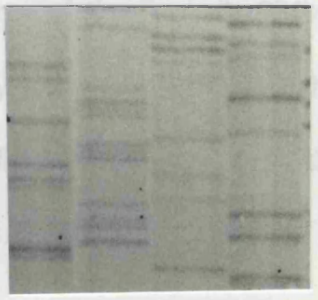
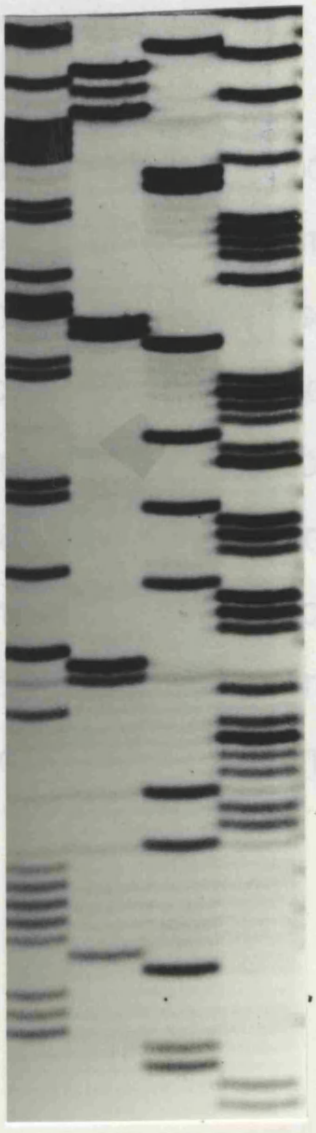


Fig 3.21 Autoradiograph of sequencing gel of 1kb insert, (subclone 4), reverse primer

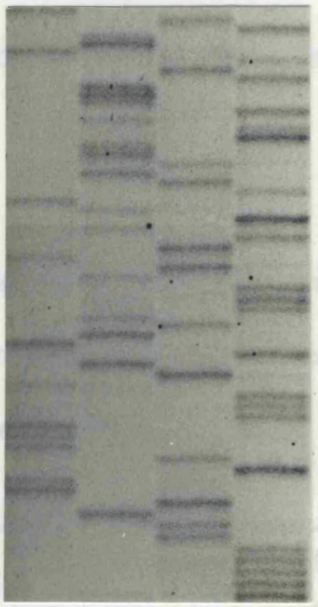


The sequence identified using the reverse primer in subclone 1 and the forward primer in subclone 6, revealed a TaqI site 284bp from the start of the sequence and thus approximately 300bp from the start of the insert and the HindIII site. This sequence is shown in figure 3.22 and an autoradiograph of part of the sequencing gel is shown in figure 3.23.

Figure 3.22 Sequence of 0.7kb insert, reverse primer (subclone 1), forward primer (subclone 6). Where it was impossible to identify whether a base was present or not, or repeated, these single bases are shown in bold. The four base motif for the TaqI cutting site is shown in bold and underlined.

CCCGG GGAGC CTCTA GAGTC GACCT GCAGC CCAAG CTTTG CCACA CCACG GTATT TCCCC GATAC CTTGT GTGCA AAATT GCATC AGATA GTTGA TAGCC TTTTG TTTGT CGTTC TGGCT GAGTT CGTGC TTACC GCAGA AGTGC AGCCA TACCG AATCC GGCTT GTGAT TGCGC CATCC CCATA GCAGC CATCA CATCA GTACC GGAAA GAGAG TCAGA AGCCG TGGCC CGTGG TGAGT CGCTC ATCAT CGGGC TTTTT GGCGA ATGAA ATTTA GCTAC GCTT**T CGA**GT CTCAT GCGCC TTCTC CCTGT ACCTG AATCA ATGTT AGGTT TCGCA GAACA CTGCG CCGGT ATCGA TATAC ATTTGG TTGGCA AACTTGA GTGTC ACTGC TGGCG TCTGA CAAGA TGACG TGTC

Fig 3.23. Autoradiograph of sequencing gel of 0.7 kb insert (subclone 1) reverse primer showing Taq I restriction site.

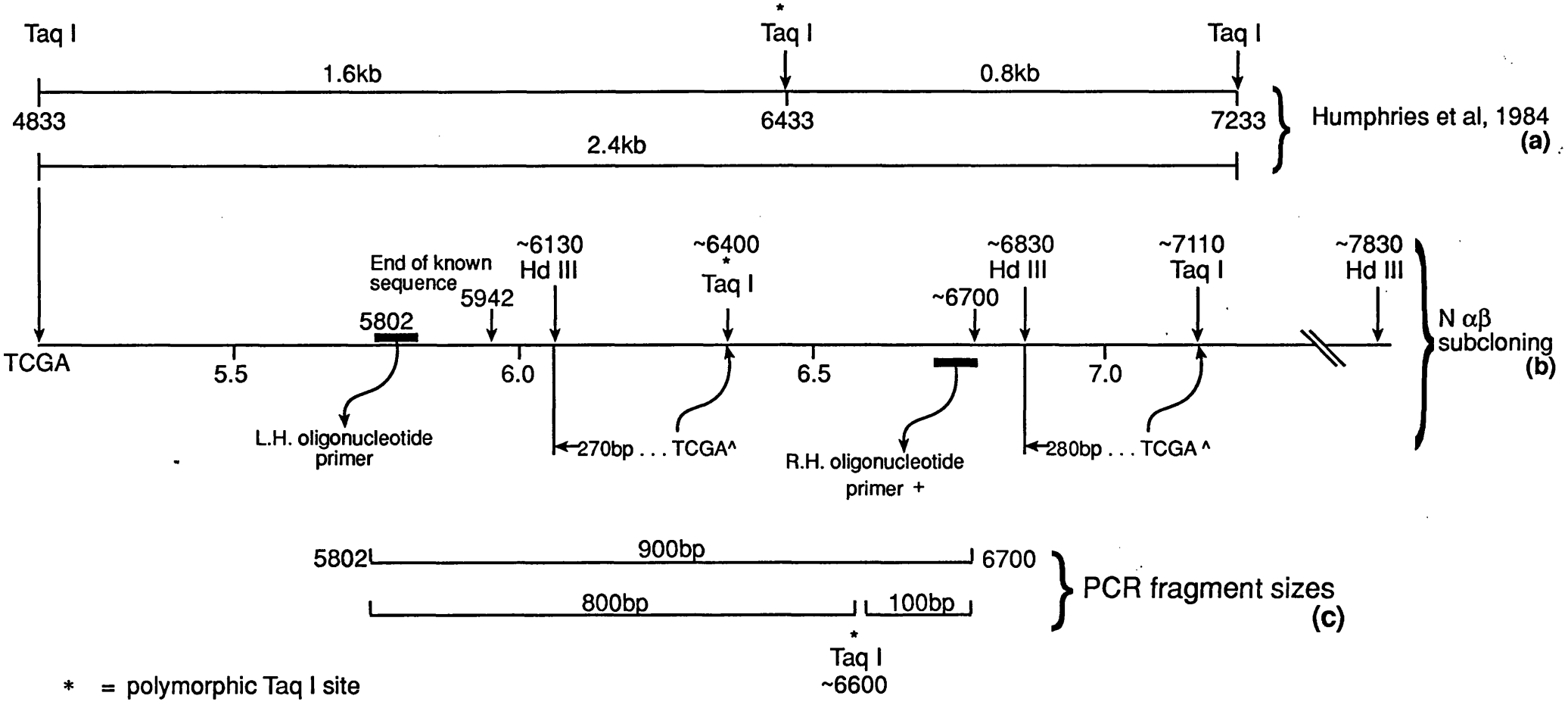


Reviewing the paper of Humphries et al, 1984 and the known sequence (Chung et al,1990) in conjunction with the results from the subcloning and sequencing done (figures 3.19, 3.22; appendices 3,4), the following restriction map has been constructed (figure 3.24a,b). The Southern blot results from Humphries et al,1984 have been compared with the predicted results from this map and the comparison shown in table 3.3.

Table 3.3 Comparison of the Southern blot results of Humphries and coworkers (1984) and those predicted from the newly constructed restriction map of the 3' end of the α-fibrinogen gene.

Restriction enzyme digest		Fragment length with α fibrinogen probe (kb)	
		Predicted	Actual
HindIII		5.0	5.0
TaqI	T-	0.75 + 2.4	0.7/0.8 + 2.4
	T+	0.75 + 1.6	0.7/0.8 + 1.6
TaqI/HindIII		0.75 + 1.2	0.8 + 1.3
TaqI/BamHI		0.75 + 1.1	0.8 + 1.0
TaqI/EcoRI	T-	0.15 + 0.45 + 0.75 + 1.7	0.4 + 0.8
	T+	0.15 + 0.45 + 0.75 + 1.0	0.4 + 0.8
TaqI/SstI	T-	0.25 + 0.35 + 0.4 + 2.4	2.4
	T+	0.25 + 0.35 + 0.4 + 1.6	1.6
HindIII/SstI		0.35 + 1.1 + 1.4	0.35 + 0.9 + 1.5

Fig. 3.24 3' end of α fibrinogen gene comparing results of Southern blotting (a), subcloning (b) and PCR (c) experiments to detect and map Taq I polymorphism and Taq I constant sites



* = polymorphic Taq I site

// = shortened map

HdIII = Hind III

^ see Fig.3.22

\+ see Appendix 4

The results from the Southern blots and the newly constructed restriction map are compatible with two exceptions. Not all the fragments expected from the TaqI/SstI digest were visible on the blots (Humphries et al, 1984) and the larger fragments from the double digest with TaqI and EcoRI are absent. This could be due to a further EcoRI site between the BamHI site and the polymorphic TaqI site.

From the map shown in figure 3.24a,b, the polymorphic TaqI site is predicted to lie within the 0.7kb fragment produced by HindIII restriction digestion of the fibrinogen αß intergenic sequence. Furthermore, it should lie approximately 300bp downstream of the HindIII site closest to the 3' end of the α-fibrinogen gene (figure 3.24a). From the subcloning and sequencing experiments, a TaqI site is found in the 0.7kb insert, 284bp from the start of the sequencing and which would be approximately 300bp in from the oligonucleotide primer used for sequencing and thus 300bp away from the HindIII restriction site (figure 3.24b). It was therefore predicted that the 0.7kb restriction fragment in subclone 6 had inserted 5'-3' while the 0.7kb restriction fragment in subclone 1 had inserted 3'-5', since subclone 6 had been sequenced with the forward primer and subclone 1 with the reverse primer, and in these orientations, the TaqI site is identified where predicted (figure 3.22). The right hand oligonucleotide primer for PCR amplification was therefore chosen from the sequencing done with subclone 1 and the forward primer/subclone 6 and the reverse primer (appendix 4) starting 125bp upstream from the HindIII restriction site (figure 3.24b) and was as follows:

5'-3' TGT CTC AGG TAC ATT TAG C

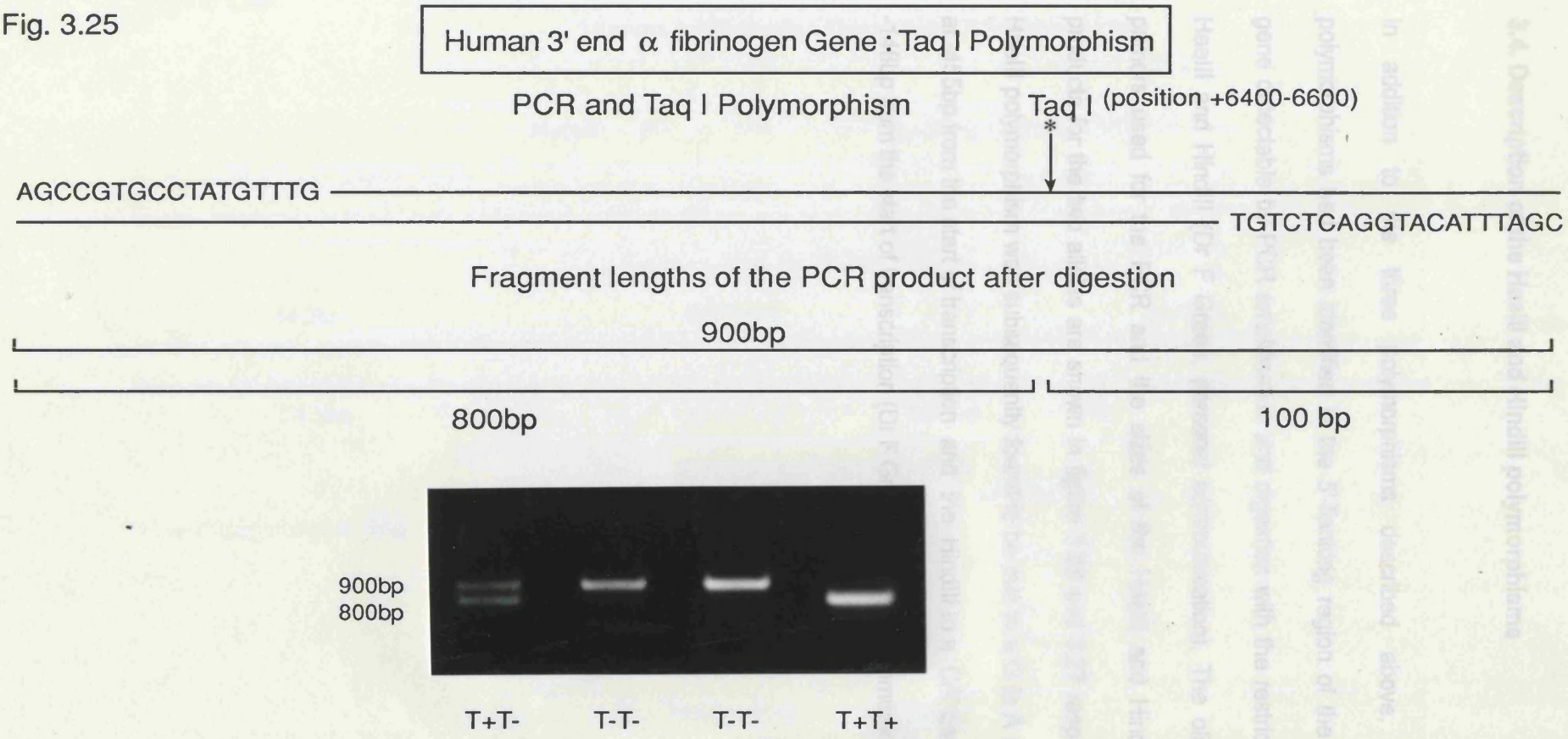
3.3.2. Amplification of the TaqI polymorphic site

The right hand oligonucleotide was chosen from the sequencing as above and the left hand oligonucleotide was chosen from known sequence of the 3' end of the α-fibrinogen gene (Chung et al,1990), starting at +5802bp and was as follows:

5'-3' AGC CGT GCC TAT CTT TG These oligonucleotides were used to amplify the intervening region of DNA which was then digested with TaqI. The undigested PCR product was approximately 900bp in length, and on PCR and digestion of DNA from an individual homozygous for the non-cutting allele, the PCR product was unchanged in size. Digestion of DNA from an individual homozygous for the cutting allele resulted in one fragment of approximately 800bp in length, the remaining fragment(s) was too small to be visualised. Digestion of DNA from an individual heterozygous for the polymorphism revealed both the 900bp and 800bp fragments (figure 3.25).

The size of the PCR fragment is 900bp and therefore the HindIII site at the 3' end of the 0.7kb restriction fragment should lie at 5802 + 900 + 125 = +6825. This is compatible with the predicted restriction map constructed from the Southern blot and sequencing results (figure 3.24b). However, the size of the fragments produced on digestion of the PCR product are predicted to be 450-500bp and 400-450bp from the sequencing and HindIII/TaqI digest of the cloned inserts but are 800bp and 100bp from the PCR digest (figure 3.25). This places the polymorphic TaqI site at 6600 rather than 6400 (figure 3.24b,c). Although such a difference would be acceptable with southern blotting, this margin of error with PCR is unexpected.

Fig. 3.25

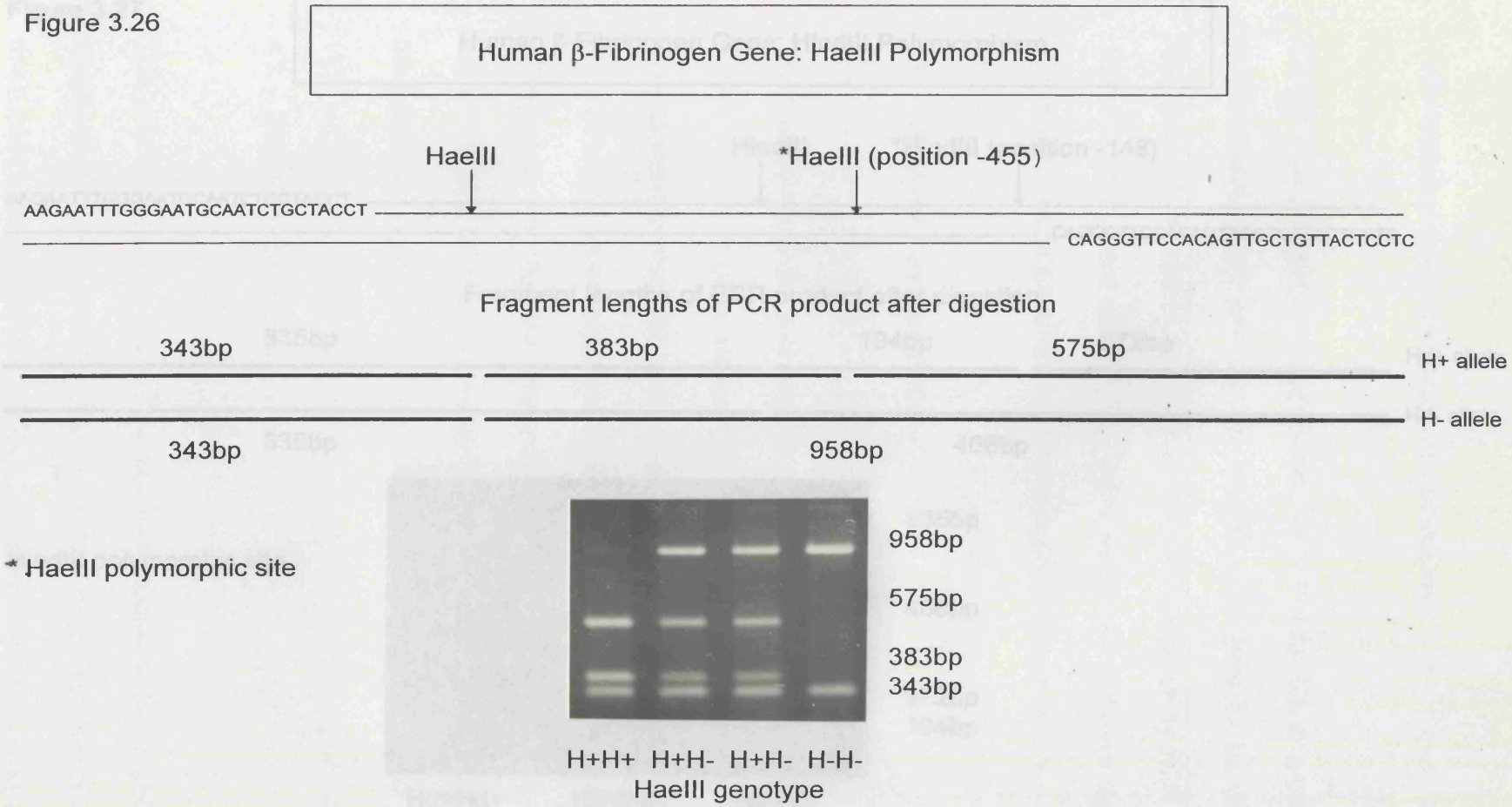


Oligonucleotide primers for PCR amplification of the Taq I polymorphic site at the 3' end of the α fibrinogen gene and the sizes of the Taq I digestion products for the two alleles

3.4. Description of the HaeIII and HindIII polymorphisms

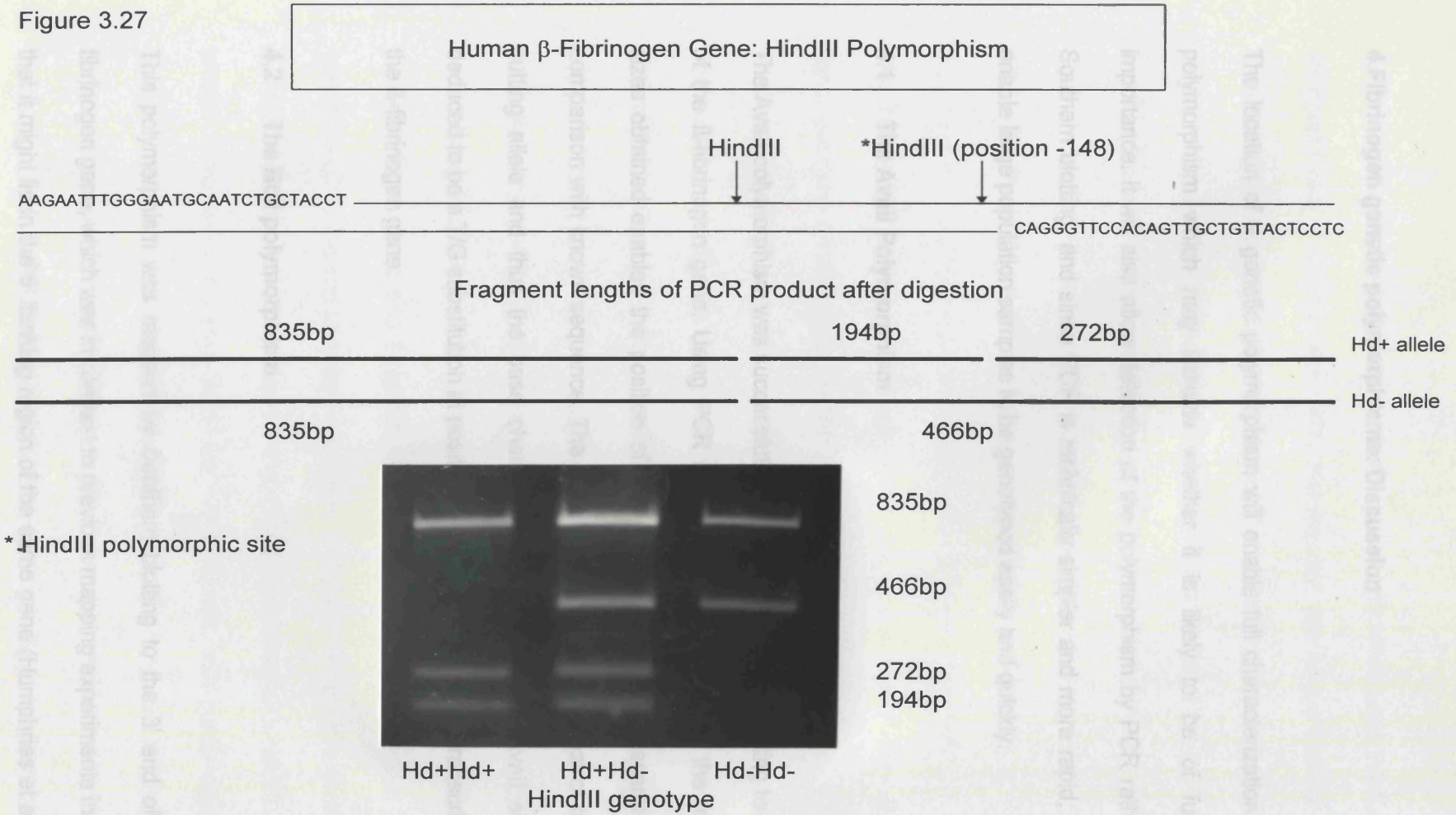
In addition to the three polymorphisms described above, two further polymorphisms had been identified in the 5'-flanking region of the ß-fibrinogen gene detectable by PCR amplification and digestion with the restriction enzyme HaeIII and HindIII (Dr F Green, personal communication). The oligonucleotide primers used for the PCR and the sizes of the HaeIII and HindIII digestion products for the two alleles are shown in figure 3.26 and 3.27 respectively. The HaeIII polymorphism was subsequently found to be due to a G to A base change at -455bp from the start of transcription and the HindIII to a C/T base change at -148bp from the start of transcription (Dr F Green, personal communication).

Figure 3.26



Oligonucleotide primers for PCR amplification of the HaeIII polymorphic site at -455bp from the start of transcription of the β-fibrinogen gene and the sizes of the HaeIII digestion products for the two alleles

Figure 3.27



Oligonucleotide primers for PCR amplification of the HindIII polymorphic site at -148bp from the start of transcription of the β-fibrinogen gene and the sizes of the HindIII digestion products for the two alleles

4.Fibrinogen genetic polymorphisms: Discussion

The location of a genetic polymorphism will enable full characterization of that polymorphism which may indicate whether it is likely to be of functional importance. It will also allow detection of the polymorphism by PCR rather than Southern blotting, and since PCR is technically simpler and more rapid, this will enable large population samples to be genotyped easily and quickly.

4.1 The AvaII Polymorphism

The AvaII polymorphism was successfully mapped by Southern blotting to intron 1 of the ß-fibrinogen gene. Using PCR amplification and digestion the fragment sizes obtained enabled the position of the polymorphism to be ascertained by comparison with known sequence. The published sequence was that of the non-cutting allele and thus the base change causing the loss of AvaII site was deduced to be a T/G substitution at position +1689 from the start of transcription of the ß-fibrinogen gene.

4.2 The BclI polymorphism

This polymorphism was mapped by Southern blotting to the 3' end of the ß-fibrinogen gene, which was in contrast to previous mapping experiments indicating that it might lie in the 5' flanking region of the same gene (Humphries et al,1987).

A clone of the αß-intergenic region was digested with HindIII and the fragments subcloned into a pUC18 vector and the relevant one sequenced to attempt demonstration of the polymorphic site by PCR amplification. The sequence did not include the polymorphic site, which lies almost in the centre of the PCR product, and thus the base change was not identified.

4.3 The TaqI Polymorphism

The TaqI polymorphism had already been mapped to the 3' end of the α-fibrinogen gene (Humphries et al,1984). However, the restriction maps available for this region varied as to the number of HindIII restriction sites present. Courtois described two HindIII sites at approximately 1.4kb and 2.4kb from the end of the sequence of α-fibrinogen cDNA, +5443bp from the start of transcription, (personal communication), while Humphries and coworkers (1984) had a single site about 0.8kb from this point and Kant and colleagues (1985) had three. The strategy used initially involved digestion of genomic DNA form individuals of known TaqI genotype with HindIII and amplification using a single oligonucleotide sited at +5801, just 5' to the end of known sequence. Two main bands were produced of 0.7 and 1.0kb in size which implied that either there were two HindIII sites at around 0.7kb and 1.0kb from the oligonucleotide used, with partial digestion of genomic DNA, or that there were at least two sites with 0.7kb and 1.0kb between them and a repeated sequence that the oligonucleotide recognised in both. The bands produced on PCR were unexpectedly bright for single-stranded DNA and

therefore a restriction digest with both EcoRI and TaqI was attempted since EcoRI sites are present in this region and it was hoped that the fragments might contain the TaqI polymorphic site. There was digestion of some of the bands implying the the PCR product was double stranded DNA. The presence or absence of further bands on digestion did not correlate with the presence or absence of TaqI cutting site in individuals whose TaqI genotype had already been determined by Southern blotting. The results were compatible with the single oligonucleotide amplification producing double-stranded DNA, and therefore posed the question as to whether there were repeat sequences 5'-3' on the complementary DNA strand, effectively enabling the left hand oligonucleotide to be the right hand olignucleotide also.

In order to investigate this further, the Nαß clone was digested with HindIII and subcloned into a pUC18 vector. These results of this showed the presence of two fragments 0.7kb and 1.0kb in length and also insertion of a 1.7kb fragment probably due to partial digestion. These results supported the map of Kant and coworkers (1985) showing three HindIII restriction sites at the 3' end of the α-fibrinogen gene and was also compatible with both Humphries and coworkers' map (Humphries et al,1984) and the map of Courtois. Sequencing of these fragments revealed two TaqI sites, one within the 1.0kb fragment at 278bp 3' to the HindIII cloning site and one within the 0.7kb fragment, 285bp from the start of the sequence and thus about 300bp from the HindIII cloning site (figure 3.24). From the mapping results (Humphries et al,1984) a constant TaqI site lies 2.4kb 3' to the known TaqI site at +4833 thus giving it a position of +7233 with the

polymorphic TaqI site at 1.6kb 3' to this first site thus giving it a position of +6433 (figure 3.24). The best fit of the results assumes that the 0.7kb fragment contains the polymorphic TaqI site and the 1.0kb fragment the constant site (figure 3.28). Of particular interest was the fact that this constant site interupts an Alu repeat sequence which was identified 197bp downstream from the 5' end of the 1.0kb fragment.

Alu sequences are short interspersed repetitive DNA elements found throughout primate genomes, including Old World monkeys and apes and man (Britten et al,1989). Most eukaryotic DNA's also contain inverted repeated sequences that consist of complementary DNA sequences covalently linked in the same DNA strand (Schmid and Jelinek,1982). There are over $5x10^5$ Alu family members per haploid genome with an average spacing of about 4kb (Shen et al,1991). In general, Alu repeats are about 280bp in length and although they are a unique feature of primate genomes they show homology to the 130bp mouse B1 repeat element (Schmid and Jelinek,1982). Each element is composed of two homologous but non-identical fragments of about 130bp, Alu-left and Alu-right, separated by an A-rich region. Members of the Alu family have related DNA sequences but because of evolutionary divergence they are not identical. There are 18 individual diagnostic mutations associated with different subfamilies and each subfamily has a sequential accumulation of mutations (Shen et al,1991). Diagnostic mutations are a more accurate measure of the age of any individual Alu family member (Shen et al,1991). A consensus Alu sequence has been

derived, and individual copies show a high degree of sequence homology. It has been suggested, because of the sequence homologies, that Alu elements are derived from 7SL RNA (Ullu and Tschudi,1984), which forms part of the signal recognition particle invloved in translocation of proteins across the endoplasmic reticulum.

The function (or functions) of the short interspersed repeats in eukaryotic DNA's has been the subject of speculation since their discovery. The Alu family is the single most abundant family of interpersed repeats in mammalian genomes. From sequence analyses, they appear to be mobile DNA elements and may have profound influences on gene expression at some of their sites of insertion into chromosomal DNA. There are reports of Alu sequences being involved in genetic recombinations (Olds et al,1993), unequal crossing-over causing sequence duplication (Lehrman et al,1987a) and exon deletions (Lehrman et al,1987b) leading to abnormal or absent protein production.

The presence of an Alu repeat at the 3' end of the α-fibrinogen gene, in conjunction with possible repeat and inverted complementary sequences, tempts one to speculate that the Alu repeat may have been involved in the duplication and inversion events that took place at the fibrinogen gene cluster. Two TaqI sites have been identified by both sequencing and Southern blotting at the 3' end of the α-fibrinogen gene and their predicted sites from each method are in agreement. However, the fragment sizes obtained on digestion of PCR product of the

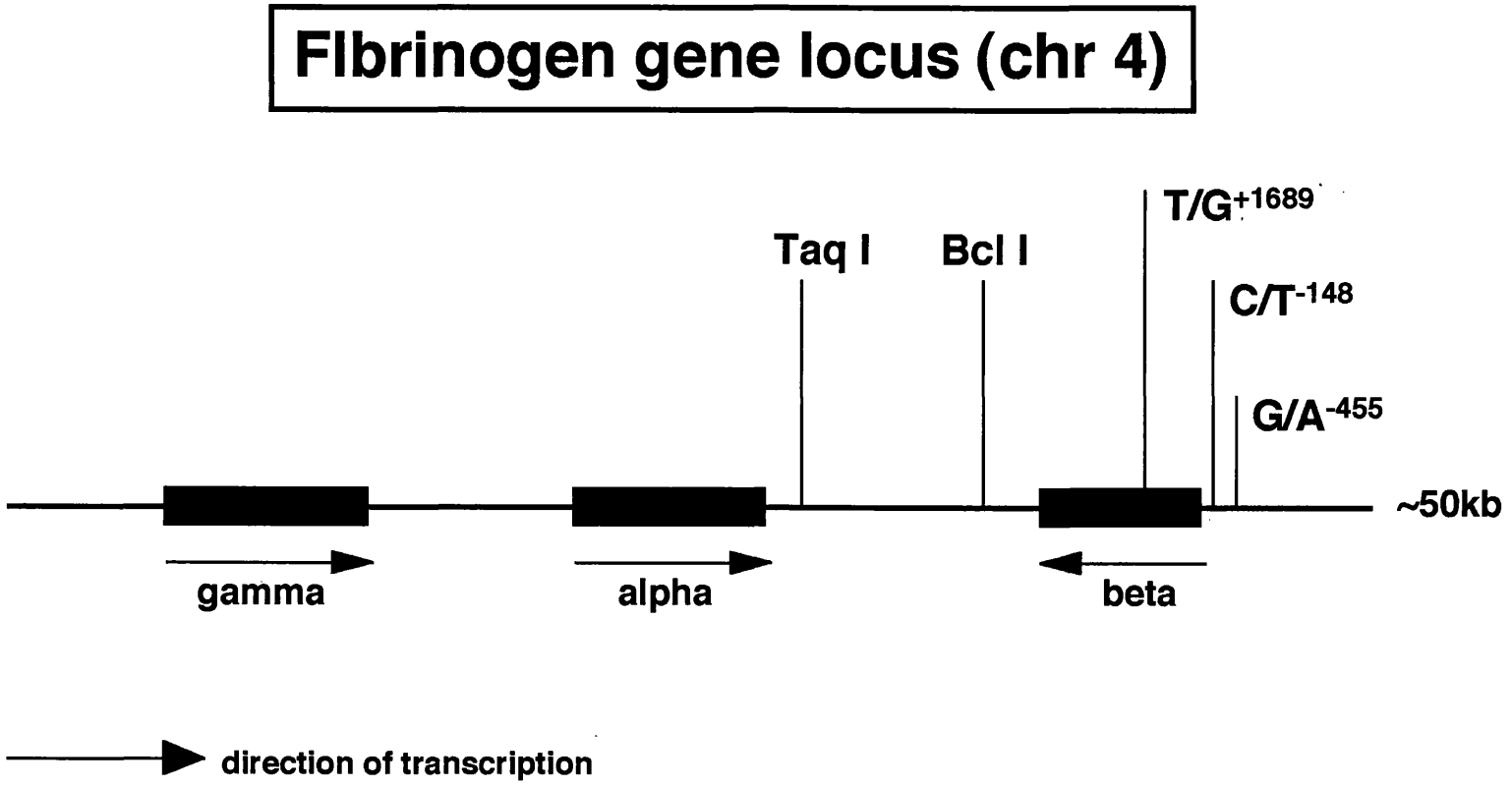
appropriate region do not correspond very well and further sequencing of the region would be needed to try to elucidate the underlying reason for this.

5. Frequency and linkage disequilibrium of the G/A^{-455}, C/T^{-148}, T/G^{+1689}, BclI and TaqI polymorphisms of the fibrinogen gene cluster in different ethnic groups: results.

Five polymorphisms of the fibrinogen gene cluster can now be investigated using the PCR technique rather than Southern blotting thus enabling large numbers of individuals to be genotyped rapidly. A sample of the general population of healthy middle aged men were studied, selected from four general practices in the Thrombosis Prevention Trial (TPT) (Meade et al,1988). Three further population samples were studied, each one of a different ethnic origin: white European Caucasian, Afrocaribbean and Gujurati Caucasian. These were recruited from a study on ethnic differences in insulin and plasma C-peptide (Cruikshank 1991). Ethnic group was determined by grandparental origin with at least three grandparents being from one ethnic group. DNA from 26 Thai individuals was sent for study by Professor Peake, Sheffield.

The locations within the fibrinogen gene cluster of the five variable sites investigated are shown in figure 5.1.

Figure 5.1


Fibrinogen gene locus (chr 4)
T/G+1689
Taq I
Bcl I
C/T-148
G/A-455
~50kb
gamma
alpha
beta
direction of transcription

5.1 Frequency and linkage disequilbrium of five fibrinogen gene alleles in a U.K. Caucasian population sample, TPT 1

The genotypes of 302 healthy middle-aged Caucasian men were determined by digestion of the appropriate PCR amplified DNA with HaeIII or BsuRI, HindIII, AvaII, BclI and TaqI. This gave their G/A^{-455}, C/T^{-148}, T/G^{+1689} and BclI ß-fibrinogen gene genotype and their TaqI α-fibrinogen gene genotype respectively. The allele frequencies were 0.81 for the G^{-455}, C^{-148} and T^{+1689} alleles, 0.85 for the B- allele and 0.73 for the T- allele. The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant differences were detected. The allele frequencies and the polymorphism information content (PIC) values are shown in table 5.1.

In this sample, the G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms of the ß-fibrinogen gene were found to be in complete linkage disequilibrium (except for one individual whose genotype was $G + A^{-455}$, $C + T^{-148}$, $T + T^{+1689}$). The ß-fibrinogen BclI polymorphism B- was in strong allelic association with the $G^{-455}C^{-148}T^{+1689}$ allele, $\Delta = 0.85$, $\chi^2 = 322$, $P < 0.001$ (table 5.2). However, linkage disequilibrium was weak between the TaqI polymorphism of the α-fibrinogen gene and all the ß-fibrinogen polymorphisms; BclI/TaqI: $\Delta = 0.01$, $\chi^2 = 1.49$, $P > 0.01$; G/A^{-455}/TaqI: $\Delta = -0.09$, $\chi^2 = 2.53$, $P > 0.01$ (table 5.2).

Table 5.1

Allele frequencies and PIC values for polymorphisms at the fibrinogen gene locus in UK Caucasian sample (TPT 1)

Polymorphism	G/A^{-455}		C/T^{148}		T/G^{+1689}		BclI		TaqI	
Allele designation	G^{-455}	A^{-455}	C^{-148}	T^{148}	T^{+1689}	G^{+1689}	B-	B+	T-	T+
Cutting site	+	-	+	-	-	+	-	+	-	+
Allele frequency	0.81	0.19	0.81	0.19	0.81	0.19	0.85	0.15	0.73	0.27
CI of rare allele*	0.16-0.22		0.16-0.22		0.16-0.22		0.12-0.18		0.23-0.20	
PIC value	0.26		0.26		0.26		0.20		0.32	
No chromosomes tested	596		596		596		586		552	

PIC = polymorphism information content

* Colton, 1974

Table 5.2

Linkage disequilibrium between polymorphisms of the fibrinogen gene cluster

Polymorphism	G/A^{-455}	C/T^{-148}	T/G^{+1689}	BclI	TaqI	
G/A^{-455}	*	1.0	1.0	0.85^{+}	$-0.09^{\$}$	
C/T^{-148}	*	*	1.0	0.85^{+}	$-0.09^{\$}$	
T/G^{+1689}	*	*	*	0.85^{+}	$-0.09^{\$}$	Δ
BclI	*	*	*	*	$0.01^{\$}$	
TaqI	*	*	*	*	*	

$^{+}$ $P < 0.001$; $^{\$}$ $P > 0.01$

5.2 Frequency and linkage disequilbrium of the G/A^{-455}, C/T^{-148} and T/G^{+1689} ß-fibrinogen gene polymorphisms in white European, Afrocaribbean, Gujurati and Thai population samples.

Almost complete linkage disequilibrium was shown between the G/A^{-455}, C/T^{-148} and T/G^{+1689} ß-fibrinogen gene polymorphisms in the white Caucasian population samples, with one allele combination four times more frequent than the other, but whether this was due to population admixture or selective advantage is unknown. Investigation of the polymorphisms in population samples of different ethnic origin would yield further information and the samples chosen were white European Caucasians, Gujurati Caucasians and Afrocaribbeans. The polymorphism frequencies and the linkage disequilibrium between them were determined in all samples and the base changes causing the polymorphisms were ascertained in the Afrocaribbeans. Allele frequency of

the G/A^{-455} and C/T^{-148} polymorphisms was determined in a small number of Thais.

5.2.1 Allele frequency in white European, Afrocaribbean, Gujurati and Thai population samples

The distribution of each of the genotypes was in Hardy-Weinberg equilibrium in each of the population samples. However, the frequency of the polymorphic alleles varied depending on the ethnic group. For the G/A^{-455} polymorphism, the Afrocaribbeans had a lower frequency of the rarer A^{-455} allele than the white Europeans and the Gujuratis, 0.02 (0.002-0.038) versus 0.17 (0.12-0.22) and 0.21 (0.16-0.26) respectively. The frequencies of the polymorphic alleles for the C/T^{-148} polymorphism and the T/G^{+1689} polymorphism were identical to those of the G/A^{-455} polymorphism in the white Europeans and Gujuratis. In the Afrocaribbeans, although the T/G^{+1689} polymorphism had the same allele frequencies as the G/A^{-455} polymorphism, the C/T^{-148} polymorphism had a rare allele frequency of 0.08 (0.04-0.12).

The G/A^{-455} and C/T^{-148} genotypes of 26 unrelated Thais (DNA kindly given by Professor Ian Peake, Sheffield) were determined, the rarer alleles both having a frequency of 0.26. The distribution of the genotypes was in Hardy-Weinberg equilibrium and was as follows: 16 with the genotype G+G^{-455}/C+C^{-148} , 11 with the genotype G+C^{-455}/C+T^{-148} and 2 with the genotype C+C^{-455}/T+T^{-148} .

Table 5.3

Allele frequencies of the G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms in different ethnic groups

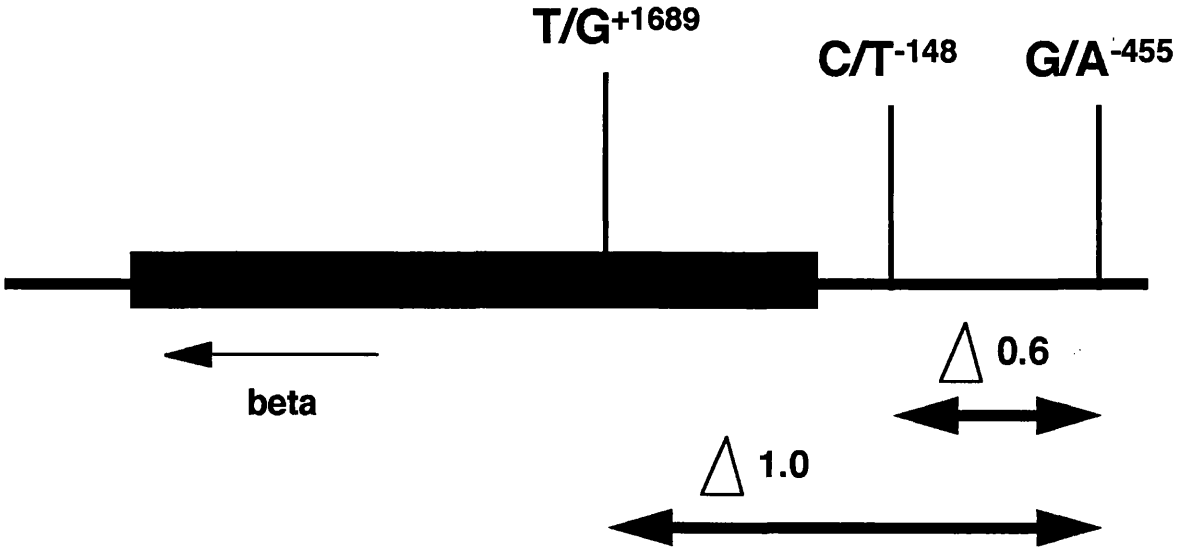
Population Sample [n]	Polymorphism frequency (CI)		
	G/A^{-455} A^{-455} allele	C/T^{-148} T^{-148} allele	T/G^{+1689} G^{+1689} allele
Afrocaribbean [112]	0.02 (0.002-0.038)	0.08 (0.04-0.12)	0.02 (0.002-0.038)
White European [94]	0.17 (0.12-0.22)	0.17 (0.12-0.22)	0.17 (0.12-0.22)
Gujurati [129]	0.21 (0.16-0.26)	0.21 (0.16-0.26)	0.21 (0.16-0.26)
Thai [26]	0.26 (0.14-0.38)	0.26 (0.14-0.38)	Not determined

5.2.2 Linkage disequilibrium of the polymorphisms in white European, Afrocaribbean, Gujurati and Thai population samples

The G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms were in complete linkage disequilibrium in the Caucasian population samples studied, that is the white European and the Gujurati groups (446 alleles), but not in the Afrocaribbean sample. In this group, 224 alleles were analysed and although the G/A^{-455} and the T/G^{+1689} polymorphisms were in complete linkage disequilibrium, the C/T^{-148} was not with the other two (figure 5.2). There were only 52 alleles available for testing in the Thai sample and the G/C^{-455} and C/T^{-148} polymorphisms were in complete linkage disequilibrium.

Figure 5.2

Beta fibrinogen gene showing T/G^{+1689}, C/T^{-148}, G/A^{-455} polymorphic sites and linkage disequiiibrium coefficients in Afrocaribbeans.



5.3 Identification of the base changes resulting in the HaeIII and HindIII polymorphisms of the ß-fibrinogen gene in Afrocaribbeans by direct sequencing

The differences in both the frequency of the G/A^{-455}, C/T^{-148} and T/G^{+1689} alleles and the different linkage disequilibrium between them in the Afrocaribbean population sample compared to the white European and Gujurati population samples prompted the question as to whether the polymorphisms were due to same base changes that had been identified in the TPT 1 sample (Dr F Green, personal communication).

The region spanning 1300bp, from -1178 to +122bp from the start of transcription, was amplified by PCR. This region was partially sequenced directly in four Afrocaribbeans of different combined G/A^{-455}, C/T^{-148} genotypes determined by the restriction enzymes HindIII and HaeIII, to identify the base changes causing the polymorphisms. The genotypes sequenced in the Afrocaribbeans were as follows:

1 $A+A^{-455}/T+T^{-148}$;

2 $G+G^{-455}/C+C^{-148}$;

3 $G+A^{-455}/C+T^{-148}$;

4 $G+G^{-455}/C+T^{148}$

The positions of the primers used, designed from known sequence (Chung et al,1983a) are shown in figure 5.3 and their sequence was as follows:

a) 5'-3' CCT GAT TGC AAC ACA CAA GTG AAC AGA C

b) 5'-3' i) [GGG] TTT AAT GGC CCC TTT TGA A

ii) [GGG] TTT AAT AGC CCC TTT TGA A

c) 5'-3' iii) [CCC] ACA TCT TCC CAG CAA AGC TTA TTT AC

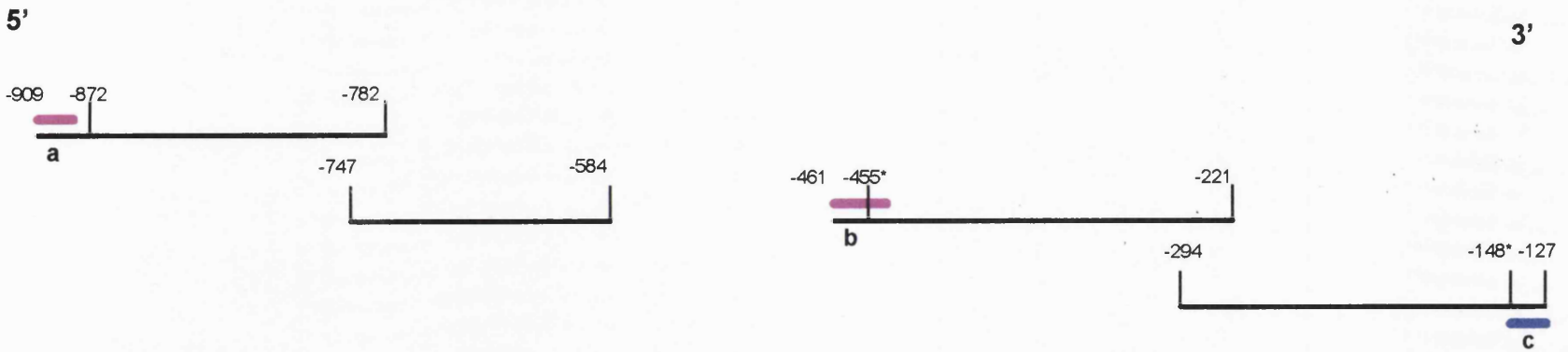
ii)[CCC] ACA TCT TCC CAG CAA AAC TTA TTT AC

[NNN] denotes part of the oligonucleotide which does not occur in wild type sequence. Two oligonucleotides, one for each of the alleles, were designed for (b) and (c). (a) and (b) are both forward primers and (c) is a reverse primer.

The sequence determined for -872bp to -584bp from the start of transcription using oligonucleotide (a) was the same as published by Chung and coworkers (Chung et al,1983a), with the exception that there were possible base pair repeats in two places. The sequence is shown at appendix 6.

Figure 5.3

Cartoon of areas sequenced in four Afrocaribbeans each of different G/A^{-455}, C/T^{-148} genotype combination with the oligonucleotide primers shown in the 5'-flanking region of the β-fibrinogen gene



-455* denotes the G/A polymorphic site

-148* denotes the C/T polymorphic site

L.H. oligonucleotide; a,b described in the text

R.H. oligonucleotide;c, described in the text

Further sequence from -294bp to -127bp using oligonucleotide (b) and -221bp to -461bp using oligonucleotide (c) (figure 5.3) was determined in individuals both homozygous and heterozygous for the G/A^{-455} and C/T^{-148} alleles, including the individual with the genotype G+G^{-455}/C+T^{-148}, and in all cases, the base changes at the polymorphic alleles were as previously described; that is, G/A^{-455} and C/T^{-148} as shown in figures 5.4, 5.5 and 5.6.

Figure 5.4 Sequence from -456 to -127 from the start of transcription (individuals 1-4). The HaeIII and the HindIII polymorphic sites are shown in bold with the base changes shown underneath the main sequence.

T**GGCC** CCTTT TGAAA TAGAA TTATG TCATT GTCAG AAAAC ATAAG
A^{-455}

CATTT ATGGT ATATC ATTAA TGAGT CACGA TTTTA GTGGT TGCCT

TGTGA GTAGG TCAAA TTTAC TAAGC TTAGA TTTGT TTTCT CACAT

ATTCT TTCGG AGCTT GTGTA GTTTC CACAT TAATT TACCA GAAAC

AAGAT ACACA TCTCT CTTTG AGGAG TGCCC TAACT TCCCA TCATT

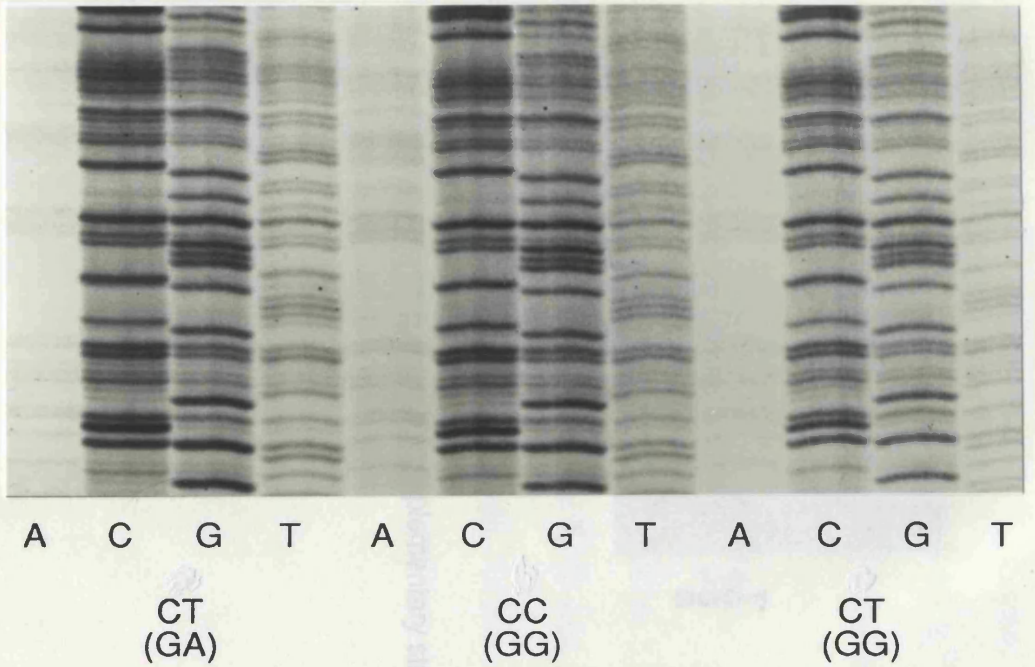
TTGTC CAATT AAATG AATTG AAGAA ATTTA ATGTT TCTAA ACTAG

ACCAA CAAAG AATAA TAGTT GTATG

ACAAG TAAAT **AAGCT T**TGCT GGGAA GATGT TGCTT
T^{-148}

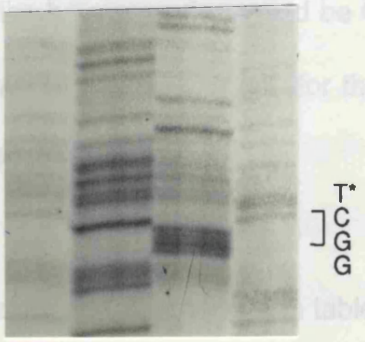
Fig 5.5

Sequencing gel of the β fibrinogen promoter in 3 Afrocaribbean individuals of known C/T $^{-148}$ genotype. The G/A $^{-455}$ genotype of the individual is shown in brackets.



* polymorphic Hind III restriction site

Fig. 5.6 Autoradiograph of the sequencing gel of the 5' flanking region of the β fibrinogen promoter in an Afrocaribbean individual homozygous for the rarer (AA) allele of the G/A $^{-455}$ polymorphism. This disrupts the restriction site recognised by the enzyme HaeIII (GGCC). The complementary strand has been sequenced.



T C G T

Genotype AA

* Polymorphic G/A $^{-455}$ site on complementary strand

5.4 Establishing the phase of the G/A^{-455} and C/T^{-148} alleles in the Afrocaribbean and Gujurati population samples.

It was of interest to determine the phase of the G/A^{-455} and C/T^{-148} alleles in individuals from the Afrocaribbean population because of the loss of complete linkage disequilibrium. In the Caucasians, since the homozygotes G+G^{-455} always occurred with the homozygotes C+C^{-148}, it could be inferred with confidence that the alleles in the heterozygotes would be G^{-455}+C^{-148}/C^{-455}+T^{-148}. However, seven Gujuratis who were heterozygotes for the two polymorphisms also had the phase of the allele determined.

The genotypes of the individuals studied are shown in table 5.3. To establish the phase of the alleles the appropriate region of DNA was digested with the restriction enzyme HaeIII and, after blotting onto Hybond-N, hybridised with oligonucleotide probes specific for the C^{-148} and T^{-148} alleles as described in chapter 2.

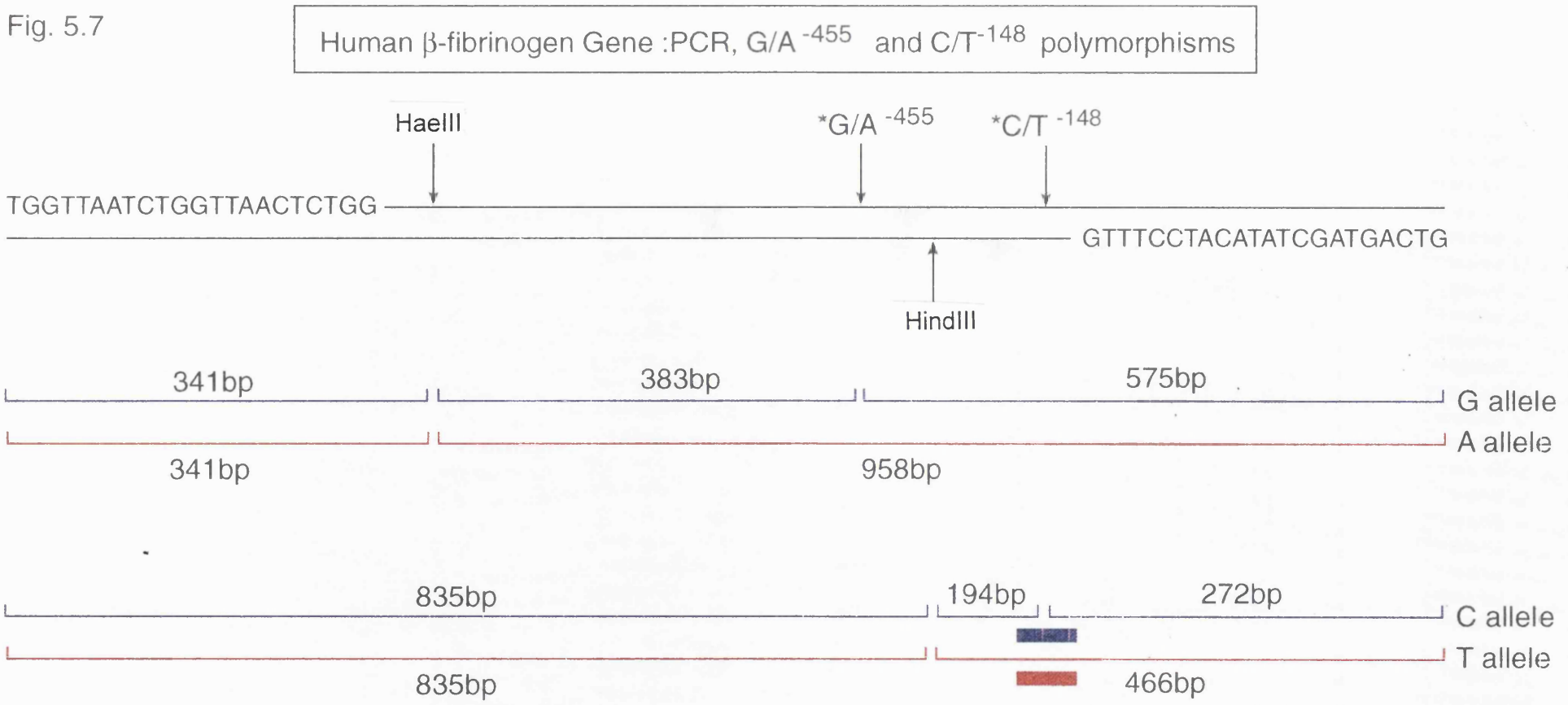
Table 5.4

Genotypes of individuals used to determine allelic phase

	-455	-148	Number
Afrocaribbean	G/G	C/T	2
Afrocaribbean	G/G	C/C	1
Afrocaribbean	A/A	T/T	1
Afrocaribbean	G/A	C/T	6
Gujurati	G/G	C/C	2
Gujurati	G/A	C/T	2
Gujurati	A/A	T/T	3

If the two alleles are in phase, then the C^{-148}-specific oligonucleotide will hybridize to the 575bp fragment (blue, figure 5.7) of the HaeIII digest, only present in those individuals possessing the G^{-455} allele. Conversely, the T^{-148}-specific oligonucleotide will hybridize to the 959bp fragment (red, figure 5.7) of the HaeIII digest, only present in those possessing the A^{-455} allele. This was the case in DNA from all individuals tested except in the Afrocaribbeans of the genotype $G+G^{-455}/C+T^{-148}$ where both the C^{-148}-specific oligonucleotide and the T^{-148}-specific oligonucleotide hybridized to the 575bp fragment of the HaeIII digest (figures 5.8 and 5.9). The six Afrocaribbeans with genotype $G+A^{-455}/C+T^{-148}$ showed that the G^{-455} always occurred with the C^{-148} allele. This was also the case for all the Gujuratis tested.

Fig. 5.7



If the polymorphisms are in phase a) the C allele probe hybridizes to the G allele fragment size 575 bp
b) the T allele probe hybridizes to the A allele fragment size 958 bp

Oligonucleotide primers for PCR amplification of the G/A^{-455} and C/T^{-148} polymorphic sites. Allele specific probes for the C/T-148 polymorphism are shown: C allele T allele

Fig. 5.8 HaeIII digested PCR product -1178bp to +122bp from the start of transcription of the β-fibrinogen gene, hybridised with a radiolabelled probe specific for the C allele of the C/T^{-148} polymorphism.
Homozygotes for the T^{-148} allele show no hybridization of the C allele probe to either fragment of the HaeIII digested PCR product.
Individuals of genotypeG+G^{-455}/ C+C^{-148} and G+G^{-455}/C+T^{-148} show hybridisation of the probe to the 575bp fragment, showing that the G^{-455} and C^{-148} alleles are in phase.

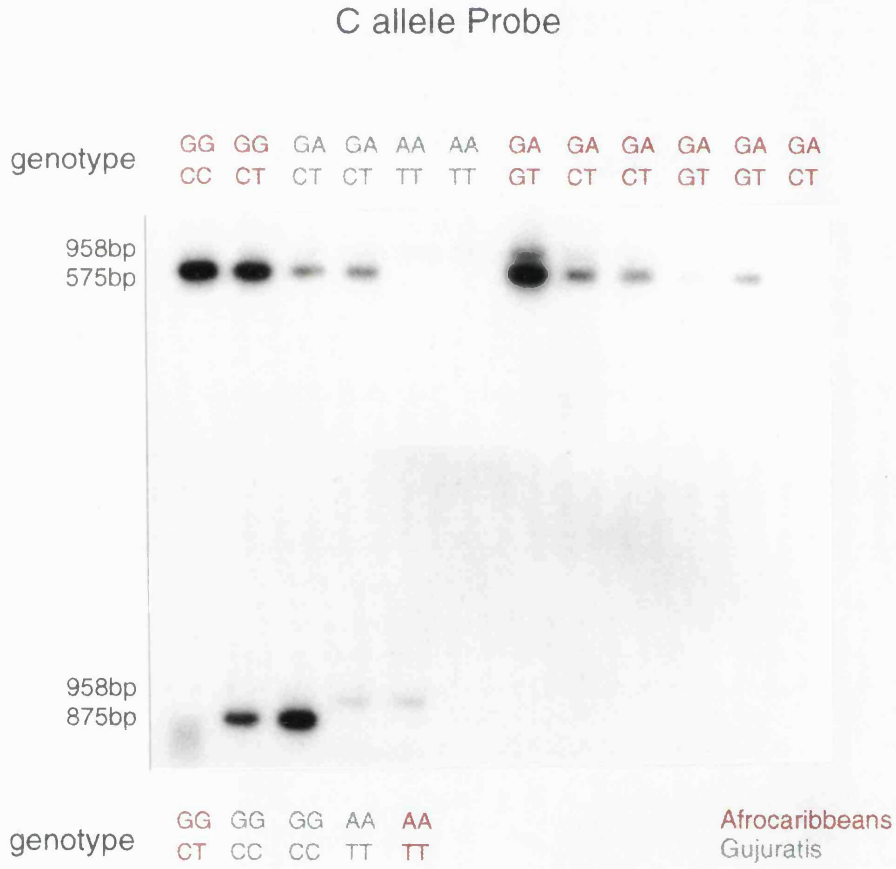
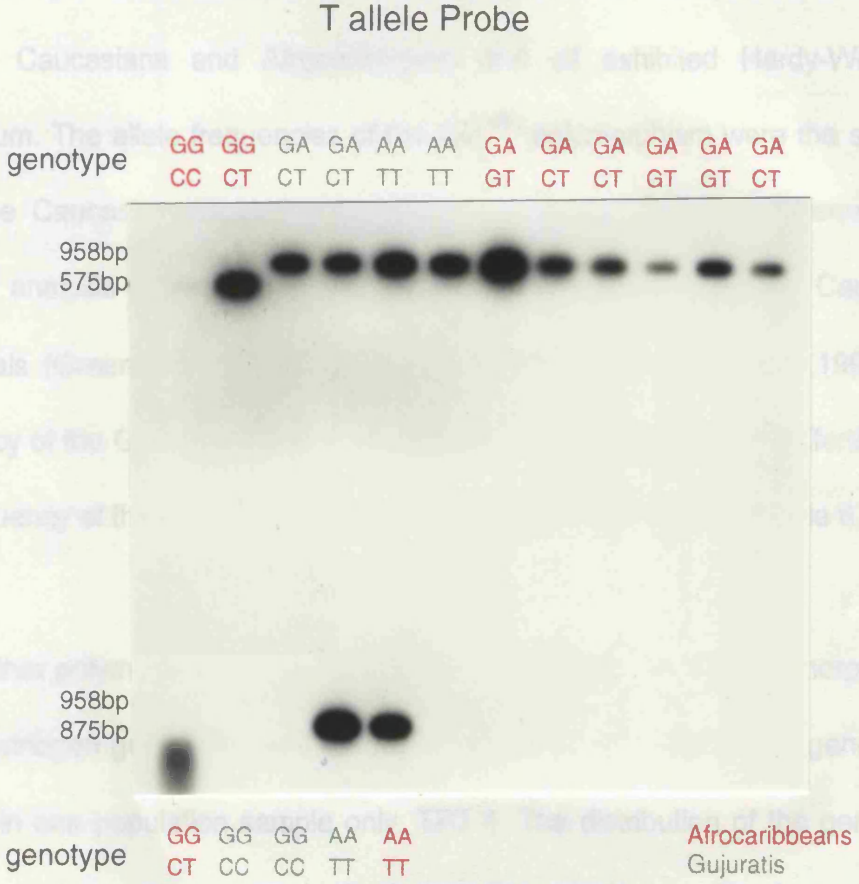


Fig. 5.9 HaeIII digested PCR product -1178bp to +122bp from the start of transcription of the β-fibrinogen gene, hybridised with a radiolabelled probe specific for the T allele of the C/T^{-148} polymorphism.
Homozygotes for the C^{-148} allele show no hybridization of the T allele probe to either fragment of the HaeIII digested PCR product.
Individuals of genotype A+A^{-455}/ T+T^{-148} and G+A^{-455}/C+T^{-148} show hybridisation of the probe to the 958bp fragment, showing that the A^{-455} and T^{-148} are in phase. The individual of genotype G+G^{-455}/C+T^{-148} (lane 2) shows hybridisation of the probe to the 575bp fragment confirming that the G^{-455} and T^{-148} alleles are in allelic association in this individual.



6. Frequency and linkage disequilibrium of polymorphisms of the fibrinogen gene cluster: discussion

6.1 Allelic frequencies of polymorphisms of the fibrinogen gene cluster

The distribution of genotypes for the G/A^{-455} polymorphism of the ß-fibrinogen gene was determined in a Caucasian population sample, TPT 1, and subsequently in three further population samples, white European Caucasians, Gujurati Caucasians and Afrocaribbeans and all exhibited Hardy-Weinberg equilibrium. The allele frequencies of the G/A^{-455} polymorphism were the same in the three Caucasian population samples studied. Comparing the frequencies, using χ^2 analysis with Yate's correction, with those of other studies in Caucasian individuals (Green et al,1993; Scarabin et al,1993; Humphries et al,1995), the frequency of the G^{-455} allele in the other studies was not significantly different from the frequency of those in the study presented in this thesis (TPT 1) (table 6.1).

Four further polymorphisms, the C/T^{-455}, the T/G^{+1689} and the BclI polymorphism of the ß-fibrinogen gene and the TaqI polymorphism of the α-fibrinogen gene, were studied in one population sample only, TPT 1. The distribution of the genotypes for all the polymorphisms were in Hardy-Weinberg equilibrium. The frequencies of the TaqI polymorphism were not significantly different from those in other studies (Humphries et al,1987; Berg and Keirulf,1989; Fowkes et al,1992) (table 6.2) but the frequencies of the BclI polymorphism were significantly different from two of

the studies (Humphries et al,1987; Berg and Keirulf,1989) but not the third (Fowkes et al,1992). All these studies were conducted in healthy males except the study of Humphries et al which included males and females.

Table 6.1

Frequency of the rare allele of the G/A^{-455} and C/T^{148} ß-fibrinogen gene polymorphisms

Study	Country/Ethnic origin	Number	G/A^{-455}	
			frequency	95% CI
TPT 1	UK all	292	0.19	0.16-0.22
Green et al,1993	Sweden all	86	0.25	0.20-0.31
Scarabin et al,1993	UK and France	648	0.21	0.19-0.23
Humphries et al,1995	Finland	180	0.21	0.16-0.25
	GB	113	0.20	0.15-0.26
	North European	308	0.23	0.20-0.26
	Middle European	240	0.22	0.18-0.26
	South European	265	0.21	0.18-0.25

Table 6.2

Frequency of the rare allele of the BclI and TaqI polymorphisms of the fibrinogen gene cluster

Study	Country (town)	Number	BclI		TaqI	
			frequency	95% CI	frequency	95% CI
Humphries et al,1987	UK (London)	91	0.25	0.19-0.32	0.28	0.22-0.35
Berg & Keirulf,1989	Norway	118	0.17	0.13-0.23	0.27	0.22-0.33
Fowkes et al,1992	UK (Edinburgh)	126	0.10	0.07-0.15	0.25	0.14-0.23
TPT 1	UK (London)	293	0.15	0.12-0.18	0.27	0.24-0.31

Comparison of BclI allele frequencies:

Fowkes v Humphries: $\chi^2 = 16.05, P < 0.001$; Fowkes v Berg: $\chi^2 = 4.62, P < 0.05$;

Fowkes v Thomas: $\chi^2 = 3.50$, NS; Thomas v Humphries: $\chi^2 = 8.48, P < 0.01$

6.2 Linkage disequilibrium at the fibrinogen gene locus

From the mapping data, three of the five polymorphisms were identified at the 5' end of the ß-fibrinogen gene, the G/A^{-455}, C/T^{-148} and T/G^{+1689} while the BclI polymorphism was at the 3' end of the ß-fibrinogen gene and the TaqI polymorphism was at the 3' end of the α-fibrinogen gene. All five polymorphisms were studied in one population sample only and the G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms were found to be in complete linkage disequilibrium (except for one individual described in the results). The BclI polymorphism was in strong allelic association with these polymorphisms but the TaqI polymorphism showed only weak disequilibrium between it and all the other ß-fibrinogen gene polymorphisms.

A population group may start with a non-random distribution of allelic variable sites at a gene locus, that is some haplotypes occur more frequently than expected based on the product of their allele frequencies, but repeated crossing over will randomize the arrangement and in the end two variable sites will be randomly associated. The time within which equilibrium will be reached depends on the genetic distance of the two sites. The closer the two sites are, the longer will be the time until equilibrium is reached. It thus follows that linkage disequilibrium can be observed when two populations homozygous for two different haplotypes have mixed a relatively short time ago and so far cross-over at a low rate has not been sufficient to lead to random distribution of the alleles. An alternative explanation is

that certain combinations of allele on closely linked gene loci have caused a selective advantage for their bearers and have therefore been preserved. In order to investigate whether the linkage disequilibrium seen for the ß-fibrinogen gene polymorphisms was more likely to be due to population admixture or selective advantage, an extended genotype in population samples from different ethnic backgrounds was determined. This study, described in chapter 5.2, showed that the rarer alleles of the β-fibrinogen gene in the Caucasian population samples, the A^{-455}, T^{-148} and G^{+1689}, had a frequency between 0.17 and 0.20 but were significantly less frequent in the Afrocaribbean sample, 0.02, 0.08 and 0.02 respectively, and that the complete linkage disequilibrium that existed in the Caucasian population samples was not so for the Afrocaribbean population sample. The frequency of the rarer alleles in the Thai population sample was not statistically different from the Caucasians and the G/A^{-455} and C/T^{-148} polymorphisms too were in complete linkage disequilibrium. In order to demonstrate that the polymorphisms were most likely to have the same origin, the 5' flanking region of the β-fibrinogen gene spanning both the G/A^{-455} and C/T^{-148} polymorphisms was sequenced in Afrocaribbeans and Caucasians.

6.3 Establishing the evolutionary origin of the polymorphisms

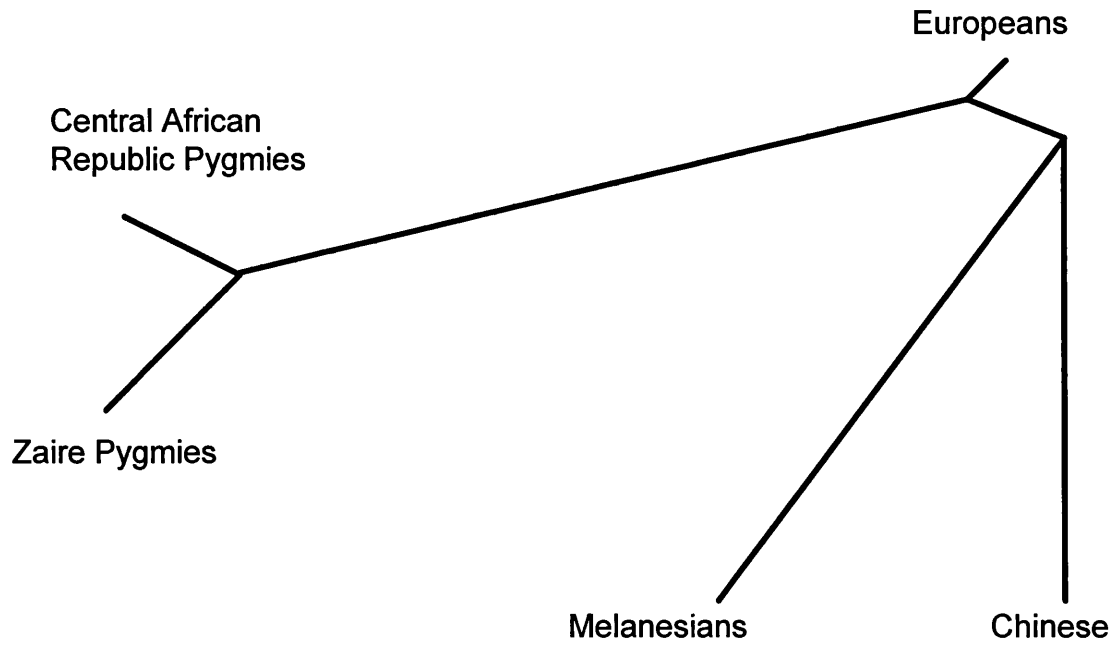
Sequencing the 5'-flanking region of the β-fibrinogen gene promoter region revealed that the base changes involved in the G/A^{-455} and C/T^{-148} polymorphisms

in the Caucasians were the same as those in the Afrocaribbeans, including individuals with the genotype $G+G^{-455}/C+T^{-148}$. This strongly supports the hypothesis that the mutations occurred only once and that there is a common ancestor. To support this hypothesis further, the phase of the alleles was established and this showed that the G^{-455} allele always occurred with the C^{-148} allele and the A^{-455} allele with the T^{-148} allele except in those individuals with the genotype $G+G^{-455}/C+T^{-148}$.

Several groups of workers have confirmed the hypothesis that the earliest divergence in human evolution separated Africans and non-Africans (Wainscoat et al,1986; Cavalli-Sforza et al,1988; Bowcock et al,1988). Further studies by Bowcock and colleagues (1988) suggest that the second fission separated Melanesians from Chinese plus Europeans. The data were then found to be consistent with admixture between a branch from the Chinese after their separation from the Melanesians and the branch leading to the two African populations. It seems likely that Europeans are a mixture of 65% Chinese ancestors and 35% African ancestors (figure 6.1). This model fits the data from the fibrinogen polymorphism study also. It is possible that there were two β-fibrinogen gene alleles, $G^{-455}\ C^{-148}\ T^{+1689}$ and $C^{-455}\ T^{-148}\ G^{+1689}$, the former being the commoner. At the point of the first separation, the rare allele as well as the common allele was present in the non-African population but at a higher frequency than in the Afrocaribbeans. At the point of admixture of the Chinese and African populations, forming the Europeans, the frequency of the alleles might change

again with a reduction of the rare allele frequency in the Europeans compared with the Chinese/Asians. However, this since this admixture was estimated to occur about 30,000 years ago, there may not yet be a demonstrable change in allele frequency. This assumes that the changes in frequency are not due to selective advantage. The differences in linkage disequilibrium between the G/A^{-455} and C/T^{-148} polymorphisms in the Afrocaribbeans can be explained by postulating that there has been a cross over between the two alleles which occurred either after the first separation or before, but that no individual with the $G+G^{-455}/C+T^{-148}$ configuration emigrated. Since the frequency of the G^{-455} T^{-148} allele is approximately 0.06 it seems more likely that it occurred early, that is before the first separation.

Figure 6.1 Cartoon of evolutionary genetic tree



Adapted from Bowcock et al,1988

The differences in allele frequency between the population samples studied could be due to either natural selection or neutral variation. If the variation is due to genetic drift alone, that is neutral variation, then although the difference in allele frequency between populations will vary from gene to gene, the extent of the variation will be predictable. However, if natural selection is the reason for the frequency variation, and if in some environments one allele is favoured while in another the other allele is favoured, then the variation for that gene frequency is expected to be higher than for a gene affected by genetic drift alone. If, on the other hand, natural selection favours the heterozygote over both the homozygotes, then the frequency variation will be lower than average. A further study of the relationship between the genotype $G+G^{-455}/C+T^{-148}$ and plasma fibrinogen levels, and in turn the effect of the latter on the health of Afrocaribbeans, may also help to identify which is the more likely explanation of the frequency variation. Furthermore, the loss of complete linkage disequilibrium between the G/A^{-455} and C/T^{-148} polymorphisms uncouples the possible synergistic action between them and this can be exploited in studies trying to determine the influences and interactions of the polymorphisms on transcription, RNA stability or levels of plasma fibrinogen.

7. Association between variation at the fibrinogen gene locus and plasma fibrinogen levels in smokers and non-smokers: results

Plasma fibrinogen level is a well-established risk factor for ischaemic heart disease. Genetic heritability has been shown to account for up to 51% of the variance in plasma fibrinogen levels in one study (Hamsten et al, 1987). This shows that there is a strong genetic component determining plasma fibrinogen levels and thus the observed variation between individuals is in part genetically determined. Furthermore, another study using genetic polymorphisms at the fibrinogen gene cluster has estimated that variation at this locus is responsible for at least 15% of the variance in plasma fibrinogen levels (Humphries et al, 1987). This present study was undertaken to confirm these findings in a larger sample and to examine the effect of extended genotype on plasma fibrinogen levels. Interaction between genetic variation and environmental factors in determination of plasma fibrinogen levels was assessed.

7.1. Thrombosis Prevention Trial Study 1 (TPT 1)

The sample population studied were healthy middle aged men, selected from four general practices in the Thrombosis Prevention Trial (TPT) (Meade et al,1988). Of note, exclusion criteria included any history of ischaemic heart disease or contraindication to warfarin therapy. Preliminary investigation of this sample had already shown that the G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms were in

complete linkage disequilibrium (except one of 596 chromosomes tested) (Fig 7.1) and therefore just one of these, the G/A^{-455}, was used. The other two polymorphisms investigated were those detectable using the restriction enzymes BclI and TaqI, of the ß-fibrinogen and α-fibrinogen genes respectively. The G/A^{-455} and BclI polymorphisms of the ß-fibrinogen gene and the TaqI polymorphism of the α-fibrinogen gene were determined by HaeIII or BsuRI, BclI and TaqI digestion respectively of the appropriate PCR amplified DNA. The primers and conditions used for PCR amplification are given in the materials and methods section as are the statistical methods used.

7.1.1 Association between G/A^{-455}, BclI and TaqI genotypes and plasma fibrinogen levels

The characteristics of the smokers and non-smokers are given in table 1 and as expected smokers have a significantly higher fibrinogen level than non-smokers, 2.93g/l versus 2.61g/l, $P<0.0001$. There is no significant difference in age or body mass index between the two groups.

Figure 7.1

Beta fibrinogen gene showlng TaqI, BclI, C/T^{-148}, G/A^{-455} polymorphlc sites and iinkage disequiiibrium coefficients in Caucasians.

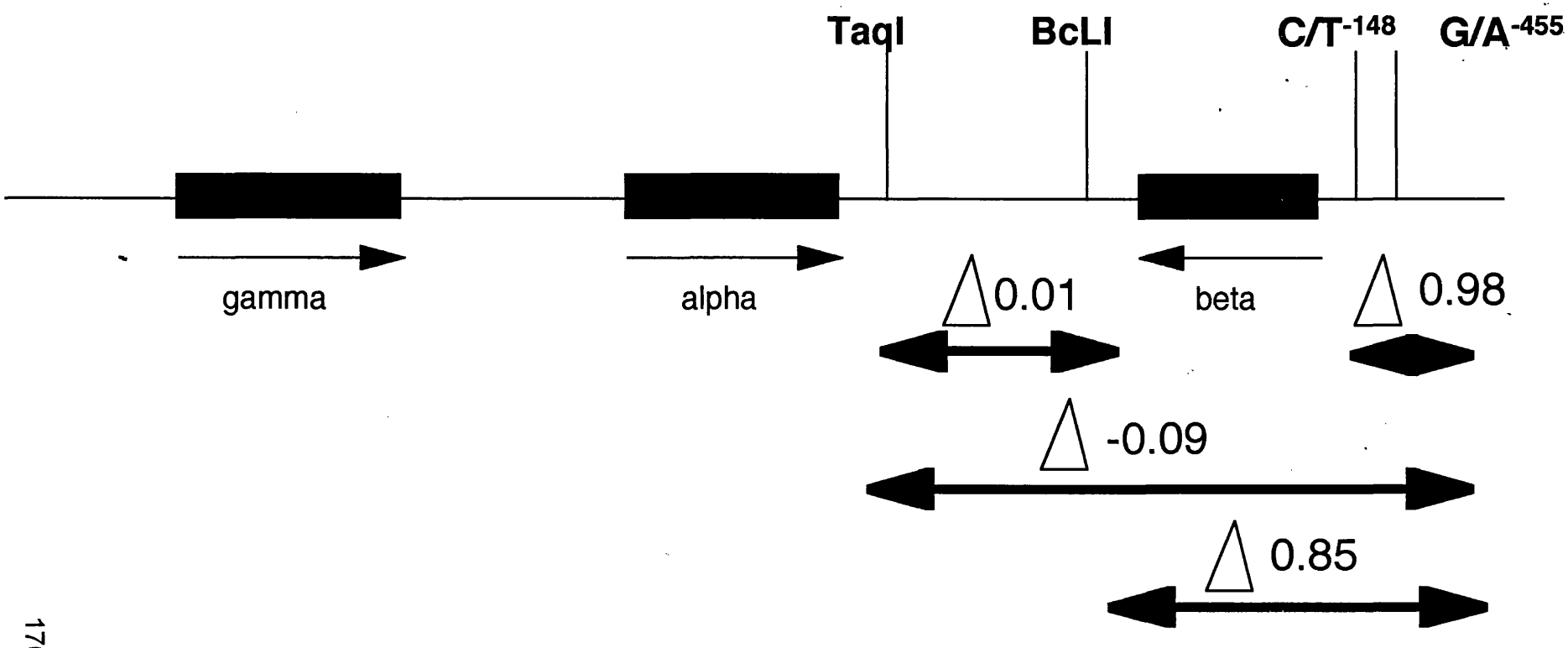


Table 7.1

Fibrinogen, Age and BMI by smoking status

Smoking status (n)	Fibrinogen (g/l) 95% CI	Age (years) (SD)	BMI (SD)
Non-smoker (172)	**2.61** 2.53-2.69	**55.5** (5.8)	**25.4** (2.8)
Smoker (120)	**2.93*** 2.81-3.05	**55.3** (5.8)	**25.6** (2.9)

* P<0.0001

Of the individual polymorphisms, only the G/A^{-455} in the non-smokers showed a significant association with unadjusted plasma fibrinogen levels, with those homozygous for the A^{-455} allele having 12.2% higher levels than those homozygous for the G^{-455} allele (P=0.03). In the smokers there was a trend, although not significant, towards higher fibrinogen levels in those possessing the A^{-455} allele, with those homozygous for this allele having fibrinogen levels 15.5% higher than those homozygous for the G^{-455} allele. (table 7.2). The effect associated with the A^{-455} allele, average excess, (Templeton, 1988) is similar in smokers and non-smokers, raising the fibrinogen by 0.17g/l and 0.18g/l respectively. For the BclI polymorphism (ß-fibrinogen) highest plasma fibrinogen levels were observed in the heterozygotes and the lowest in those homozygous for the non-cutting allele for both smokers and non-smokers (table 7.2). The numbers were small in those homozygous for the cutting allele. This is similar to the trend seen in a previous study (Humphries et al,1987).

Table 7.2

Unadjusted plasma fibrinogen levels according to genotype and smoking status

RFLP	Smoking status	Fibrinogen g/l 95% CI [n]			P* value
G/A^{-455}	Smoker	GG	GA	AA	0.13
		2.84 2.70-2.98 [75]	**3.07** 2.84-3.33 [42]	**3.28** 1.60-6.76 [3]	
	Non-smoker	**2.54** 2.45-2.63 [115]	**2.74** 2.58-2.92 [49]	**2.85** 2.29-3.55 [8]	**0.03**
BclI	Smoker	B-B-	B-B+	B+B+	0.16
		2.85 2.72-2.99 [83]	**3.12** 2.85-3.41 [35]	**2.96** 2.01-4.36 [2]	
	Non-smoker	**2.55** 2.46-2.64 [123]	**2.77** 2.57-2.98 [37]	**2.61** 1.92-3.57 [4]	0.10
TaqI	Smoker	T-T-	T-T+	T+T+	0.52
		2.93 2.77-3.10 [62]	**2.97** 2.78-3.18 [48]	**2.67** 2.26-3.15 [7]	
	Non-smoker	**2.66** 2.54-2.78 [88]	**2.55** 2.41-2.69 [62]	**2.49** 2.24-2.76 [9]	0.37

* One way analysis of variance

For the TaqI polymorphism (α-fibrinogen), no significant association was shown with plasma fibrinogen levels in smokers or in non-smokers. However, in both

groups a trend toward lower levels in those with one or two cutting alleles was observed, with those homozygous for T+ having 8.9% lower levels than those homozygous for T- in smokers and 6.4% lower levels in non-smokers (table 7.2).

7.1.2 Association between extended genotype and plasma fibrinogen levels

The three polymorphisms investigated in this study span approximately 23kb of DNA which includes the intergenic region between the α- and ß-fibrinogen genes, the ß-fibrinogen gene and the 5'-flanking region of the ß-fibrinogen gene and are illustrated in figure 5.1. To what extent further information about plasma fibrinogen levels is gained using an extended genotype has been assessed.

Combined genotypes were devised by counting the number of alleles in any given genotype combination that were associated higher fibrinogen levels. Although some associations did not reach significance in the TPT 1 population sample, the trend was similar to that seen in a previous study (Humphries et al,1987) and the designated 'fibrinogen-raising alleles' determined from this. These were the A^{-455}, the BclI cutting and the TaqI non-cutting alleles. Thus, for an extended genotype involving two different polymorphisms, there are five categories having 0,1,2,3 or 4 'fibrinogen-raising alleles'. For the extended genotype involving all three polymorphisms, there are seven possible categories. The association between plasma fibrinogen levels and combined genotypes was investigated and the most significant effect was seen with the combination of the G/A^{-455} and TaqI genotypes

(table 7.3). This reached significance in the non-smokers but not in the smokers. The number of categories of genotypes was reduced to investigate whether the presence of 3-4 'fibrinogen-raising alleles' compared with the presence of 0-2 'fibrinogen-raising alleles' showed a more significant difference. In the non-smokers, fibrinogen levels were 17% higher (2.95g/l versus 2.52g/l, P=0.0001) and in smokers, levels were 10% higher (3.15g/l versus 2.87g/l, P=0.07).

Table 7.3

Mean* plasma fibrinogen level by combined HaeIII and TaqI genotype and smoking status

G/A^{-455}/TaqI genotype (No fibrinogen raising alleles)	Fibrinogen g/l 95% CI [n]			
	Smoker		Non-smoker	
AA/T-T- (4)	**3.28** 2.17-4.98 [3]	**3.15** 2.83-3.50 [26]	**3.10** 2.20-4.36 [4]	**2.95** 2.72-3.20 [33]
AA/T-T+ GA/T-T- (3)	**3.13** 2.78-3.53 [23]		**2.93** 2.68-3.20 [29]	
GG/T-T- AA/T+T+ GA/T-T+ (2)	**2.86** 2.70-3.03 [55]	**2.87** 2.75-3.00 [91]	**2.52** 2.43-2.62 [74]	**2.52** 2.44-2.61 [126]
GG/T-T+ GA/T+T+ (1)	**2.95** 2.70-3.22 [29]		**2.52** 2.35-2.70 [46]	
GG/T+T+ (0)	**2.67** 2.62-3.15 [7]		**2.57** 2.20-3.00 [6]	
P value+	0.35	0.07	**0.0036**	**0.0001**

* geometric, unadjusted

+ from analysis of variance on log-transformed, unadjusted fibrinogen values

The combination of the G/A^{-455} and BclI genotype did not show any significant association with plasma fibrinogen levels although a trend towards higher levels with increasing numbers of 'fibrinogen-raising alleles' was seen both in the

smokers and non-smokers (table 7.4). However, when the number of genotype categories was reduced to two, comparing the presence of 2-4 'fibrinogen-raising alleles' with 0-1 'fibrinogen-raising alleles', in the non-smokers the fibrinogen levels were 8% higher (2.75g/l versus 2.55g/l, $P<0.05$) and in the smokers 9% higher (3.10g/l versus 2.85g/l, $P=0.06$) (table 7.4).

Table 7.4

Mean* plasma fibrinogen levels by combined G/A^{-455} and BclI genotype and smoking status

G/A^{-455}/BclI genotype (No fibrinogen raising alleles)	Fibrinogen g/l 95% CI [n]			
	Smoker		Non-smoker	
AA/B+B+ (4)	**3.05** - [1]	**3.10** 2.86-3.36 [38]	**2.61** 1.92-3.57 [4]	**2.75** 2.65-2.84 [42]
GA/B+B+ AA/B-B+ (3)	**3.38** 0.42-27.0 [2]		**3.53** 0.62-20.12 [2]	
AA/B-B- GG/B+B+ GA/B-B+ (2)	**3.09** 2.83-3.37 [35]		**2.72** 2.53-2.93 [36]	
GA/B-B- GG/B-B+ (1)	**3.01** 2.26-4.01 [7]	**2.85** 2.72-2.99 [82]	**2.77** 2.40-3.20 [12]	**2.55** 2.46-2.64 [122]
GG/B-B- (0)	**2.84** 2.70-2.98 [75]		**2.53** 2.43-2.62 [110]	
P value^{+}	0.40	0.06	0.052	**<0.05**

* geometric, unadjusted

$^{+}$ from analysis of variance on log-transformed, unadjusted fibrinogen values

The combination of the BclI and TaqI genotypes showed a trend towards higher levels with increasing numbers of 'fibrinogen-raising alleles' which reached significance in the non-smokers, P=0.04 (table 7.5). When the number of genotype categories was reduced to two, comparing the presence of 3-4

'fibrinogen-raising alleles' with 0-2 'fibrinogen-raising alleles', in the non-smokers the fibrinogen levels were 16% higher (2.95g/l versus 2.54g/l, P=0.0017) and in the smokers 10% higher (3.17g/l versus 2.88g/l, P=0.095) (table 7.5).

Table 7.5

Mean* plasma fibrinogen level by combined BclI and TaqI genotype and smoking status

<table>
<tr><td rowspan="2">BclI/Taq1 genotype (No fibrinogen raising alleles)</td><td colspan="4">Fibrinogen g/l
95% CI
[n]</td></tr>
<tr><td colspan="2">Smoker</td><td colspan="2">Non-smoker</td></tr>
<tr><td>B+B+/T-T-
(4)</td><td>3.05
-
[1]</td><td rowspan="2">3.17
2.82-3.56
[20]</td><td>3.00
-
[1]</td><td rowspan="2">2.95
2.66-3.27
[23]</td></tr>
<tr><td>B-B+/T-T-
B+B+/T-T+
(3)</td><td>3.18
2.80-3.60
[19]</td><td>2.95
2.64-3.28
[22]</td></tr>
<tr><td>B-B+/T-T+
B+B+/T+T+
B-B-/T-T-
(2)</td><td>2.88
2.72-3.06
[60]</td><td rowspan="3">2.88
2.76-3.02
[97]</td><td>2.56
2.46-2.67
[79]</td><td rowspan="3">2.54
2.46-2.63
[133]</td></tr>
<tr><td>B-B+/T+T+
B-B-/T-T+
(1)</td><td>2.94
2.70-3.20
[30]</td><td>2.51
2.35-2.69
[48]</td></tr>
<tr><td>B-B-/T+T+
(0)</td><td>2.67
2.26-3.15
[7]</td><td>2.57
2.20-3.00
[6]</td></tr>
<tr><td>P value+</td><td>0.44</td><td>0.095</td><td>0.040</td><td>0.0017</td></tr>
</table>

*geometric, unadjusted

+ from analysis of variance on log-transformed, unadjusted fibrinogen values

An extended genotype using the G/A^{-455}, BclI and TaqI genotypes and the association with plasma fibrinogen levels was analyzed and although a trend was seen of increasing plasma fibrinogen levels with increasing numbers of `fibrinogen-raising alleles' this did not reach significance in either the non-smokers or the smokers (table 7.6). However, when the 7 separate categories were reduced to two and the presence of 3-6 'fibrinogen-raising alleles' was compared to 0-2 'fibrinogen-raising alleles, fibrinogen levels were 12% higher in the non-smokers (2.82g/l versus 2.51g/l, P=0.0015) and 9% higher in the smokers (3.10g/l versus 2.84g/l, P<0.05) (table 7.6). The G/A^{-455}/BclI/TaqI genotype was coded in this table according to number of alleles associated with higher fibrinogen levels

0 = GG/B-B-/T+T+;

1 = GG/B-B-/T-T+, GG/B-B+/T+T+, GA/B-B-/T+T+;

2 = GG/B-B-/T-T-, GG/B+B+/T+T+, GA/B-B+/T+T+, GA/B-B-/T-T+, AA/B-B-/T+T+, AA/B-B+/T-T+;

3 = AA/B-B+/T+T+, AA/B-B-/T-T+, GA/B-B+/T-T+, GA/B+B+/T+T+, GG/B-B+/T-T-, GG/B+B+/T-T-;

4 = AA/B+B+/T+T+, AA/B-B-/T-T-, GG/B+B+/T-T-, GA/B-B+/T-T-, GA/B+B+/T-T+, AA/B-B+/T-T+;

5 = AA/B+B+/T-T+, AA/B-B+/T-T-, GA/B+B+/T-T-;

6 = AA/B+B+/T-T-

Table 7.6

Mean* plasma fibrinogen levels by combined G/A $^{-455}$, BclI and TaqI genotype and smoking status

** Coded G/A^{-455}/BclI/TaqI genotype	Fibrinogen g/l 95% CI [n]			
	Smoker		Non-smoker	
6	**3.05** - [1]	**3.10** 2.87-3.35 [44]	**3.00** - [1]	**2.82** 2.64-3.02 [45]
5	**3.98** - [1]		**3.02** 1.36-6.72 [3]	
4	**3.12** 2.77-3.53 [19]		**2.91** 2.62-3.24 [21]	
3	**3.05** 2.71-3.42 [23]		**2.70** 2.46-2.97 [20]	
2	**2.78** 2.61-2.97 [37]	**2.84** 2.70-2.98 [73]	**2.50** 2.40-2.61 [63]	**2.51** 2.42-2.61 [111]
1	**2.95** 2.70-3.22 [29]		**2.52** 2.34-2.73 [42]	
0	**2.67** 2.26-3.15 [7]		**2.57** 2.20-3.00 [6]	
P value^{+}	0.33	**<0.05**	0.065	**0.0015**

* geometric, unadjusted

$^{+}$ from analysis of variance on log-transformed, unadjusted fibrinogen values

A multiple regression analysis was performed for all combinations of genotypes having first entered BMI then age as covariates. In non-smokers, BMI made a contribution to variance of 5.4% and age a smaller contribution of 2.3% (table 7.7). BMI and age made no significant contribution in the smokers. In the non-smokers, genotype explained 2.4% ($P<0.05$) and 3.6% ($P<0.01$) of the variance in plasma fibrinogen levels for the BclI polymorphism and the G/A^{-455} respectively. The TaqI polymorphism made a smaller contribution of 1.4% which did not reach significance. However, the combination of the G/A^{-455} and TaqI polymorphisms together explained 8.9% of the variance ($P<0.005$) (table 7.7). The combination of the three genotypes together did not increase the contribution.

Table 7.7

Contribution to variance in plasma fibrinogen levels using a multiple regression model

Smoking status	Variable					
	BMI [n]	Age [n]	Polymorphism			
			BclI [n]	G/A^{-455} [n]	TaqI [n]	G/A^{-455}/TaqI [n]
Non-smoker	**5.4%$^{\#}$** [172]	**2.3%$^{\$}$** [172]	**2.4%$^{\$}$** [164]	**3.6%$^{\#}$** [172]	1.4%* [159]	**8.9%$^{+}$** [159]
Smoker	0.1%* [120]	1.5%* [120]	2.5%* [120]	**3.8$^{\$}$** [120]	0.3%* [117]	4.4%* [117]

BMI and age were entered first into the regression model and then genotype entered and the additional contribution shown in the table.

* Not significant $^{\$}$ $P<0.05$, $^{\#}$ $P<0.01$, $^{+}$ $p=0.005$

In the smokers, using multiple regression analysis, G/A^{-455} genotype explained 3.8% variance in plasma fibrinogen levels ($P<0.05$) but no other genotype or combination made a significant contribution (table 7.7). When the group was analyzed as a whole, the effect of smoking contributed 6.6% of the variance in the plasma fibrinogen levels.

7.1.3 Interaction between genotype and smoking

With evidence for both genetic and environmental influences determining plasma fibrinogen levels, it is of interest to consider gene-environment interaction.

Three different methods of analysis were used to investigate whether a genotype-smoking interaction could be demonstrated in this study. Interaction terms were computed for the different genotypes and smoking and entered into a multiple regression model with smoking, BMI, age and genotype. There was no significant interaction between any of the genotypes and smoking (data not shown).

One way analysis of variance with genotype and smoking as main effects and BMI and age as covariates was performed and again no significant two-way interaction was seen between smoking and genotype. The P values for the two-way interaction are shown in table 7.8.

Table 7.8

Genotype-smoking interaction using one way analysis of variance

Genotype-smoking interaction	
Genotype	P value
G/A^{-455}	1.0
BclI	0.96
TaqI	0.62
G/A^{-455},BclI	0.99
G/A^{-455},TaqI	0.80
BclI,TaqI	0.78
G/A^{-455},BclI,TaqI	0.94

Finally, the mean plasma fibrinogen levels were compared for each allele combination between smokers and non-smokers in a paired fashion. For example, the mean plasma fibrinogen of smokers with the genotype $G+G^{-455}$ was compared with non-smokers of the genotype $G+G^{-455}$ using Student's t-test. Smoking always had a raising effect on fibrinogen levels (table 7.9) and was not dependent on the presence of a particular allele. Thus for the G/A^{-455} polymorphism the smoker versus non-smoker effect was 0.3g/l for the $G+G^{-455}$ individuals, 0.34g/l for the $G+A^{-455}$ individuals and 0.43g/l for the $A+A^{-455}$ individuals. These differences reached significance for those homozygous for the common alleles and heterozygotes (table 7.9).

Table 7.9

Comparison of unadjusted plasma fibrinogen levels between smokers and non-smokers according to genotype

RFLP		Fibrinogen g/l 95% CI [n]		
G/A^{-455}	Genotype	GG	GA	AA
	Smoker	**2.84**$^{+}$ 2.70-2.98 [75]	**3.07*** 2.84-3.33 [42]	**3.28**$^{\$}$ 1.60-6.76 [3]
	Non-smoker	**2.54** 2.45-2.63 [115]	**2.74** 2.58-2.92 [49]	**2.85** 2.29-3.55 [8]
BclI	Genotype	B-B-	B-B+	B+B+
	Smoker	**2.85**$^{+}$ 2.72-2.99 [83]	**3.12*** 2.85-3.41 [35]	**2.96**$^{\$}$ 2.01-4.36 [2]
	Non-smoker	**2.55** 2.46-2.64 [123]	**2.77** 2.57-2.98 [37]	**2.61** 1.92-3.57 [4]
TaqI	Genotype	T-T-	T-T+	T+T+
	Smoker	**2.93**$^{\lozenge}$ 2.77-3.10 [62]	**2.97**$^{+}$ 2.78-3.18 [48]	**2.67**$^{\$}$ 2.26-3.15 [7]
	Non-smoker	**2.66** 2.54-2.78 [88]	**2.55** 2.41-2.69 [62]	**2.49** 2.24-2.76 [9]

$^{\$}$ Not significant; * $P < 0.05$; $^{\lozenge}P < 0.01$; $^{+}$ $P \leq 0.001$

7.2 Association of plasma fibrinogen levels with age, BMI, ethnic origin and $G+A^{-455}/C+T^{-148}$ genotype

The analysis of the TPT 1 study described in the preceeding section confirms that genetic variation at the fibrinogen gene locus is implicated in determining an individual's basal plasma fibrinogen levels and that an extended genotype can give further information. Whether the polymorphisms themselves are functional or simply markers for other functional changes cannot be determined. However, three of the polymorphisms, the G/A^{-455}, the C/T^{-148} and the T/G^{+1689}, are in complete linkage disequilibrium in three different Caucasian population samples but not in an Afrocaribbean population sample studied, described in chapter 5. The association of plasma fibrinogen levels with ß-fibrinogen genotype was therefore investigated in the Afrocaribbean population sample.

7.2.1 General characteristics of the sample

There is no smoking data on the individuals in this study and therefore for the purposes of analysis it has had to be assumed that smoking has an equal effect on fibrinogen levels in the different sexes. The characteristics of the sample are shown in table 7.10. The mean plasma fibrinogen level was 3.39g/l which is higher than that reported in the study with Caucasian individuals described in 7.1. This might partly be explained by the higher BMI of these individuals.

Table 7.10

General characteristics of the Afrocaribbean group

Population [n]	Fibrinogen g/l (95% CI)	Age years (SD)	BMI (SD)
Afrocaribbean [70]	3.39 (3.21-3.59)	55.3 (4.9)	27.5 (4.3)

7.2.2 Association between G/A^{-455}, C/T^{-148} and T/G^{+1689} genotypes and plasma fibrinogen levels

The relationship between both the G/A^{-455} and the C/T^{-148} polymorphisms and plasma fibrinogen levels was investigated. Fibrinogen levels were adjusted for age, sex and BMI before analysis and the results shown in table 7.11. For the G/A^{-455} polymorphism, those homozygous for the G^{-455} allele had higher plasma fibrinogen levels than the heterozygotes, 3.40 g/l versus 3.19g/l (6.6%) but this did not reach statistical significance. However, there were only three individuals of genotype G+A^{-455} and no individual homozygous for the A^{-455} allele. For the C/T^{-148} polymorphism, those who possessed one T^{-148} allele had higher plasma fibrinogen levels than those homozygous for the C^{-148} allele, 3.65g/l versus 3.34g/l, (9.3%) but again this did not reach statistical significance. When an extended genotype was analysed, the highest fibrinogen level was seen in those with the genotype G+G^{-455},C+T^{-148}, 3.83g/l versus 3.34g/l (14.8%) for those of genotype

$G+G^{-455}$,$C+C^{-148}$ and 3.83g/l versus 3.19g/l (19.2%) for those with the genotype $G+A^{-455}$,$C+T^{-148}$. Neither of these differences reached statistical significance.

Table 7.11

Mean* plasma fibrinogen levels and association with G/A^{-455} and C/T^{-148} genotype in Afrocaribbeans

Polymorphism	Fibrinogen* g/l (95% CI) [n]		
G/A^{-455}	GG	GA	AA
	3.40 (3.21-3.59) [67]	3.19 (3.01-4.47) [3]	-
C/T^{-148}	CC	CT	TT
	3.34 (3.15-3.55) [59]	3.65 (3.15-4.22) [11]	-
G/A^{-455},C/T^{-148}	GG/CC	GG/CT	GA/CT
	3.34 (3.15-3.55) [59]	3.83 (3.20-4.60) [8]	3.19 (2.27-4.47) [3]

* Adjusted for age, BMI and sex

7.3 Thrombosis Prevention Trial Study 2 (TPT 2)

As a result of the TPT 1 study, this second study was set up to examine the relationship between plasma fibrinogen levels, G/A^{-455} genotype and smoking habit in more detail, in particular whether a genotype-environmental interaction could be demonstrated.

The sample population studied were healthy middle aged men, selected from three general practices in the Thrombosis Prevention Trial (TPT) (Meade et al,1988), different from those used in TPT 1. Again, exclusion criteria included any history of ischaemic heart disease or contraindication to warfarin therapy.

7.3.1 General characteristics of the sample and relationship of plasma fibrinogen to age and BMI

The characteristics of the subjects are shown in table 7.12. 31% of those studied were current smokers, smoking on average 14 cigarettes/day (range 1-60) and 69% were non-smokers. 73.2% had smoked at sometime with only 26.8% in the never-smoked group. The mean plasma fibrinogen level for the whole group was 2.80g/l which is similar to that in the TPT screenees as a whole (Meade et al,1988). There was no significant difference in BMI when comparing the smoking and non-smoking groups but the smokers were significantly younger, 54.9 years versus 56.2 years, $P<0.03$. When analysed separately, the ex-smokers were significantly older compared to never smokers and current smokers (57.1 years versus 54.7 years and 54.9 years respectively, ($P<0.0001$) with a higher mean BMI compared to never smokers and current smokers (26.8 versus 26.0 and 26.1, $P=0.007$). The plasma fibrinogen levels were significantly different from each other ($P<0.0001$) in the different smoking groups, with the current smokers, as expected, having the highest levels (2.92g/l) followed by ex-smokers (2.73g/l), with never-smokers having the lowest value (2.66g/l).

Table 7.12

Characteristics of subjects and their unadjusted mean fibrinogen values

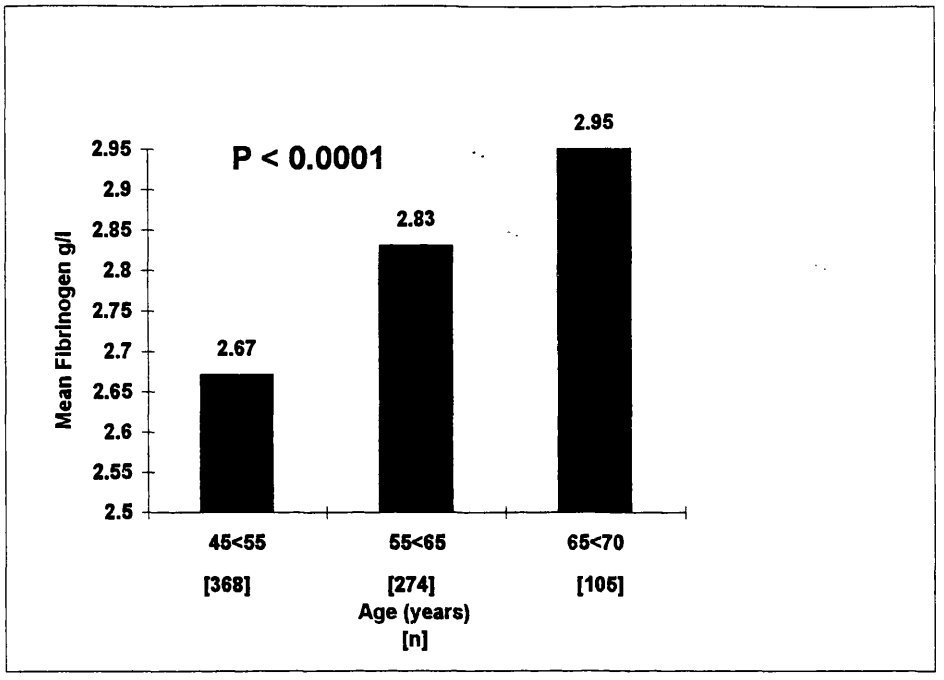
	Fibrinogen g/l (SD) [n]		BMI (SD) [n]		Age (SD) [n]	
Never smoker	**2.66** (0.49) [194]	**2.70** (0.49) [505]	**26.0** (3.6) [211]	**26.5** (3.4) [545]	**54.7** (6.8) [214]	**56.2** (7.1) [553]
Ex-smoker	**2.73** (0.49) [311]		**26.8** (3.3) [334]		**57.1** (7.1) [339]	
Current smoker	**2.92** (0.56) [234]		**26.1** (3.6) [241]		**54.9** (6.9) [245]	
*P value	**<0.0001**	**<0.0001**	**0.007**	NS	**<0.0001**	**<0.03**

* Oneway analysis of variance

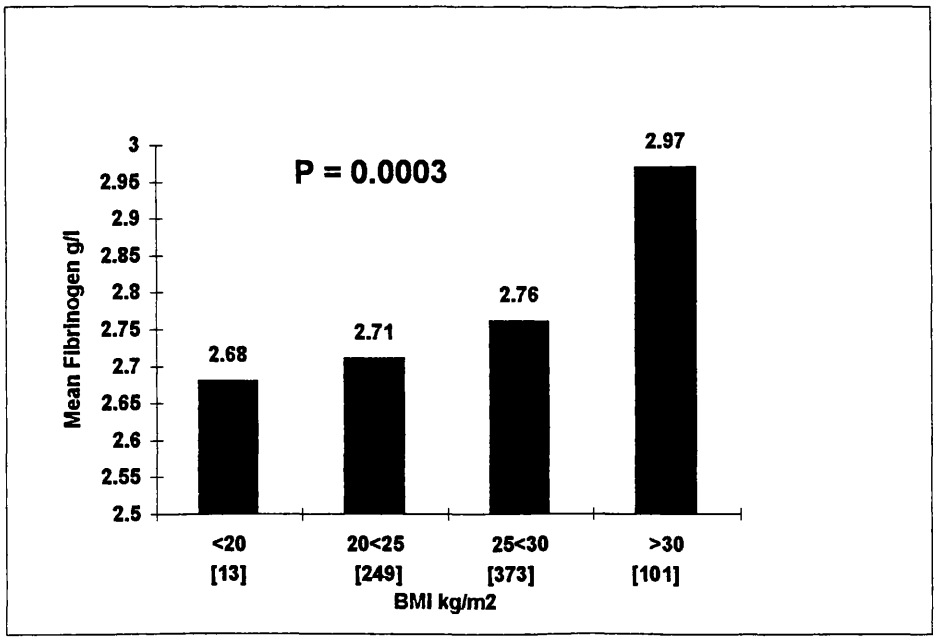
Plasma fibrinogen levels were compared between individuals grouped by decade of age, 45 < 55 years, 55 < 65 years and ≥ 65 years, and showed a significant rise with increasing age; 2.67g/l , 2.83g/l and 2.95g/l, P < 0.0001 (figure 7.2). Fibrinogen levels were also compared between individuals grouped according to BMI, <20, 20<25, 25<30 and >30, and showed a significant rise in plasma fibrinogen level with increasing BMI; 2.68g/l, 2.71g/l, 2.76g/l and 2.97g/l, P=0.0003 (figure 7.2)

Figure 7.2

a. Plasma fibrinogen by age in TPT 2 men



b. Plasma fibrinogen by BMI in TPT 2 men



7.3.2 Frequency of alleles across different age groups and in different smoking groups

For this analysis, 482 individuals in the sample had their G/A^{-455} genotype determined; all smokers were included and an equal number of non-smokers who were age and BMI matched. Genotype distribution within groups of different age and smoking habit was compared.

The frequency of the rarer A^{-455} allele in the subjects was 0.20 in the whole group and overall was not significantly different between the smokers and non-smokers. The distribution of genotypes was in Hardy-Weinberg equilibrium in the group as a whole and in the smokers and non-smokers separately. Allele frequency was also examined in individuals grouped by decade of age as well as in groups of different smoking habit. The frequency of the A^{-455} allele was significantly lower in smokers than in non-smokers in subjects aged >65 years ($F = 5.38$, $P < 0.05$) but not in younger men, 0.94 versus 0.77 (table 7.13). In addition, the difference in frequency of the A^{-455} allele between smokers and non-smokers varied significantly with age ($\chi^2 = 9.32$, $P < 0.01$) (table 7.13).

Table 7.13

Allele frequency of the G/A^{-455} polymorphism in different age groups by smoking habit

Age (years)	45 < 55		55 < 65		≥ 65	
Allele frequency	G^{-455} [n]	A^{-455} [n]	G^{-455} [n]	A^{-455} [n]	G^{-455} [n]	A^{-455} [n]
Smoker	**0.79** [201]	**0.21** [53]	**0.78** [121]	**0.22** [35]	**0.94** [49]	**0.06** [3]
Non-smoker	**0.81** [229]	**0.19** [53]	**0.83** [133]	**0.17** [27]	**0.77** [46]	**0.23** [14]

Multiple logistic regression: the difference in frequency of the A^{-455} allele between smokers and non-smokers varied significantly with age (χ^2 = 9.32, P < 0.01).

The frequency of the A^{-455} allele was significantly lower in smokers than in non-smokers in subjects aged >65 years (F = 5.38, P < 0.05) but not in younger men.

In the smaller sample, TPT 1, the same trend in allele frequency was seen but this did not reach significance (table 7.14).

Table 7.14

Allele frequency of the G/A^{-455} polymorphism in different age groups by smoking habit in TPT 1

Age (years)	45 < 55		55 < 65		≥ 65	
Allele frequency	G^{-455} [n]	A^{-455} [n]	G^{-455} [n]	A^{-455} [n]	G^{-455} [n]	A^{-455} [n]
Smoker	**0.79** [82]	**0.21** [22]	**0.79** [107]	**0.21** [29]	**1.00** [6]	**0.0** [0]
Non-smoker	**0.79** [134]	**0.21** [36]	**0.84** [129]	**0.16** [25]	**0.85** [22]	**0.15** [4]

Multiple logistic regression: the frequency of the A^{-455} allele is not significantly lower in smokers than in non-smokers.

7.3.3 Association between G/A^{-455} genotype and plasma fibrinogen levels

Plasma fibrinogen level adjusted for age and BMI was used to analyse the association of plasma fibrinogen and G/A^{-455} genotype, because of the differences in age and BMI found between the different smoking groups and their known association with plasma fibrinogen levels. Because of the small numbers of individuals homozygous for the rarer A^{-455} allele, heterozygotes and A+A^{-455} homozygotes were grouped together. As shown in table 7.16, in the never smokers there was a significant association between G/A^{-455} genotype and plasma fibrinogen level, with those possessing one or two of the A^{-455} alleles having significantly higher plasma fibrinogen levels than those homozygous for

the G^{-455} allele, 2.78g/l versus 2.57g/l (8.2%, P=0.026). An effect of similar size associated with genotype was seen in the ex-smokers, 2.90g/l versus 2.66g/l (9.0%, P=0.0064). However, in the smokers, the effect associated with genotype was much smaller, 2.97g/l versus 2.90g/l (2.4%, P>0.05) and this was not significant.

Table 7.15

Mean fibrinogen values in different G/A^{-455} genotype groups adjusted for age and BMI

G/A^{-455} genotype	Fibrinogen g/l (SD)		
	Never smokers [n]	Ex-Smokers [n]	Smokers [n]
GG	**2.57** (0.40) [55]	**2.66** (0.49) [105]	**2.90** (0.57) [145]
GA AA	**2.78** (0.51) [42]	**2.90** (0.50) [45]	**2.97** (0.53) [82]
* P value	**0.026**	**0.016**	NS

Note that only subjects where fibrinogen, genotype, age and BMI are known are included in this analysis * One way analysis of variance

Noting the differences in allele frequency between the different age groups in smokers and non-smokers described in 7.3.2., association of plasma fibrinogen level and genotype was also investigated in these different age groups and the results shown in table 7.16. Unadjusted mean fibrinogen levels were used since

no significant difference in BMI was shown between smokers and non-smokers. For the non-smokers in the youngest age group, 45 < 55 years, there was a significant association between G/A^{-455} genotype and plasma fibrinogen levels, with those with one or more A^{-455} alleles having levels 11.6% higher than those with only the G^{-455} allele ($P<0.005$). By contrast, in the men in the older age groups, the effect associated with genotypes became smaller, being 5.6% in those 55 < 65 years ($P=0.02$) and 4.2% in those > 65 years ($P=0.5$).

In the smokers, the raising effect associated with the A^{-455} allele was smaller in the men in the two youngest age groups (5.0% and 1.0% respectively) but not in the oldest (and smallest) group (4.4%). However, none of these differences reached statistical significance.

Table 7.16

Unadjusted mean fibrinogen levels by G/A^{-455} genotype in different age groups

Age (years)	45 < 55		55 < 65		≥ 65	
G/A^{-455} genotype	Fibrinogen (SD) g/l		Fibrinogen (SD) g/l		Fibrinogen (SD) g/l	
	Non-smoker [n]	Smoker [n]	Non-smoker [n]	Smoker [n]	Non-smoker [n]	Smoker [n]
GG	**2.49** (0.51) [91]	**2.79** (0.57) [79]	**2.69** (0.43) [55]	**2.93** (0.51) [46]	**2.88** (0.29) [17]	**3.18** (0.66) [23]
GA AA	**2.78** (0.50) [50]	**2.93** (0.48) [48]	**2.84** (0.44) [25]	**2.99** (0.48) [32]	**3.00** (0.66) [13]	**3.32** (1.21) [3]
*P value	**0.0013**	0.18	0.16	0.57	0.52	0.75

* One way analysis of variance

7.3.4. Relationship between plasma fibrinogen level and environmental factors

7.3.4.a Smoking history

The association between smoking and plasma fibrinogen levels has already been described in 7.3.1, with the highest fibrinogen levels found in current smokers, the lowest levels in never smokers with ex-smokers having intermediate levels 2.66g/l, 2.92g/l and 2.73g/l respectively, $P<0.0001$ (table 7.12).

In this study a questionnaire (appendix 7) on smoking habit was completed and the relationship between number of cigarettes smoked and plasma fibrinogen levels was examined in the current smoking group. There was a weak, positive correlation between the two, $r=0.09$, but this did not reach significance.

7.3.4.b Plasma cotinine levels

Plasma cotinine was measured in indivduals in this trial as an objective measure of nicotine exposure. There was a positive correlation between serum cotinine levels and plasma fibrinogen levels, $r=0.18$, $P=0.001$. The correlation between serum cotinine levels and number of cigarettes smoked was investigated in the

smokers. There was a weak, positive correlation between the two, r=0.13, but this did not reach significance.

Using a multiple regression model, the contribution of age, BMI, $\log_{10}$ cotinine and G/A^{-455} genotype to fibrinogen levels was entered in a stepwise fashion. The variables together explained 12.1% of the variance in plasma fibrinogen levels with cotinine levels contributing 4.8% of the variance, age 3.5%, HaeIII genotype 2.2% and BMI 1.5% (table 7.17) . No interaction between cotinine levels and G/A^{-455} genotype could be shown using a multiple regression model. In the same way, the contribution to fibrinogen levels of age, BMI, smoking status (that is whether a current smoker or not) and G/A^{-455} genotype was entered in a stepwise fashion into a multiple regression model. The variables together explained 12.0% of the variance in plasma fibrinogen level, with smoking contributing 4.7% of the variance, age 3.8%, HaeIII genotype 2.3% and BMI 1.3%. If smoking status were entered as current, ex- or never-smoker using dummy variables instead of simply whether the individual was a current smoker or not, no further information was gained, (that is there was no significant increase in R^2) the total contribution of the variables being 12.1% with the contribution from the smoking information being 4.7%. Where the smoking details were replaced by the number of cigarettes smoked, this variable made no significant contribution to the variance in plasma fibrinogen levels (table 7.17).

Table 7.17

Contribution to variance in plasma fibrinogen levels using a stepwise multiple regression model

Details of smoking habit	Variable				Total
	Age	BMI	G/A^{-455} genotype	Smoking details	
Smoker Y/N	3.8%	1.3%	2.3%	4.7%	12%*
Smoker current, ex-never	3.8%	1.3%	2.3%	4.7%	12.1%
Cotinine	3.5%	1.5%	2.2%	4.8%	12.1%*
Cigs	4.2%	1.0%	3.3%	**	8.6%*

* Totals differ slightly due to rounding up or down of individual variable's percentages

** Not included in stepwise regression model because contribution to the variance was not significant

The interaction between smoking, fibrinogen and genotype was investigated using two different methods. Using a multiple regression model where interaction terms were computed for both smokers/non-smokers and G/A^{-455} genotype and current, ex- and never-smokers and G/A^{-455} genotype no significant interaction was shown. Using one way analysis of variance, again no interaction was shown

8. Association between variation at the fibrinogen gene locus and plasma fibrinogen levels in smokers and non-smokers: discussion

8.1 Thrombosis Prevention Trial 1 (TPT 1)

8.1.1 Association between G/A^{-455}, BclI and TaqI genotypes and plasma fibrinogen levels

The distribution of genotypes for the G/A^{-455}, BclI and TaqI polymorphisms of the β-fibrinogen gene all exhibit Hardy-Weinberg equilibrium in this sample. It is likely that the sample is representative of healthy men in the U.K. due to the selection procedure used. The data show that of the three polymorphisms, the G/A^{-455} was associated with the greatest effect on plasma fibrinogen level and accounted for 3.6% and 3.7% of the variance in non-smokers and smokers respectively. A trend of higher fibrinogen level with the B+ genotype and lower fibrinogen level with the T+ genotype was seen in both smokers and non-smokers but only the BclI polymorphism made a statistically significant contribution of 2.4% to the variance in plasma fibrinogen levels and only in the non-smokers. A previous smaller study using the BclI and TaqI polymorphisms showed that the B+ allele was associated with a raising effect on plasma fibrinogen level and the T+ allele a lowering effect, with these polymorphisms explaining 9.0% and 4.2% of the phenotypic variance respectively (Humphries et al,1987). These estimates were made in a younger population sample (mean age 46 years versus 55.4 years), the population was not

selected for lack of IHD and smokers and non-smokers were analysed together. Because of the marked contribution to variance in plasma fibrinogen level that smoking makes, analysing the smokers and non-smokers together would tend to decrease the influence of genotype on plasma fibrinogen level. However, since those with a history of IHD were not specifically excluded and the average age of the individuals was lower, this would tend to increase the influence of genotype on plasma fibrinogen level and may account for the greater contribution to variance found. Importantly, the trend in the TPT 1 study described in 7.1 is the same as reported in that of Humphries and colleagues (1987).

Due to marked within-individual variation that can occur with fibrinogen levels (Thompson et al,1987) contributions to the variance in plasma fibrinogen levels of these polymorphisms or their combinations are likely to be a minimum estimate since a single fibrinogen measurement was used in this study which does not give a precise estimate of an individual's typical value. Repeat determinations of plasma fibrinogen can help to overcome this problem. In addition, because ex-smokers have been included in the non-smoking group this will, if anything, attenuate the relationship between smoking habit and fibrinogen but will have no effect on the association between genotype and fibrinogen assuming that there is no interaction between genotype and smoking.

The studies by Connor and coworkers (1992) and Berg and Keirulf (1989) showed no significant effect of genotype on plasma fibrinogen levels but correction for BMI

was not made in either study. In this study, both BMI and age made a significant contribution in the non-smokers accounting for 5.4% and 2.3% of the variance respectively. In the smokers, neither BMI nor age made a significant contribution, possibly because the effect of smoking is so strong that it overrides lesser effects in studies of this size and with a relatively narrow age and BMI range.

8.1.2 Association between extended genotype and plasma fibrinogen levels

Using an extended genotype at the fibrinogen gene locus, the most significant effect was seen with the combination of the G/A^{-455} and TaqI genotypes. The combination in the non-smokers accounted for 8.9% of the variance of plasma fibrinogen levels but this did not differ significantly from the G/A^{-455} alone (P=0.09). The G/A^{-455}/BclI combination did not contribute any further to the variance in plasma fibrinogen levels than the G/A^{-455} polymorphism alone. These results are not surprising since the G/A^{-455} and the BclI polymorphisms are in strong linkage disequilibrium (Δ = 0.85, P<0.001), whereas the G/A^{-455} and the TaqI polymorphisms are not (Δ = -0.09, P>0.1) (figure 7.1). It is possible that the TaqI polymorphism is a marker for a functional site in the α- or γ-fibrinogen gene promoters since they also have IL-6 and HNF elements. Since the TaqI and the G/A^{-455} polymorphisms are in linkage equilibrium, there are four different haplotype combinations which could allow finer control of plasma fibrinogen level possibly through an interaction between the two polymorphisms which is suggested by the

results. A combination of all three genotypes contributed no further information than the G/A^{-455}/TaqI combination alone.

8.1.3 Interaction between genotype and smoking

In this sample, the effects of genotype and smoking were additive, there being no statistically significant interaction when fibrinogen levels and genotype were compared in smokers and non-smokers. In contrast, Green and colleagues (1993) found significant interaction between smoking status and genotype in determining plasma fibrinogen levels in healthy Swedish controls, but not in the patient group, who consisted of young survivors of myocardial infarction. Scarabin and colleagues (1993) also found an interaction between smoking and genotype but in cases as well as controls. In this study, the difference between the plasma fibrinogen level in smokers and non-smokers was more marked in those of the AA genotype than those of the GG genotype, 0.61g/l in both cases and controls versus 0.02g/l and 0.31g/l in cases and controls respectively. The reasons for this are not clear, but Scarabin's study included a wider age range than the study described here and included cases, who consisted of survivors of recent myocardial infarcts. There may be an interaction between smoking, genotype and disease status which may in part involve both acute and more chronic, low grade stimulators of the acute phase response. It is possible that the acute and chronic stimulators are not additive in raising plasma fibrinogen levels in individuals with the GG genotype and thus there is no difference between plasma fibrinogen levels

in non-smoking and smoking cases, but effects are additive in those of the AA genotype resulting in an increased fibrinogen in smokers of this genotype group. In the controls, there is no acute stimulation, only the chronic low grade stimulation in the smokers alone and thus a rise in fibrinogen is seen in all genotype groups when smokers are compared to non-smokers.

In the study described in this thesis, those who had a history of coronary artery disease were excluded. Since a high fibrinogen level has consistently been shown to be a predictor of coronary events, it seems likely that a disproportionate number of people with higher fibrinogen levels would have been excluded. This would decrease the chances of showing an association between genotype and plasma fibrinogen level and might explain why the fibrinogen level is lower than expected in the BclI B+B+ group which has previously been shown to be associated with higher fibrinogen levels (Humphries et al,1987). There is also a significantly lower percentage of smokers in the oldest age group, 19% in those $\geq$ 65 years versus 47% in the 55<65 year olds and 38% in the 45<55 year olds (likelihood ratio χ^2=5.86, P=0.04) which would decrease the likelihood of showing a smoking-genotype interaction.

8.2 Ethnic population sample

In the Afrocaribbean population sample studied, linkage disequilibrium between the G/A^{-455} and C/T^{-148} polymorphisms was not complete. This allowed the chance

to investigate the relationship between plasma fibrinogen levels and these polymorphisms in different haplotypes.

8.2.1 Association of genotypes and plasma fibrinogen levels in Afrocaribbeans

Unfortunately, no smoking data were available for the sample studied and the numbers were small and included males and females, although sex was adjusted for in the analysis. No statistically significant differences between the plasma fibrinogen levels in the various genotype groups could be demonstrated but the highest fibrinogen levels were found in those of the genotype $C+T^{-148}$ and the extended genotype $G+G^{-455},C+T^{-148}$. Only three individuals had the genotype $G+A^{-455}$ and they had the lowest mean fibrinogen levels. Although numbers are small, these results would be compatible with the hypothesis that the T alleleof the C/T^{-148} polymorphism is the fibrinogen raising allele, rather than the A allele of the G/A^{-455} polymorphism. Investigation of a larger sample of Afrocaribbean individuals in whom the smoking data are known would help to clarify this point. The hypothesis could also be examined in vitro by testing the promoter strength of different allelic combinations using a CAT assay. However, it may be necessary to test these different combinations in both DNA obtained from Afrocaribbean and Caucasian individuals, if other sequence differences exist in the region of the gene.

8.3 Thrombosis Prevention Trial 2 (TPT2)

The results of this study confirm that increasing age, smoking and the A^{-455} allele of the G /A^{-455} genotype are all associated with higher plasma fibrinogen levels as has been shown in TPT 1 and other previous studies (Balleisen et al,1985; Kannel et al,1987; Meade et al,1987; Humphries et al,1987; Heinrich et al,1995; Humphries et al,1994; Scarabin et al,1993). In the group of men studied here, smoking was associated with a difference in plasma fibrinogen level between smokers and non-smokers of 0.22g/l which compares with a difference of 0.26g/l in the study of Balleisen and colleagues (1985) and 0.15g/l in the study of Meade and colleagues (1987). Ex-smokers had fibrinogen levels intermediate between never smokers and current smokers as was shown by Meade and colleagues (1987).

8.3.1 Frequency of the A^{-455} polymorphic allele versus age

Fibrinogen increases with age, in this study approximately 0.2g/l with every decade. This is similar to the rise noted by Balleisen and colleagues (1985) and is slightly greater than in the Framingham study (Kannel et al,1987). However, in those who possess at least one A^{-455} allele, the rise is less, 0.06g/l in both smokers and non-smokers between the 45<55 years and the 55<65 years age bands and 0.14g/l in the non-smokers between the 55<65 years and ≥ 65 years age bands. One explanation for the smaller rise in plasma fibrinogen levels in

those who possess at least one A^{-455} allele could be that those with a greater rise and consequently higher plasma fibrinogen level are no longer healthy and have been excluded from the study. In support of this, there is a significant decrease in frequency of the A^{-455} allele in smokers $\geq$ 65 years both when compared to non-smokers of the same age group or smokers of a younger age group. The same analysis was performed on allele frequency in smokers and non-smokers according to decade of age in the individuals in TPT 1 but although the trend was the same, it did not reach statistical significance in this smaller sample.

Since higher plasma fibrinogen levels have been shown to be associated with an increased risk of ischaemic heart disease and plasma fibrinogen levels rise significantly with age, smoking and A^{-455} genotype, it is possible that older smokers possessing an A^{-455} allele, would be more likely to have had an ischaemic event and be excluded from the study. This interaction between fibrinogen, age and smoking may explain previously reported inconsistencies between fibrinogen genotype and fibrinogen levels in studies of older individuals (Fowkes et al,1992; Connor et al, 1992). The findings in the study presented here support the hypothesis that age, smoking and genotype have additive effects on lack of health.

8.3.2 Association of genotype with plasma fibrinogen level

A similar fibrinogen-raising effect associated with the A^{-455} allele was seen in both non-smokers and ex-smokers, the fibrinogen level being 0.21g/l and 0.24g/l higher respectively in those either heterozygous or homozygous for this allele compared to those with the genotype GG. No significant effect was seen in smokers. This is in contrast to the TPT 1 study described in chapter 7.1 where the increase in fibrinogen associated with the A^{-455} allele was similar in healthy smokers and non-smokers and in contrast to two case-control studies where the higher fibrinogen level associated with A^{-455} genotype was seen in the smokers only (Scarabin et al,1993; Green et al,1993). In the study of Scarabin and coworkers, the difference in plasma fibrinogen level between smokers and non-smokers was most marked in those of the AA genotype compared with those of the GG genotype, 0.61g/l in both cases and controls versus 0.02g/l and 0.31g/l in cases and controls respectively. The reasons for this are not clear, but Scarabin's study included individuals of a wider age range than the TPT 1 and TPT 2 studies described in this thesis and included cases who were survivors of recent myocardial infarcts and controls.

There may be an interaction between smoking, genotype and disease status which may in part involve both acute and more chronic, low grade stimulators of the acute phase response. It is possible that in those of the GG genotype there is only a single site at which both chronic and acute stimulators of the acute phase

response can act and thus there is no difference in plasma fibrinogen level between smokers and non-smokers in the cases but there is in the controls. In those of the AA genotype, it is possible that a repressor protein which binds to the G^{-455} allele is unable to bind to the A^{-455} allele, possibly allowing additive effects of acute and chronic stimulators, and thus there is an even greater difference in plasma fibrinogen level between smokers and non-smokers in this genotype group. This greater difference between smokers and non-smokers in those homozygous for the A^{-455} allele is also seen in the controls although the mean fibrinogen levels are lower than those of the cases. Band shift assays using these two alleles and hepatic nuclear extracts, have shown differential binding of a protein, as yet unidentified, compatible with this hypothesis (Dr F Green, personal communication).

The difference in allele frequency with age prompted investigation of the effect of genotype on plasma fibrinogen levels in the different age groups. The greatest effect was seen in non-smokers in the 44<55 year age band where the plasma fibrinogen level in those homozygous for the G^{-455} allele was significantly lower when compared to those with at least one A^{-455} allele. However, the effect diminshed with age in both the non-smokers and the smokers except for smokers $\geq$ 65 years. This again may be due to the interaction between age, genotype and smoking outlined in 8.3.1.

8.4 Relationship between plasma fibrinogen level and environmental factors

Current smoking status defined by whether an individual was a current smoker, an ex-smoker or a non-smoker appeared to be a significant determinant of plasma fibrinogen level which supports the findings of Meade and coworkers (1987) in their detailed study of effects of changes in smoking on fibrinogen level. However assessment of number of cigarettes smoked was not a significant predictor of fibrinogen level and showed only a weak positive correlation between the two. This may be because number alone is not a good indication of blood nicotine level achieved due to the different nicotine content of the cigarettes smoked and the 'puff patterns' of smokers. It has been shown that when smokers switch to lower-yield cigarettes, many alter their smoking behaviour to maintain their usual intake of nicotine; they may increase the number of puffs per cigarette, the volume of each puff or the duration of each puff (Sutton et al,1982).

8.4.1 Relationship between plasma cotinine level and plasma fibrinogen level

There was a significant but weak positive correlation between plasma cotinine level and plasma fibrinogen level. However, when the relationship between number of cigarettes smoked and plasma cotinine level was examined no

significant association was seen and again the differences in smoking behaviour between individuals could explain this finding.

Smoking habit was the most important determinant of plasma fibrinogen levels, explaining between 4.7-4.8% of the variance. Details of habit, whether given as current smoker or not; current, ex- or non-smoker or as cotinine level, did not influence the contribution to the variance. Number of cigarettes smoked was not a significant contributory factor in the variance of plasma fibrinogen level, almost certainly for the reasons already outlined. G/A^{-455} genotype explained between 2.2-2.3% of the variance with age and BMI together accounting for about 5.0% of the variance.

Smoking is the most important environmental factor known to affect plasma fibrinogen level and in both TPT 1 and TPT 2, G/A^{-455} genotype accounts for about half the amount of variance that smoking does. In neither of the studies presented in this thesis could an interaction between smoking and genotype be shown.

9 Final conclusions

Elevated plasma fibrinogen is a well established predictor of IHD (Wilhelmsen et al,1984; Stone and Thorp,1985: Meade et al,1986; Kannel et al,1987) but several others are known such as high levels of plasma cholesterol and factor VIIc. IHD is a multifactorial polygenic disorder, with both genetic and environmental factors involved to varying extents in causing the disease in different individuals in the general population (Goldbourt and Neufeld,1986). Genetic variation in a single gene, or even a number of genes, is not likely to be the sole cause of IHD in an individual. It is more likely that the propensity for an individual to develop atherosclerosis is due to interaction between a number of genes and the environment.

The studies presented in this thesis are concerned with genetic variation at the fibrinogen gene locus and gene-environment interaction and their association with plasma fibrinogen level. Of the polymorphisms examined, the G/A^{-455} substitution in the 5' flanking region of the β-fibrinogen gene is associated with the most consistent differences in plasma fibrinogen level in both smokers and non-smokers, accounting for between 3.1-3.6% of the variance seen. A significant association between the G/A^{-455} polymorphism and fibrinogen level has also been shown in studies from Sweden (Green et al,1993), Greenland Inuit (de Maat et al,1995), in healthy men and MI survivors in the ECTIM study (Scarabin et al, 1993) and in young men and women in the EARS study (Humphries et al,1995).

Certain of these studies have shown gene-environment interactions between ß-fibrinogen genotype and sex (de Maat et al,1995; Humphries et al,1995) with genotype having a greater effect in males than females, and ß-fibrinogen genotype and smoking (Green et al,1993; Scarabin et al,1993) where the higher fibrinogen level associated with A^{-455} genotype was seen in the smokers only. There was no evidence of interaction between smoking and genotype in the studies presented here but the population was selected to be healthy and with no history of IHD and this may have altered the relationship. Since higher plasma fibrinogen levels have been shown to be associated with an increased risk of ischaemic heart disease and plasma fibrinogen levels rise significantly with age, smoking and A^{-455} genotype, it is possible that older smokers possessing an A^{-455} allele would be more likely to have had an ischaemic event and be excluded from the study. This is supported by the interaction shown between age, smoking and genotype frequency, where there was a significant decrease in frequency of the A^{-455} allele in older smokers. This interaction between fibrinogen, age and smoking may explain previously reported inconsistencies between fibrinogen genotype and fibrinogen levels in studies of older individuals (Berg and Kierulf,1989 ; Fowkes et al,1992; Connor et al,1992). The findings in this study support the hypothesis that age, smoking and genotype have additive effects on lack of health.

It is possible to propose a molecular mechanism for the effect on fibrinogen levels associated with G/A^{-455} genotype. It has been shown using pulse-chase

experiments and transfection of the Bβ chain cDNA into HepG2 cells that synthesis of the Bβ chain is the rate limiting step in the production of mature fibrinogen (Yu et al,1983; Yu et al,1986; Roy et al,1990) and it is therefore reasonable to propose that changes in the rate of transcription of the β-fibrinogen gene will alter the rate of production of the protein. Interleukin-6 (IL-6) is the major regulator of the acute phase response (Castell et al,1989) and monocytes, macrophages, fibroblasts and endothelial cells are probably the major sources of IL-6 in the inflammatory state. IL-6 induces a liver-specific nuclear protein to bind to the promoter region of the acute phase genes (Poli and Cortese,1989). The region up to 150bp upstream of the start of transcription of the β-fibrinogen gene contains the information necessary for liver specific transcription of the β-fibrinogen gene (Courtois et al,1987) while a region between -150 and -82bp is responsible for the IL-6 induction (Huber et al,1990; Dalmon et al,1993). In addition, it has been demonstrated that a domain between -2900 and -1500bp from the start of transcription confers dexamethasone inducibility (Huber et al,1990). The G/A variable site at -455bp from the start of transcription lies very close to the IL-6 responsive element and it is possible that the substitution itself may be a functional change, which affects the affinity of a nuclear protein involved in the transcription of the gene. The sequence change underlying the G/A^{-455} polymorphism has been found, in band shift assays, to affect the binding of a hepatic nuclear protein (Dr F Green personal communication), where the G^{-455} allele binds the protein whereas the A^{-455} allele does not. Work presented in this thesis has shown that the A^{-455} sequence change is in complete allelic association

in all Caucasian populations so far studied with a C/T^{-148} change located close to the consensus sequence of the IL-6 element (Dalmon et al,1993). Fibrinogen transcription is markedly increased by cytokines such as IL-6 which are induced in response to injury (Castell et al,1989) with one likely source of such cytokines being from macrophges recruited to the lungs as a result of damage from smoking. It has been shown, again using band shift assays, that the T^{-148} sequence binds a nuclear protein that the C^{-148} sequence does not (Lane et al,1993). This raises the possibilty that the G/A change is acting as a neutral marker for the functional C/T change or that the two sites may interact with each other and the environment in determining plasma fibrinogen level.

It is possible that influences on plasma fibrinogen level may involve both acute and more chronic, low grade stimulators of the acute phase response. The mechanism of the raising effect of the A^{-455} allele might be the release of a repressor protein from or binding of a positive transcription factor to a cis-acting element in the promoter region or possibly both. Environmental factors such as smoking may interact with the transcription factors, altering their affinity with the cis-acting elements to a degree dependent on the sequence of such elements thus altering the rate of transcription of the gene in a sequence specific way. It is also possible that interaction between different transcription factors is affected to a greater or lesser extent by the sequence of the cis-acting elements and therefore variations in plasma fibrinogen level are accentuated or diminshed accordingly. Polymorphisms other than the C/T^{-148} have been shown to be in linkage

disequilibrium with the G/A^{-455} polymorphism, one of which is the T/G^{+1689} in intron 1 of the ß-fibrinogen gene, described in this thesis, and the other in the coding region of the ß fibrinogen gene which results in the substitution of $arginine_{448}$ for lysine (Baumann and Henschen,1994). This is a relatively conservative substitution and results in a novel peptide.whose sequence corresponds to that of bovine fibrinogen which is not dysfunctional (Schmelzer et al,1988). However, it remains a possibilty that this amino acid change may be functional and that the other ß-fibrinogen polymorphisms are acting as markers for it. Other polymorphisms in non-coding regions of the fibrinogen gene locus have also been described in this thesis. One, in the 3'-flanking region of the ß-fibrinogen gene, detected with the restriction enzyme BclI, is in linkage disequilibrium with the G/A^{-455} polymorphism and could itself be functional or a marker for another functional change in the 3' untranslated region since this region can be important in regulation of gene transcription (Semenza et al,1991). In addition there is a polymorphism in the 3'-flanking region of the α-fibrinogen gene, detectable with the restriction enzyme TaqI, which is in linkage equilibrium with the G/A^{-455}, C/T^{-148} and T/G^{+1689} polymorphisms of the β-fibrinogen gene and in an extended genotype with the G/A^{-455} contributes more to the variance in plasma fibrinogen levels in non-smokers than either does alone. This implies that there is an interaction either between the two polymorphisms themselves or between other changes for which they are markers.

Environmental and genetic influences both appear to affect an individual's plasma fibrinogen level. As an individual ages, environmental influences will have had a longer time to exert their effects and it may be more informative, in order to reduce the selection bias that might otherwise occur, to determine fibrinogen genotype and plasma fibrinogen level in a younger population than studied here. However, in utero environmental effects may be exerting a greater influence on those who are younger and there is some evidence that foetal and infant growth are related to adult plasma fibrinogen level (Barker et al,1992). Not only may environmental influences confound genetic influence, but because of the complete linkage disequilibrium that exists between three the β-fibrinogen gene polymorphisms studied here, it is not possible to identify which, if any, of the polymorphisms may be exerting the most important effect. The difference in linkage disequilibrium between these same polymorphisms that occurs in the Afrocaribbean population sample studied could well be exploited in trying to determine more precisely their effect and interactions.

Understanding of the mechanism of the interaction between genetic polymorphisms and environmental factors will be important in determining the aetiology of high plasma fibrinogen levels and may be useful in identifying those smoking individuals who would lower their risk of IHD substantially by cesssation of smoking. It has to be said, however, that because smoking is associated with many other effects, such as emphysema, increased blood pressure and cancer, it

would not be appropriate to advise anyone of a 'favourable genotype' that smoking was not harmful.

Although not possible at present, once the mechanisms controlling changes in plasma fibrinogen level are better understood, it might be possible to develop directed therapeutic strategies that will reduce risk in a genotype-specific manner. For instance, it might be possible to block an allele-specific positive transcription factor using an antagonist or partial agonist which would compete for the specific binding site thereby suppressing transcription of the gene in a genotype-specific way.

Future studies

1. In order to try to elucidate which of the β-fibrinogen gene polymorphisms is having the greatest effect, it would be of interest to determine the β-fibrinogen and α-fibrinogen genotype along with plasma fibrinogen level in a larger population sample of Afrocaribbeans whose smoking status was known.

2. To try to prevent selection bias which might obscure gene-environment interactions, determination of fibrinogen genotype and plasma fibrinogen level in a younger population sample, at the age before the overt onset of IHD, would be helpful. It would then be of interest to follow such a sample in a longitudinal study to determine if smokers possessing the A^{-455} allele were significantly less healthy than the non-smokers or those homozygous for the G^{-455} allele, especially with regard to IHD.

3. Concurrently, it would be of interest to determine promoter strength of the different alleles and their combinations in CAT assays and the effect of various cytokines on promoter strength in the different constructs. Bandshift studies would help to identify differential binding of nuclear proteins to the various alleles. These experiments are being pursued by Dr Fiona Green.

4. Examine the fibrinogen gene locus for other polymorphisms, both in coding and non-coding regions, and repeat similar association studies using those which look most promising.

Appendix 1

Sequence of 2.8kb insert (subclone 13), forward primer

The sequence of the pUC18 vector is underlined with the oligonucleotides subsequently identified for further sequencing or PCR shown in bold. Bases that could not be uniquely identified are shown in italics. There is a gap in the sequencing with the forward primer as indicated by .../.../...

<u>TAGAG TCGAC CTGCA GCCCA AGCTT</u> ATCCT CTTAA TCTTT TCATG TAAGC AAAGC TCATT AATTT CTGTC TTGGA AATGC TACTA CTCTC TTTAA TTACT CACCA AATCC AACTT TAACT TTTG**A CCTGG TTTCT CTGCC ACAAG** TTCTG TCCCA CTGGA GCCCA TACTC ACCTA CCGCT TTGCC ATCA*X* A*KX*TA ACAGA AAACT CTTAT CAACT TA**CTT CCTGC TTAAC ATTAG CTCC**T T*S*CTA TCTAT A.../.../...TTTTA GCTGG AGTAA AAATG GTCCC AGTAC CATTT CCTGT TCCCT TCACT ATAAC CTACA TTTTT GTCAC ATTAA GTTTT TCCCT ATTCC AGGAC AGGTC AGGCC CTTTA AAAAT TTCAA CAGCT TTATT GAGAT ATAAT TGATA TAATT TAAA*X XX*TC**C** TGCAC ATGTG TCATG CTGGA GCC**C**T ATTGA TTCCA ACAGG GATGG CGCCT TGTCC AAGAA GAGAC CCAGA GCCAG TGAAT GGGAC ATAGG GTTTA TTTAG GACTT AAATA CAGAT GTGGT CCAGT GGCAG TGGGC TGGAC AGGAC CGCTA CTATT TGTAA AGAGT ATGTA GTTAT ATACA TTCTA CTTAG CACTT **CACTA GCACT TCACT CGCAC TCA**TT ACCAA TAGCT AGC*X*C

K = T/G; S = G/C; X = unknown

Appendix 2

Sequence of 2.8kb insert (subclone 13) reverse primer

The sequence chosen for an oligonucleotide for PCR is shown in bold. Single bases that could not be uniquely identified are shown in italics. Where it was impossible to identify whether a base was present or not, or repeated, these single bases are shown in bold.

GTGCA TACAT CCATT ATAGT TTGTT GCAGA CTTTC AGTCC TAATT GTATA ATTAT ATATA CATAT ATATA ATTAT ATATG TGTAT ATATA TATAT GTATA TATAT ATATA AATAA **AATAG TTCTC ATACC ACAGT GT**GGA TTAAA AACTT TCAAC TAA*K*C TTTCT ATTAT CATCT TAAAA TCATG AGTTA TACAA TCTTG **C**TTGTTACTG TTTTA TATAT **TG**CTA AATAG GG**G**A*M* G**C**TAC CTTTT TTATT ATATT CAAAA TTG*Y*G *MK*GGC AAAAT CAGAC ACATA ACA

K = T/G; M = C/A; Y = C/T

Appendix 3

Sequence of 1.0kb insert (subclones 4 and 16) reverse primer

The sequence of the pUC18 vector is underlined. Bases that could not be uniquely identified are shown in bold. Where it was impossible to identify whether a base was present or not, or repeated, these single bases are also shown in bold.

AAGCT TCTCG CTSCA A**CCTT** GAAGA CTATT GGTTT GAGAA CTTCT CTTCC CATAC CACCC AAAAT CATAA TGCCA TTGGA AAGCA AAAAG TTGTT TTATC CATTT GATTT GAATT GTTTT AAGCC AATAT TTTAA GGTAA AACTC ACTGA ATCTA ACCAT AGCTG ACCTT TGTAG TAGAA TTTAC AACTT ATAAT TACAA TGCAC AATTT ATAAT TACAA TATGT ATTTA TGTCT TTTGC TATGG AGCAA ATCCA GGAAG GCAAG AGAAA CATTC TTTCC TAAAT ATAAA TGAAA ATCTA TCCTT TAA**A**C TCTTC **C**ACTA GACG**T** TGTAA TGCAC ACTTA T

S = G/C

Appendix 4

Sequence of 0.7kb insert, forward primer (subclone 1), reverse primer (subclone 6)

The sequence of the pUC18 vector is underlined with the oligonucleotides subsequently identified for further sequencing or PCR amplification shown in bold. Bases that could not be uniquely identified are shown in italics. Where it was impossible to identify an whether a base was present or not, or repeated, these single bases are shown in bold.

AAGCT TCTCT CTGCC ACTTT C**C**TGA TGTAC TGAAT GGCCC TTGGT ATATC TTTAT AAAAA TGTTA AAACC CACAG AGCTA TTTAA AGATA AAAAA AACCT TACAT AACTT T*M*TTT TTCAA AGACT AATA**T GTCTC AGGTA CATTT AGC**TA CAGTA CAAAG GATAA GAAA**A** TAGCA CCTAG G**G**AA**A** TTTTT T*X*G*R*T TGT**TA** GAGA**A** T*S*TCA **A**CTGC TTACG TTGAT TCTCT C

M = C/A; R = A/G; S = G/C

Appendix 5

Sequence of 0.7kb insert, reverse primer (subclone 1), forward primer (subclone 6)

Bases that could not be uniquely identified are shown in italics. Where it was impossible to identify an whether a base was present or not, or repeated, these single bases are shown in bold. The four base motif for the TaqI cutting site is shown in bold and underlined.

CCCGG GGAGC CTCTA GAGTC GACCT GCAGC CCAAG CTTTG CCACA CCACG GTATT TCCCC GATAC CTTGT GTGCA AAATT GCATC AGATA GTTGA TAGCC TTTTG TTTGT CGTTC TGGCT GAGTT CGTGC TTACC GCAGA AGTGC AGCCA TACCG AATCC GGCTT GTGAT TGCGC CATCC CCATA GCAGC CATCA CATCA GTACC GGAAA GAGAG TCAGA AGCCG TGGCC CGTGG TGAGT CGCTC ATCAT CGGGC TTTTT GGCGA ATGAA ATTTA GCTAC GCTT**T CGA**GT CTCAT GCGCC TTCTC CCTGT ACCTG AATCA ATGTT AGGTT TCGCA GAACA CTGCG CCGGT ATCGA TATAC ATTTGG TTGGCA A**A**CTTGA GTGTC ACTGC TGGCG TCTGA CAAGA TGACG TGTC

Appendix 6

Sequence from -872 to -584 from the start of transcription (individuals 1-3).

Where it was unclear as to the number of bases in a repetitive sequence, the bases are shown in bold .

AAATT TTGTG GCTTG TGGGA AATGA AGGAA AATGG GCCTC ATTTA GTCTG TGAGC ATACT AATTG AAATA GATAT ATGAA GACTT CACCA GTGTT AAAAT AACAT TGTTT TTATA AATCA TATGA TATAA ACTAT ATAAC AATAA AATAG AATGT TAAAC ATGTA TTTAA TCATC ATCAT AATTT TGATT CAGAA ATCTA TAATT TATTA GTTAT CTTAA TAATG TTTAG AATTT GTTGA ACATT TTACC TTATG TGAAT TAAGG ACAAA ATATT AAAGC TATTC AGCAC A

CURRENT SMOKERS ONLY: PLEASE CONTINUE BY ANSWERING THE FOLLOWING QUESTIONS

10. How old were you when you first started smoking regularly? |__|__| years old 47

11. Which of the following do you now smoke? (You may need to tick more than one box)

		Amount per day	OFFICE USE	
CIGARETTES	|__|	.....	|__|__|__|	49
CIGARS	|__|	.....	|__|__|	53
CHEROOTS OR SMALL CIGARS	|__|	.....	|__|__|	56
PIPE	|__|	.....	|__|__.__|	59
OTHER	|__|	.....	|__|__|	63

12. Which brand(s) do you currently smoke? (Please be as accurate as you can)

.....

OFFICE USE |__|__| 66

IF YOU SMOKE CIGARETTES: PLEASE CONTINUE BY ANSWERING THE FOLLOWING QUESTIONS

13. Are these usually filter tipped? YES |__| NO |__| 68

14. Is the tar yield usually: (please tick)

LOW |__| LOW/MIDDLE |__| MIDDLE |__| DON'T KNOW |__| 69

15. Is the size usually:

SUPERKING|__| KING|__| REGULAR|__| SMALL|__| HANDROLLED |__| 70

16. Do you normally inhale? YES |__| NO |__| 71

17. Do you smoke your cigarette to the stub? YES |__| NO |__| 72

18. Have you changed to low tar cigarettes? YES |__| NO |__| 73

19. If so, how long ago was this? |__|__|years |__|__|months 74

THANK YOU FOR YOUR HELP. PLEASE HAND THIS FORM BACK TO THE NURSE. 233

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Variation in the Promoter Region of the β Fibrinogen Gene Is Associated with Plasma Fibrinogen Levels in Smokers and Non-Smokers

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Summary

We investigated the association between fibrinogen levels and a HaeIII restriction fragment length polymorphism located at −453 bp from the start of transcription of the β fibrinogen gene. 292 healthy men aged 45 to 69 years, recruited from general practices throughout Britain, were studied. None had a history of ischaemic heart disease. 41.1% (120) were smokers and fibrinogen levels were higher in this group. The frequency of the non-cutting allele (designated H2) was 0.19 and was the same in smokers and non-smokers. The H2 allele was associated with elevated levels of fibrinogen in both smokers and non-smokers and the effect of genotype was similar in both groups. After smoking, HaeIII genotype was the strongest predictor of fibrinogen levels and explained 3.1% of the variance in fibrinogen levels. These results confirm earlier studies that variation at the fibrinogen locus contributes to the between-individual differences in plasma fibrinogen level.

Introduction

Several prospective studies have now confirmed the predictive value of plasma fibrinogen levels for vascular disease, including ischaemic heart disease (1–4). Fibrinogen is an acute phase protein that is synthesised in the liver and whose plasma level rises acutely after infection, injury and other trauma (5). Use of the oral contraceptive pill (6, 7), pregnancy (8) and the menopause (6) are also associated with a raised fibrinogen level, and in both men and women levels increase with age and obesity (6, 9–11). Diabetics are known to have higher levels than non-diabetics (12). However, levels of fibrinogen can be raised chronically and are affected by a variety of environmental factors, most particularly smoking (1, 2, 5, 6, 9, 13).

Genetic heritability has been shown to account for 51% of the variance of the plasma fibrinogen level in one study (14). This shows there is a strong genetic component determining plasma fibrinogen levels and thus the observed variation between individuals is in part genetically determined. Another study which used DNA polymorphisms at the fibrinogen gene cluster has estimated that variation at this locus is responsible for at least 15% of the variance in fibrinogen levels (15). These estimates of the contribution of various factors to fibrinogen level are almost certainly lower than their true contribution due to the marked within-individual variation that occurs, which in one study accounted for up to 27% of the sample variance in standardised assays (16).

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The fibrinogen molecule has a molecular weight of 340 kDa and is made up of two identical subunits joined together by disulphide bonds. Each subunit consists of three polypeptide chains Aα, Bβ and γ (17). Each chain is coded for by a separate gene (18–20) and these genes lie within a 50 kb segment of DNA in the distal third of the long arm of chromosome 4 (20–22). The β gene lies in the opposite transcriptional orientation to the other two genes.

It has been shown using pulse-chase experiments and more recently by transfection of the Bβ chain cDNA into HepG2 cells that synthesis of the Bβ chain is the rate limiting step in the formation of fibrinogen (23–25) and it is therefore reasonable to assume that changes in the rate of transcription of the β fibrinogen gene will alter the rate of production of the protein. The region up to 150 base pairs upstream of the start of transcription has been shown to contain the information necessary for liver specific transcription of the β fibrinogen gene (26) while a region between −150 and −82 base pairs is responsible for IL6 induction (27). Further upstream a sequence between −2,900 to −1,500 has been shown to be necessary for dexamethasone induction (27).

It is likely that a substantial part of the relationship between smoking and ischaemic heart disease is mediated through the rise in fibrinogen levels (9). A possible mechanism by which smoking causes this rise in levels is that persistent stimulation of lung macrophages by cigarette smoke causes them to produce IL6 which is a known mediator of the acute phase response (28–30). This circulating IL6 would result in chronic stimulation of fibrinogen synthesis through binding of positive transcription factors (31, 32) to the IL6 responsive element in the β fibrinogen gene (27).

Recently, using the restriction enzyme HaeIII we have detected a common DNA polymorphism in the 5′ flanking region of the β fibrinogen gene with the variable site 453 bp upstream from the start of transcription. We have investigated the association between this polymorphism and fibrinogen levels in a sample of healthy men, both smokers and non-smokers. We also investigated whether the elevation in fibrinogen levels associated with fibrinogen genotype was the same in smokers and non-smokers.

Materials and Methods

Individuals participating in this study were selected from four general practices in the Thrombosis Prevention Trial (TPT) (33). The methodology of the trial has been previously described (33). Men with a history of ischaemic heart disease and those who had contraindications to warfarin or aspirin therapy were excluded from the trial. This rendered about a third of those registered with the chosen practices ineligible. The remainder were invited to be screened for TPT. The men were classified by smoking status as current smokers or non-smokers. Ex-smokers were included in the latter group as risk assessment in TPT is based on current smoking habit. A sample of current smokers was then selected at random

across the distribution of plasma fibrinogen levels available from the screening and were matched for age and body mass index (BMI) with a sample from the group of non-smokers. Age and BMI were controlled for in this way as they are known to affect fibrinogen levels.

Each of the 292 men was informed of the purpose of the study and a blood sample was taken, 4.5 ml into 0.5 ml of 3.8% sodium citrate and 10 ml as two aliquots into EDTA. The citrated sample was sent as plasma to Northwick Park Hospital and assayed for fibrinogen concentration by the Clauss method (34). All assays were performed in mixed batches with the routine TPT samples. The EDTA samples were stored in liquid nitrogen pending transfer frozen to the Charing Cross Sunley Research Centre for genotyping. On arrival there, they were stored at −20° C. DNA was extracted (after a maximum storage time of 2.5 years) using the "Triton-X100" lysis method (35). A portion of the β fibrinogen gene from −1178 bp from the start of transcription to +122 bp was amplified by the polymerase chain reaction (PCR) technique (36), using 100–400 ng of genomic DNA and thermostable Taq polymerase (Perkin Elmer-Cetus) under conditions recommended by the manufacturer. The PCR reactions were performed in a Cambio "Intelligent Heating Block". The initial cycle consisted of steps at 93° C for 3 min, 55° C for 1 min and 72° C for 2 min. The 30 subsequent cycles were 93° C for 1 min, 55° C for 1 min and 72° C for 2 min. The oligonucleotide primers were:

(5′-3′) AAGAATTTGGGAATGCAATCTCTGCTACCT

and

(5′-3′) CTCCTCATTGTCGTTGACACCTTGGGAC

on the coding and non-coding strands of the DNA respectively. The fragment amplified was therefore 1.3 kb in length (Fig. 1). 10–30 µl of PCR product were digested with the restriction enzyme HaeIII (Boehringer Mannheim) or its isoschizomer BsuRI (Anglian Biotec Limited) at 37° C using 10–20 U/reaction. The DNA fragments were separated on 2% agarose gels by electrophoresis in 40 mM Tris-acetate 1 mM EDTA pH 7.7 containing 0.5 µg/ml ethidium bromide and visualized under ultra violet light.

Table 1 Characteristics of subjects

Subjects	No.	Age (SD) (years)	BMI (SD)	Fibrinogen (SD) (g/l)
Non smokers	172	54.8 (5.7)	25.4 (2.7)	2.66 (0.56)
Smokers	120	54.9 (5.7)	25.5 (2.9)	3.01 (0.74)*
All	292	54.8 (5.7)	25.5 (2.8)	2.80 (0.66)

*p <0.001
BMI = body mass index.

Statistical Analysis

The distribution of fibrinogen was found, in common with other studies, to be skewed to the right. Logarithmic transformation normalized the distribution and transformed values were used in the analyses. Age, body mass index and plasma fibrinogen level were compared between genotype groups and smokers and non-smokers using one way analysis of variance and Student's unpaired t-test. Observed numbers of each genotype were compared with those expected if the sample were in Hardy-Weinberg equilibrium using chi-squared analysis. Allele frequencies in different groups were compared by gene counting and chi-squared analysis.

In order to evaluate the relative contribution to the plasma fibrinogen of the genotype, smoking status, age and body mass index, each of these was entered into a stepwise multiple regression analysis, which allows for the assessment of any significant interaction between the variables. A regression analysis was also performed separately for smokers and non-smokers. The effect associated with the H2 allele of the HaeIII polymorphism (average excess) was calculated by the method of Templeton

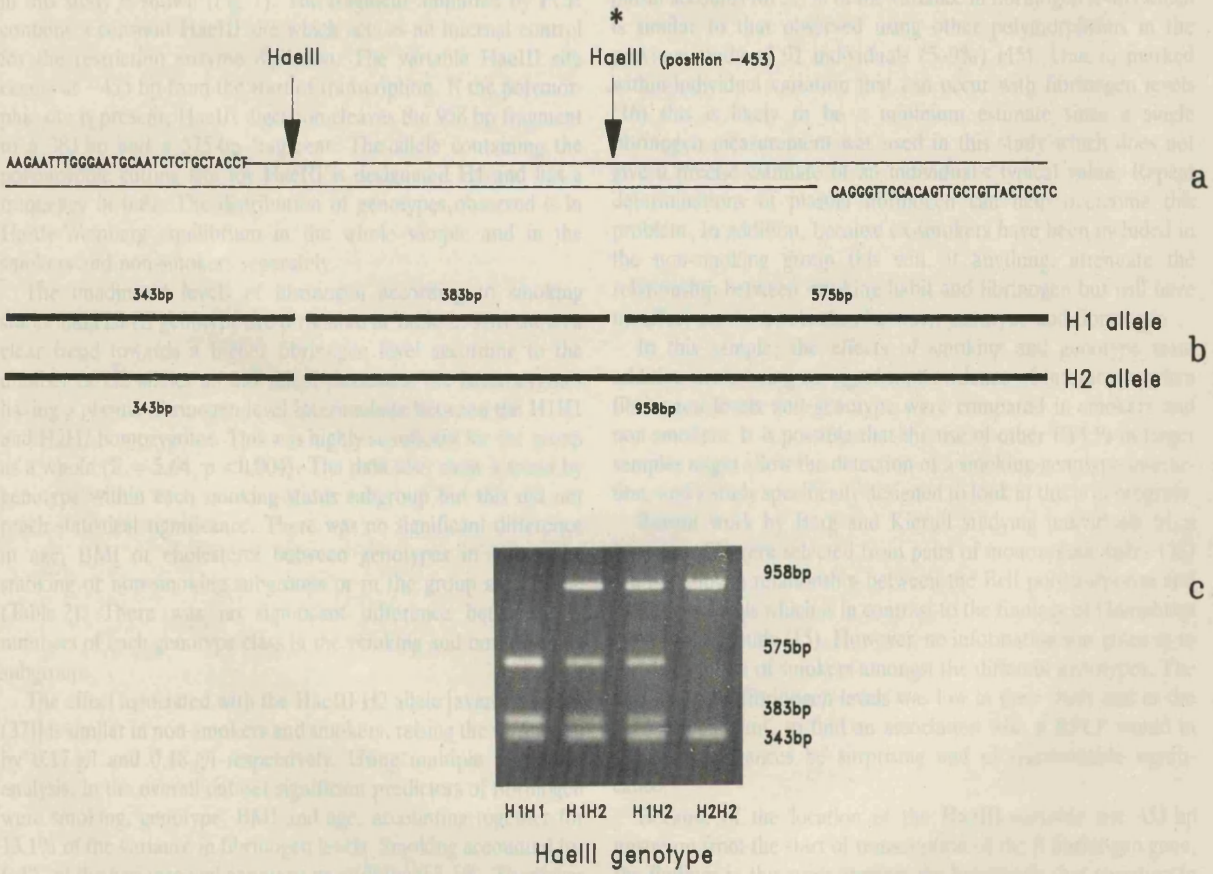

Fig. 1 A schematic representation of the HaeIII polymorphism within the 5′ flanking region of the β fibrinogen gene: a: The sequence of the oligonucleotide primers and relative positions of the constant and polymorphic (*) HaeIII cutting sites. b: The fragment lengths of the PCR product after digestion. c: HaeIII digested products from individuals of different genotypes separated by agarose gel electrophoresis

Table 2 Mean fibrinogen values in groups with different HaeIII genotypes

Genotype	No.	Mean Fgn (SD) (g/l)	Age (SD) (years)	BMI (SD)	Cholesterol (SD) (mmol/l)
Non smokers					
H1H1	115	2.58 (0.51)	55.3 (5.8)	25.2 (2.7)	5.87 (1.09)
H1H2	49	2.81 (0.64)	53.8 (5.3)	25.9 (2.7)	5.74 (0.90)
H2H2	8	2.91 (0.62)	53.8 (6.7)	25.4 (2.4)	5.58 (1.22)
Smokers					
H1H1	75-	2.90 (0.64)	55.0 (5.6)	25.4 (2.8)	5.98 (1.01)
H1H2	42	3.18 (0.87)	54.9 (6.0)	25.7 (3.2)	5.79 (1.03)
H2H2	3	3.32 (0.58)	53.7 (6.1)	28.4 (1.8)	5.13 (0.32)
All					
H1H1	190	2.71 (0.59)*	55.6 (5.7)	25.3 (2.7)	5.91 (1.06)
H1H2	91	2.98 (0.77)	54.3 (5.6)	25.8 (2.9)	5.76 (0.95)
H2H2	11	3.02 (0.61)	53.7 (6.3)	26.2 (2.6)	5.46 (1.06)

*Analysis of variance: $F = 5.64$, $p < 0.004$.

(37). Untransformed means and standard deviations are given in the tables for simplicity.

Results

The characteristics of the population studied are shown in Table 1. The mean (SD) fibrinogen level of the whole group was 2.80 (0.66) g/l which is similar to that previously reported (3). As expected, there was no significant difference in age and BMI between each of the smoking and non-smoking subgroups but there was a highly significant difference in plasma fibrinogen levels, being 2.66 (0.56) g/l in non-smokers compared with 3.01 (0.74) g/l in smokers ($p < 0.001$).

A schematic representation of the HaeIII polymorphism used in this study is shown (Fig. 1). The fragment amplified by PCR contains a constant HaeIII site which acts as an internal control for the restriction enzyme digestion. The variable HaeIII site occurs at −453 bp from the start of transcription. If the polymorphic site is present, HaeIII digestion cleaves the 958 bp fragment to a 383 bp and a 575 bp fragment. The allele containing the polymorphic cutting site for HaeIII is designated H1 and has a frequency of 0.81. The distribution of genotypes observed is in Hardy-Weinberg equilibrium in the whole sample and in the smokers and non-smokers separately.

The unadjusted levels of fibrinogen according to smoking status and HaeIII genotype are presented in Table 2. This shows a clear trend towards a higher fibrinogen level according to the number of H2 alleles an individual possesses, the heterozygotes having a plasma fibrinogen level intermediate between the H1H1 and H2H2 homozygotes. This was highly significant for the group as a whole ($F = 5.64$, $p < 0.004$). The data also show a trend by genotype within each smoking status subgroup but this did not reach statistical significance. There was no significant difference in age, BMI or cholesterol between genotypes in either the smoking or non-smoking subgroups or in the group as a whole (Table 2). There was no significant difference between the numbers of each genotype class in the smoking and non-smoking subgroups.

The effect associated with the HaeIII H2 allele [average excess (37)] is similar in non-smokers and smokers, raising the fibrinogen by 0.17 g/l and 0.18 g/l respectively. Using multiple regression analysis, in the overall dataset significant predictors of fibrinogen were smoking, genotype, BMI and age, accounting together for 13.1% of the variance in fibrinogen levels. Smoking accounted for 6.4% of the variance and genotype an additional 3.1%. There was no evidence of a significant interaction between smoking and genotype in their effects on fibrinogen levels.

Discussion

The distribution of genotypes for the HaeIII polymorphism of the β fibrinogen gene exhibits Hardy-Weinberg equilibrium in this sample. Because of the selection procedure used, it is likely that the sample is representative of healthy men in the U.K. The data show that there is a significant association between HaeIII genotype and plasma fibrinogen concentrations. The results of this study support previous findings of an association in healthy individuals between RFLPs at the fibrinogen gene locus and fibrinogen levels (15). The strongest predictor of fibrinogen levels was smoking accounting for 6.4% of the variance. Age and BMI together explained 3.6% of the variance but both these parameters had been controlled for in this study. The HaeIII polymorphism accounts for 3.1% of the variance in fibrinogen levels which is similar to that observed using other polymorphisms in the previous study of 91 individuals (5–9%) (15). Due to marked within-individual variation that can occur with fibrinogen levels (16) this is likely to be a minimum estimate since a single fibrinogen measurement was used in this study which does not give a precise estimate of an individual's typical value. Repeat determinations of plasma fibrinogen can help overcome this problem. In addition, because ex-smokers have been included in the non-smoking group this will, if anything, attenuate the relationship between smoking habit and fibrinogen but will have no effect on the association between genotype and fibrinogen.

In this sample, the effects of smoking and genotype were additive there being no significant evidence of interaction when fibrinogen levels and genotype were compared in smokers and non-smokers. It is possible that the use of other RFLPs in larger samples might allow the detection of a smoking-genotype interaction, and a study specifically designed to look at this is in progress.

Recent work by Berg and Kierulf studying individuals from Norway who were selected from pairs of monozygotic twins (38) did not show a relationship between the BclI polymorphism and fibrinogen levels which is in contrast to the findings of Humphries et al. in their study (15). However, no information was given as to the distribution of smokers amongst the different genotypes. The heritability of fibrinogen levels was low in their study and as the authors comment, to find an association with a RFLP would in these circumstances be surprising and of questionable significance.

Because of the location of the HaeIII variable site 453 bp upstream from the start of transcription of the β fibrinogen gene, the findings in this study support the hypothesis that variation in the 5′ flanking region of this gene is important in the control of fibrinogen production. The base change that creates the HaeIII

polymorphism may itself be a functional change which affects the affinity of a nuclear protein involved in control of transcription of the gene. Experiments to test this possibility are in progress. However, it is also possible that the HaeIII site is in linkage disequilibrium with functionally important sequence changes in another region of the gene cluster. Our data show that both genotype and smoking status strongly and independently predict fibrinogen level in healthy men and, as expected, the worst possible combination is the possession of the H2 allele with a smoking habit.

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MUTATION IN BRIEF

Linkage Disequilibrium Across the Fibrinogen Locus As Shown by Five Genetic Polymorphisms, G/A^{-455} (*Hae*III), C/T^{-148} (*Hin*dIII/*Alu*I), T/G^{+1689} (*Ava*II), and *Bcl*I (β-Fibrinogen) and *Taq*I (α-Fibrinogen), and Their Detection by PCR

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INTRODUCTION

Elevated plasma fibrinogen level is a risk factor for cardiovascular disease (reviewed in Ernst, 1993) and several studies have shown that genetic variation at the fibrinogen locus is implicated in determining an individual's basal or inducible fibrinogen level (Humphries et al., 1987; Thomas et al., 1991a, Green et al., 1993). We have assessed linkage disequilibrium at the fibrinogen locus as revealed by five polymorphisms within or adjacent to the α- and β-fibrinogen genes, namely G/A^{-455} (*Hae*III), C/T^{-148} (*Hin*dIII/*Alu*I), T/G^{+1689} (*Ava*II), and *Bcl*I (β-fibrinogen) and *Taq*I (α-fibrinogen). The *Ava*II, *Bcl*I (β-fibrinogen), and *Taq*I (α-fibrinogen) polymorphisms, shown to be associated with differences in plasma fibrinogen level (Humphries et al., 1987) and with peripheral arterial disease for the *Bcl*I polymorphism (Fowkes et al., 1992), were previously detectable only by the relatively laborious process of Southern blotting and we describe here simple protocols for their rapid detection by PCR. The G/A^{-455} polymorphism in the 5′-flanking region of the β-fibrinogen gene is detected by PCR and digestion with *Hae*III and has also been shown to be associated with differences in plasma fibrinogen level (Thomas et al., 1991a; Green et al., 1993). We also report the identification of a C to T base change at position −148 in the β-fibrinogen promoter, detected by PCR with the same primers used for detection of the G/A^{-455} polymorphism but followed by digestion with *Hin*dIII (or *Alu*I).

METHODS

Mapping of Polymorphic Restriction Enzyme Cutting Sites

The polymorphic *Ava*II site was mapped to intron 1 and the polymorphic *Bcl*I site to the 3′ end of the β-fibrinogen gene by comparison with a *Hin*dIII restriction map of the fibrinogen locus (Kant et al., 1985), contrary to our previous map (Humphries et al., 1987). The polymorphic *Taq*I site has previously been mapped to the 3′ end of the α-fibrinogen gene (Humphries et al., 1984) (Fig. 1).

Identification of PCR Primers for *Bcl*I and *Taq*I Polymorphisms

The nucleotide sequence of the region, between the α- and β-fibrinogen genes, to which these polymorphic sites had been mapped was unavailable. Therefore, in order to obtain sufficient sequence to design PCR primers, a bacteriophage lambda clone Nαβ (kind gift of Dr Giles Courtois, INSERM U217, Grenoble, France), containing the 3′ ends of the α- and β-fibrinogen genes and the intergenic region, was digested with *Hin*dIII and subcloned into pUC18. *Bcl*I and *Taq*I sites could be demonstrated within the predicted fragments (2.8-kb and 0.7-kb fragments respectively; see Figure) and these fragments were partially sequenced. This, together with published sequenced from the 3′ end of the α-fibrinogen gene (Chung et al., 1990), enabled the construction of primers to PCR amplify across the *Bcl*I and *Taq*I sites, both of which were found to be polymorphic.

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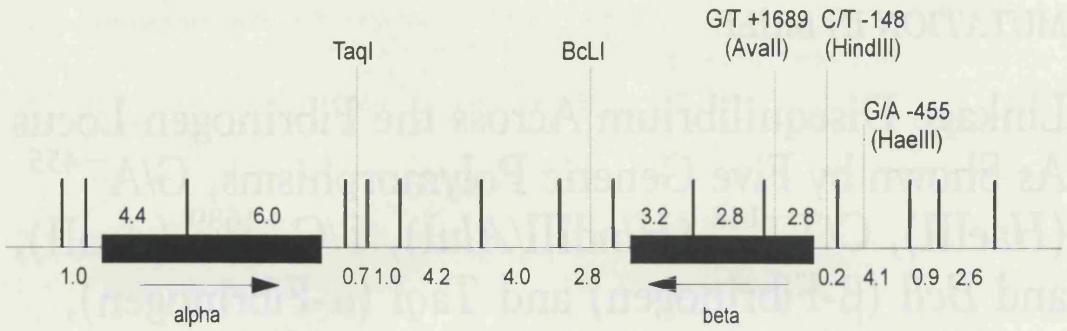

FIGURE 1. A map of the α- and β-fibrinogen genes (not to scale) showing the relative positions of the polymorphic sites described in this report (dotted vertical lines), together with *Hin*dIII cutting sites (solid vertical lines) with approximate distances between adjacent *Hin*dIII sites given in kilobase pairs. The *Hin*dIII map was derived from Kant et al. (1985), Chung et al. (1990), Courtois et al. (personal communication), and Thomas et al. (unpublished data). The direction of transcription of the two genes is indicated by *arrows*.

Identification of Base Changes Causing *Hin*dIII (*Alu*I) and *Ava*II Polymorphisms

Direct sequence analysis of the PCR fragment revealed that the *Hin*dIII (AluI) polymorphism (Thomas et al., 1991b) was caused by the presence of either a C or a T at position −148 from the start of transcription of the β-fibrinogen gene. Examination of the published nucleotide sequence (Chung et al., 1990) revealed two possible polymorphic AvaII sites in the appropriate position, differing from the recognition sequence by a single nucleotide. The most 3′ of these at +1,689 bp from the start of transcription was found to be polymorphic, with either a T or a G at this position.

PCR Conditions

Each 50-μl PCR reaction contained 100–400 ng genomic DNA, 200 ng of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 0.01% gelatin, 200 μM dNTPs, 1 unit Taq polymerase and 0.05% W1 (GIBCO BRL, UK). The reactions were incubated at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, in a Cambio "Intelligent Heating Block" (with "plate" temperature control), except for the *Taq*I polymorphism where forty cycles were required.

PCR Primers (5′–3′)

C/T^{-148} (*Hin*dIII/*Alu*I)	AAG AAT TTG GGA ATG CAA TCT CTG CTA CCT
	CTC CTC ATT GTC GTT GAC ACC TTG GGA C
T/G^{+1689} (*Ava*II)	TGG TTA ATC TGG TTA AGT CTG G
	GTC AGT AGC TAT ACA TCC TTT G
*Bcl*I	ACC TGG TTT CTC TGC CAC AAG
	AAT AGT TCT CAT ACC ACA GTG T
*Taq*I	AGC CGT GCC TAT CTT TG
	TGT CTC AGG TAC ATT TAG C

Restriction Enzyme Digestion

Ten μl of the PCR reaction were digested with 10 units of the appropriate restriction enzyme according to the standard procedures. PCR and digestion fragment sizes are shown in Table 1.

RESULTS AND DISCUSSION

Allele frequencies and PIC values for the five polymorphisms in a sample of 302 healthy middle-aged Caucasian men (Thomas et al., 1991a), are shown in Table 1. In this sample, the G/A^{-455}, C/T^{-148}, and T/G^{+1689} polymorphisms of the β-fibrinogen gene were found to be in complete linkage disequilibrium (except for one individual whose genotype was $G + A^{-455}C + T^{-148}T + T^{+1689}$). The β-fibrinogen *Bcl*I polymorphism B1 allele was in strong allelic association with the $G^{-455}\,C^{-148}\,T^{+1689}$ allele ($\Delta = 0.85$, $\chi^2 = 322$, $P < 0.001$). However, linkage disequilibrium was weak between the *Taq*I polymorphism of the α-fibrinogen gene and all the β-fibrinogen polymorphisms (*Bcl*I/*Taq*I: $\Delta = 0.01$, $\chi^2 = 1.49$, $P > 0.1$; G/A^{-455}/*Taq*I: $\Delta = -0.09$, $\chi^2 = 2.53$, $P > 0.1$). Thus, in studies of association between fibrinogen genotype and fibrinogen level or cardiovascular disease, determination of genotype for one of the β-fibrinogen $G/A^{-455}\,C/T^{-148}$, or T/G^{+1689} polymorphisms, the β-fibrinogen *Bcl*I polymorphism and the α-fibrinogen *Taq*I polymorphism will provide maximal information.

Increasing interest has been shown recently in the polymorphisms of the fibrinogen gene locus because they are associated with interindividual differences in plasma fibrinogen level (Humphries et al., 1987; Thomas et al., 1991a; Green et al., 1993) and because they may also determine indi-

TABLE 1. Allele Frequencies, PIC Values, and Fragment Size of PCR and Digestion Products for Polymorphisms at the Fibrinogen Gene Locus

Polymorphism	G/A^{-455} (*HaeIII*)		C/T^{-148} (*HindIII/AluI*)		T/G^{+1689} (*AvaII*)		*BclI*		*TaqI*	
PCR fragment size (bp)	1,301		1,301		710		2,500		900	
Allele designation	G^{-455}	A^{-455}	C^{-146}	T^{-146}	T^{+1689}	G^{+1689}	B1	B2	T1	T2
Restriction enzyme cutting site	+	–	+	–	–	+	–	+	+	+
Digestion fragment size (bp)	–	958	835	835	400	400	2500	–	←	900
	575	–	–	466	310	–	–	1400	800	→
	383	–	272	–	–	250	–	1100	100	→
	343	343	194	–	–	60	–	–	–	–
Allele frequency	0.81	0.19	0.81	0.19	0.81	0.19	0.85	0.15	0.73	0.27
PIC value	0.26		0.26		0.26		0.2		0.32	
No. chromosomes tested	596		596		596		586		552	

PIC, polymorphism information content; PCR, polymerase chain reaction; bp, base pairs. Discrepancy in numbers of chromosomes tested is due to a small number of failed PCRs.

vidual-specific effects of environmental factors such as smoking (Green et al., 1993) on fibrinogen level. In one study, fibrinogen genotype has been shown to be predictive of peripheral arterial disease risk (Fowkes et al., 1992) and may also contribute to coronary heart disease risk. We report here that the previously identified *Hin*dIII polymorphism in the β-fibrinogen promoter (Thomas et al., 1991b) is caused by a C to T base change at −148 bp from the start of transcription of the β-fibrinogen gene. The position of this base change, within a putative transforming growth factor-β responsive element and adjacent to interleukin-6 responsive elements, suggests that it may influence cytokine and/or growth factor modulated transcription from the β-fibrinogen promoter, a possibility which is currently being investigated in this laboratory. Preliminary analysis of the binding of hepatic nuclear proteins to the β-fibrinogen promoter in vitro showed that binding was influenced to some extent by genotype at both the C/T^{-148} and the G/A^{-455} polymorphic sites (Green F, Lane A, unpublished data). The finding that there is strong association between the alleles at these two polymorphic sites, both of which appear to influence binding of nuclear proteins, makes it tempting to suggest that there may be some interaction between these two sites and/or their binding proteins, perhaps in the control of transcription.

Detection of these fibrinogen genetic polymorphisms using PCR rather that Southern blotting is both technically simpler and more rapid, enabling large population samples to be genotyped easily and quickly. This will facilitate analysis of the role of gene–environment interaction in determining plasma fibrinogen levels in the general population and in determining an individual's risk of cardiovascular disease.

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Original articles

The association of combined α and β fibrinogen genotype on plasma fibrinogen levels in smokers and non-smokers

A E Thomas, F R Green, H Lamlum, S E Humphries

Abstract

Objective **– To determine in healthy men: (1) whether an extended genotype of the fibrinogen gene cluster using the G/A^{-455} and the *BclI* polymorphism of the β fibrinogen gene and *TaqI* of the α fibrinogen gene explains a significantly larger proportion of variance in plasma fibrinogen levels in either smokers or non-smokers than a single polymorphism (G/A^{-455}); (2) whether there is any evidence for genotype-smoking interaction in the determination of fibrinogen levels.**

Design **– A cross sectional study of healthy, white men recruited at the screening for entry into the Thrombosis Prevention Trial.**

Setting **– The subjects were drawn from four general practices in the United Kingdom.**

Results **– The frequency of the rare alleles in the sample was 0·19 for the G/A^{-455} polymorphism (A^{-455}), 0·15 for *BclI* (B+), and 0·27 for *TaqI* (T+) alleles. BMI and age made significant contributions to the variance in plasma fibrinogen levels only in non-smokers of 5·4% and 2·3% respectively and, in the group as a whole, smoking accounted for 6·6% of the variance. In the non-smokers, of the individual polymorphisms only the G/A^{-455} showed a significant association with plasma fibrinogen levels (p=0·03). The mean fibrinogen in non-smokers homozygous for the G^{-455} allele was 2·54 g/l *v* 2·85 g/l in those homozygous for the A^{-455} allele, with the polymorphism explaining 3·6% of the variance in plasma fibrinogen levels in this group. On investigation of the association of fibrinogen levels with combined genotypes, the most significant effect was seen with the combination of the G/A^{-455} and *TaqI* polymorphisms, with those with no "fibrinogen raising alleles" having a mean fibrinogen of 2·57 g/l *v* 3·10 g/l for those with four "fibrinogen raising alleles" (p=0·0036), and this combination explained 8·9% of the variance in plasma fibrinogen levels (p<0·005). Although the contribution to variance was greater with the G/A^{-455}/*TaqI* combination than the G/A^{-455} polymorphism alone (8·9% *v* 3·6%), this did not reach significance (p=0·09). By contrast, in the smoking group, the only significant contribution to the difference in plasma fibrinogen levels was the G/A^{-455} genotype alone which, after adjustment for BMI and age, contributed 3·8% to the variance (p<0·05). No interaction was shown between smoking and genotype.**

Conclusion **– These data suggest that in non-smokers an extended genotype using the G/A^{-455} β fibrinogen gene polymorphism and the *TaqI* α fibrinogen gene polymorphism explains a larger proportion of the variance in plasma fibrinogen levels than any one polymorphism alone, but that smoking has an overriding effect so that other variables such as age and BMI make little additional contribution.**

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Several prospective epidemiological studies have shown that plasma fibrinogen level is an important predictor of ischaemic heart disease and stroke.[1–6] The risk of developing peripheral vascular disease is also increased with increasing plasma fibrinogen levels.[7–9] Fibrinogen is an acute phase protein whose levels rise in response to infection, inflammation, and trauma.[10] Other factors which influence levels include age, obesity, hormonal changes, and diabetes.[11–15] Fibrinogen can be raised as a result of a variety of environmental factors, most particularly smoking.[1 2 10–12 16] It is likely that a substantial part of the relationship between smoking and ischaemic heart disease is mediated through its effect on plasma fibrinogen levels.

Fibrinogen consists of two each of three separate chains, Aα, Bβ, and γ,[17] each encoded by a separate gene.[18–20] The fibrinogen gene loci lie in a cluster spanning approximately 50 kb on the long arm of chromosome 4.[20–22] The synthesis of the Bβ chain is the rate limiting step for fibrinogen production[23–25] and this has prompted investigation of genetic variation at these loci, especially the β fibrinogen promoter region, for association between such variation

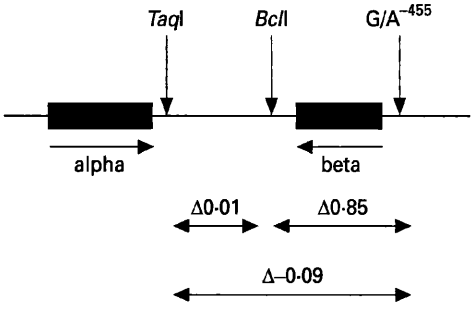
and differences in plasma fibrinogen levels. Several reports have shown such an association,[26–29] but this has not been seen in all studies.[30 31] More recently there has been a report of a gene-environment interaction between smoking and fibrinogen genotype[28] but other studies have not shown this finding.[26 29]

In this report, the role of genetic variation at the fibrinogen gene locus has been studied in more detail. Recently, detection of five different polymorphisms involving both the α and the β fibrinogen gene loci using PCR has been described.[32] It is therefore relatively quick and simple when compared to Southern blotting to elucidate the extended genotype of a person. To what extent further information is gained by using the different polymorphisms or an extended genotype has been investigated. Possible gene-environment interaction has also been sought.

Materials and methods

The 292 healthy men participating in this study were from four general practices being screened for entry into the Thrombosis Prevention Trial[33] and the selection procedures have previously been described.[26] Samples were collected and DNA extracted as described by Thomas *et al.*[26] Three of the polymorphisms described by Thomas *et al*,[32] G/A^{-455}, C/T^{-148}, and T/G^{+1689}, are in complete linkage disequilibrium (except one of 596 chromosomes tested) and therefore just one of these, G/A^{-455}, was used. The other two polymorphisms investigated were those detectable using the restriction enzymes *BclI* and *TaqI*, of the β fibrinogen and α fibrinogen genes respectively. The primers and conditions used for PCR amplification are given in Thomas *et al.*[32]

The data were analysed using the statistical package SPSS.[34] Fibrinogen levels were $\log_{10}$ transformed to normalise the data. Combined genotypes were devised by counting the number of alleles in any given genotype combination that were associated with higher fibrinogen levels. Although some associations did not reach significance in this study, the trend seen was similar to that in a previous study[27] and the designated "fibrinogen raising alleles" determined from this. These were the A^{-455}, the *BclI* cutting, and *TaqI* non-cutting alleles. Thus, for an extended genotype involving two different polymorphisms there are five categories having 0, 1, 2, 3, or 4 "fibrinogen raising alleles". For the extended genotype involving all three polymorphisms there are seven possible categories. A quadratic term was calculated for each of the genotype groups and tested with the linear term in a multiple regression analysis. There was no evidence of non-linearity in any of the genotype groups except for the combined G/A^{-455}/*TaqI* in non-smokers. The genotypes were therefore entered into any regression analysis as n categories except for the G/A^{-455}/*TaqI* combination when dummy variables were used. In order to evaluate the relative contribution to the variance in plasma fibrinogen levels of the genotypes and their combinations, multiple regression analysis was used, first adjusting for BMI and age which are known to affect plasma fibrinogen levels and then entering the various genotypes or their combinations to see if any further significant effect was seen. The analysis was performed separately in smokers and non-smokers. Interaction terms were calculated between smoking and age, smoking and BMI, and smoking and genotype and entered into the multiple regression model.



A map of the α and β fibrinogen genes (not to scale) showing relative positions of the TaqI, BclI, and G/A^{-455} polymorphic sites and linkage disequilibrium coefficients. The transcription of the two genes is indicated by arrows above the text alpha and beta.

Results

The locations within the fibrinogen gene cluster of the three variable sites used in the study are shown in the figure. The characteristics of the PCR fragments of each of the polymorphisms have been previously described.[32] The overall frequencies of the polymorphic alleles in the subjects were 0·81 and 0·19 for the G/A^{-455} polymorphism, 0·85 and 0·15 for the *BclI* non-cutting (B−) and cutting (B+) allele respectively, and 0·73 and 0·27 for the *TaqI* non-cutting (T−) and cutting (T+) allele respectively. These frequencies are similar to those found in previous studies.[27 30 31] The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium and no significant differences were detected.

The characteristics of the smokers and non-smokers are given in table 1 and, as expected, smokers have a significantly higher fibrinogen than non-smokers (2·93 g/l *v* 2·61 g/l, p<0·0001). Of the individual polymorphisms, only the G/A^{-455} in the non-smokers showed a significant association with unadjusted plasma fibrinogen levels, with those homozygous for the A^{-455} allele having 12·2% higher levels than those homozygous for the G^{-455} allele (p= 0·03). In the smokers there was a trend, although not significant, towards higher fibrinogen levels in those possessing the A^{-455}

Table 1 Fibrinogen, age, and BMI by smoking status

Smoking status (No)	*Fibrinogen (g/l) (95% CI)*	*Age (y) (SD)*	*BMI (SD)*
Non-smoker (172)	2·61 (2·53–2·69)	55·5 (5·8)	25·4 (2·8)
Smoker (120)	2·93* (2·81–3·05)	55·3 (5·8)	25·6 (2·9)

*p<0·0001.

Table 2 Unadjusted plasma fibrinogen levels according to genotype and smoking status

RFLP	*Smoking status*	*Fibrinogen (g/l) 95% CI (No)*			*p value*
		GG	*GA*	*AA*	
G/A^{-455}	Smoker	2·84 2·70–2·98 (75)	3·07 2·84–3·33 (42)	3·28 1·60–6·76 (3)	0·13
	Non-smoker	2·54 2·45–2·63 (115)	2·74 2·58–2·92 (49)	2·85 2·29–3·55 (8)	0·03
		B−B−	*B−B+*	*B+B+*	
BclI	Smoker	2·85 2·72–2·99 (83)	3·12 2·85–3·41 (35)	2·96 2·01–4·36 (2)	0·16
	Non-smoker	2·55 2·46–2·64 (123)	2·77 2·57–2·98 (37)	2·61 1·92–3·57 (4)	0·10
		T−T−	*T−T+*	*T+T+*	
TaqI	Smoker	2·93 2·77–3·10 (62)	2·97 2·78–3·18 (48)	2·67 2·26–3·15 (7)	0·52
	Non-smoker	2·66 2·54–2·78 (88)	2·55 2·41–2·69 (62)	2·49 2·24–2·76 (9)	0·37

One way analysis of variance.

Table 3 Mean (geometric, unadjusted) plasma fibrinogen level by combined G/A^{-455} and TaqI genotype and smoking status

G/A^{-455}/TaqI genotype (No of "fibrinogen raising alleles")	*Fibrinogen (g/l) 95% CI (No)*			
	Smoker		*Non-smoker*	
AA/T−T− (4)	3·28 2·17–4·98 (3)		3·10 2·20–4·36 (4)	
AA/T−T+ GA/T−T− (3)	3·13 2·78–3·53 (23)	3·15 2·83–3·50 (26)	2·93 2·68–3·20 (29)	2·95 2·72–3·20 (33)
GG/T−T− AA/T+T+ GA/T−T+ (2)	2·86 2·70–3·03 (55)		2·52 2·43–2·62 (74)	
GG/T−T+ GA/T+T+ (1)	2·95 2·70–3·22 (29)	2·87 2·75–3·00 (91)	2·52 2·35–2·70 (46)	2·52 2·44–2·61 (126)
GG/T+T+ (0)	2·67 2·62–3·15 (7)		2·57 2·20–3·00 (6)	
p value*	0·35	0·07	0·0036	0·0001

* From analysis of variance on log transformed, unadjusted fibrinogen values.

Table 4 Contribution to variance in plasma fibrinogen levels using a multiple regression model

Smoking status	*Variable*					
			Polymorphism			
	BMI (No)	*Age (No)*	*BclI (No)*	*G/A^{-455} (No)*	*TaqI (No)*	*G/A^{-455}/TaqI (No)*
Non-smoker	5·4%‡ (172)	2·3%† (172)	2·4%† (164)	3·6%‡ (172)	1·4%* (159)	8·9%§ (159)
Smoker	0·1%* (120)	1·5%* (120)	2·5%* (120)	3·8%† (120)	0·3%* (117)	4·4%* (117)

BMI and age were entered first into the regression model and then genotype entered and the additional contribution shown in the table.
* Not significant, † $p<0·05$, ‡ $p<0·01$, § $p=0·005$.

allele, with those homozygous for this allele having fibrinogen levels 15·5% higher than those homozygous for the G^{-455} allele (table 2). For the *BclI* polymorphism (β fibrinogen) highest plasma fibrinogen levels were observed in the heterozygotes and the lowest in those homozygous for the non-cutting allele for both smokers and non-smokers (table 2). The numbers were small in those homozygous for the cutting allele. This is similar to the trend seen in a previous study.[27] For the *TaqI* polymorphism (α fibrinogen), no significant association was shown with plasma fibrinogen levels in smokers or in non-smokers. However, in both groups a trend toward lower levels in those with one or two cutting alleles was observed, with those homozygous for T+ having 8·9% lower levels than those homozygous for T− in smokers and 6·4% lower levels in non-smokers (table 2).

The association between plasma fibrinogen levels and combined genotypes was investigated and the most significant effect was seen with the combination of the G/A^{-455} and *TaqI* genotypes (table 3). This reached significance in the non-smokers but not in the smokers. The number of categories of genotypes was reduced to investigate whether the presence of 3–4 "fibrinogen raising alleles" compared with the presence of 0–2 "fibrinogen raising alleles" showed a more significant difference. In the non-smokers, fibrinogen levels were 17% higher (2·95 g/l *v* 2·52 g/l, $p=0·0001$) and in smokers, levels were 10% higher (3·15 g/l *v* 2·87 g/l, $p=0·07$).

A multiple regression analysis was performed for all combinations of genotypes having first entered BMI then age. In non-smokers, BMI made a contribution to variance of 5·4% and age a smaller contribution of 2·3% (table 4). BMI and age made no significant contribution in the smokers. In the non-smokers, genotype explained 2·4% ($p<0·05$) and 3·6% ($p<0·01$) of the variance in plasma fibrinogen levels for the *BclI* polymorphism and the G/A^{-455} respectively. The *TaqI* polymorphism made a smaller contribution of 1·4% which did not reach significance. However, the combination of the G/A^{-455} and *TaqI* polymorphisms together explained 8·9% of the variance ($p<0·005$) (table 4). The combination of the three genotypes together did not increase the contribution.

In the smokers, using multiple regression analysis, G/A^{-455} genotype explained 3·8% variance in plasma fibrinogen levels ($p<0·05$) but no other genotype or combination made a significant contribution. When the group was analysed as a whole, the effect of smoking contributed 6·6% of the variance in the plasma fibrinogen levels. No statistically significant interaction could be shown between genotypes or their combinations and smoking in determining plasma fibrinogen levels.

Discussion

Of the three polymorphisms, the G/A^{-455} was associated with the greatest effect on plasma fibrinogen level and accounted for 3·6% and 3·8% of the variance in non-smokers and smokers respectively. The combination of the G/A^{-455}/*TaqI* polymorphisms in the non-smokers accounted for 8·9% of the variance in plasma fibrinogen levels but this did not differ significantly from the G/A^{-455} alone ($p=0·09$). The G/A^{-455}/*BclI* combination did not contribute any further to the variance in plasma fibrinogen levels than the G/A^{-455} polymorphism alone. These results are not surprising since the G/A^{-455} and the *BclI*

polymorphisms are in strong linkage disequilibrium ($\Delta = 0{\cdot}85$, $p < 0{\cdot}001$), whereas the G/A^{-455} and the *Taq*I polymorphisms are not ($\Delta = -0{\cdot}09$, $p > 0{\cdot}1$).[32] However, the *Taq*I polymorphism on its own was not associated with a significant effect on plasma fibrinogen levels although a trend of lower fibrinogen level with the T+ genotype was seen in both smokers and non-smokers. A previous smaller study using the *Taq*I and *Bcl*I polymorphisms showed that the T+ allele was associated with a lowering effect on plasma fibrinogen level and the B+ allele a raising effect, with these polymorphisms explaining 4·2% and 9·0% of the phenotypic variance respectively.[27] The effect of the combination of these two genotypes on fibrinogen level was additive explaining 14·7% in total of the variance,[27] which is similar to the size of the effect seen here with the combination of the G/A^{-455} and *Taq*I genotypes.

The studies by Connor *et al*[30] and Berg and Keirulf[31] showed no significant effect of genotype on plasma fibrinogen levels but correction for BMI was not made in either study; in this study it made a significant contribution in the non-smokers. In the smokers BMI made no significant contribution and neither did age, possibly because the effect of smoking is so strong that it overrides lesser effects in studies of this size and with a relatively narrow age and BMI range. No statistically significant interaction was seen between genotype and smoking. In contrast, Green *et al*[28] found a significant interaction between smoking status and genotype in determining plasma fibrinogen levels in healthy Swedish controls, but not in the patient group, who consisted of young survivors of myocardial infarction.

In this study, those who had a history of coronary artery disease were excluded. Since a high fibrinogen level has consistently been shown to be a predictor of coronary events it seems likely that a disproportionate number of people with higher fibrinogen levels would have been excluded. This would decrease the chances of showing an association between genotype and plasma fibrinogen level and might explain why the fibrinogen level is lower than expected in the *Bcl*I B+B+ group, which has previously been shown to be associated with higher fibrinogen levels.[27] There is also a significantly lower percentage of smokers in the oldest age group, 19% in those ≥ 65 years *v* 47% in the 55<65 year olds and 38% in the 45<55 year olds (likelihood ratio $\chi^2 = 5{\cdot}86$, $p = 0{\cdot}04$) which would decrease the likelihood of showing a smoking-genotype interaction.

Detection of these fibrinogen genetic polymorphisms using PCR rather than Southern blotting is both technically simpler and more rapid, enabling large population samples to be genotyped easily and quickly. Using the extended genotype G/A^{-455} and *Taq*I explains a greater proportion of the sample variance in plasma fibrinogen levels than G/A^{-455} alone, but extending the genotype to include the *Bcl*I polymorphism does not contribute any further information. Further study in a younger population before the onset of ischaemic heart disease should help to clarify the relationship between genetic variation and plasma fibrinogen levels. Gene-environment interaction needs further investigation, paying attention to different age groups and with a detailed history of smoking characteristics.

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Spinocerebellar ataxia 1 (SCA1) in the Japanese in Hokkaido may derive from a single common ancestry

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Abstract

Spinocerebellar ataxia 1 (SCA1) is caused by expansion of an unstable CAG triplet repeat located on the short arm of chromosome 6. Precise mapping has shown a positional relationship to closely linked markers in the order of D6S109-D6S274-D6S288-SCA1-AM10GA-D6S89-EDN1 from centromere to telomere. The haplotype which cosegregated with the disease was determined in 12 Japanese pedigrees with SCA1. Although the alleles of the SCA1 haplotype varied from pedigree to pedigree, depending on the distance from the SCA1 locus, the affected and presymptomatic subjects carried the same alleles at D6S288 and D6S274. All the families with SCA1 had migrated from either Miyagi or Yamagata Prefectures, neighbouring areas in the Tohoku District, the northern part of Honshu which is the main island of Japan. It seems highly likely that SCA1 in the Japanese, at least those residing in Hokkaido, derives from a single common ancestry.

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The dominantly inherited spinocerebellar ataxias are a cluster of genetically heterogeneous neurodegenerative disorders. Recent advances in molecular genetics have led to the identification of loci and specific gene abnormalities. Six different gene loci have been determined: SCA1 on chromosome 6p24-p23,[1] SCA2 on 12q23-q24,[2] SCA3 or Machado-Joseph disease (MJD) on 14q24-32,[3 4] SCA4 on 16q24-ter,[5] SCA5 on 11q,[6] and dentatorubropallidoluysian atrophy (DRPLA) on 12p.[7] Among these disorders, abnormal expansions of the CAG trinucleotide repeat have been identified in patients with SCA1,[8] DRPLA,[7] and most recently with MJD.[9]

The gene locus for SCA1 was first assigned on the basis of linkage with HLA.[10] After demonstration of a tight linkage with D6S89,[11] SCA1 was precisely mapped to chromosome 6p24-p23. The order of the gene is defined as D6S109-D6S274-D6S288-SCA1-AM10GA-D6S89-EDN1 from centromere to telomere.[8 12] Based on linkage analysis and the CAG triplet repeat causing SCA1 when expanded, SCA1 was considered to be a major disorder in dominant OPCA in the Japanese.[13] Most SCA1 pedigrees in Hokkaido originate from the same area, Miyagi and Yamagata Prefectures, thereby suggesting a common origin, and we searched for haplotypes carrying the SCA1 gene in each pedigree. A comparison of these haplotypes with those of healthy populations suggested that SCA1 in the Japanese, at least those who migrated to Hokkaido from these Prefectures, may derive from a single common ancestry.

Materials and methods

SCA1 PEDIGREES

Of the 12 pedigrees studied, 10 families were living in Hokkaido and two were from Yamagata Prefecture (table 1). The former are descendants of Japanese who migrated from Honshu, the main island of Japan. The original residence in Honshu could be traced in six of 10 Hokkaido pedigrees. In each pedigree, affected subjects of either gender were distributed over successive generations. There were 46 affected subjects, 56 at risk subjects, and 23 spouses. Although the number of mem-

Table 1 SCA1 pedigrees

Family No	Subjects sampled				Mean age at onset (SD)	Collected from
	Affected	At risk	Spouses	Total		
P4	4	2 (1)*	2	8	33·8 (2·9)	Hokkaido
P9	4	3 (0)	0	7	46·0 (5·3)	Hokkaido, Tokyo
P10	16	25 (7)	5	46	38·0 (8·9)	Hokkaido
P11	2	2 (0)	1	5	31·5 (0·7)	Hokkaido (Hakodate)
P13	2	1 (0)	1	4	35·0 (0·0)	Hokkaido, Tokyo
P16	4	4 (0)	4	12	22·8 (7·5)	Hokkaido (Hakodate)
P26	2	2 (1)	1	5	30·5 (6·4)	Hokkaido (Hakodate)
P38	2	1 (0)	0	3	52·0 (0·0)	Hokkaido
P39	1	3 (0)	2	6	52·0 (0·0)	Hokkaido
P51	5	6 (2)	2	14	31·8 (10·0)	Yamagata, Tokyo
P52	2	4 (1)	2	8	36·0 (2·8)	Yamagata, Tokyo
P61	2	3 (1)	3	8	29·0 (1·4)	Hokkaido
Total	46	56 (13)	23	126	35·6 (9·4)	

* Figures in parentheses indicate number of presymptomatic subjects.

Gender-Related Association Between β-Fibrinogen Genotype and Plasma Fibrinogen Levels and Linkage Disequilibrium at the Fibrinogen Locus in Greenland Inuit

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Abstract Elevated plasma fibrinogen levels represent an increased risk for cardiovascular disease, but the mechanism explaining this association is still not clear. Genetic differences may play a role, because it has been shown that individuals who carry the rare alleles of polymorphisms in the genes for the Bβ-chain (*Bcl* I and G/A^{-455}) and the Aα-chain (*Taq* I) of fibrinogen have higher plasma fibrinogen levels and that patients with peripheral arterial disease have a higher frequency of the rare allele of the *Bcl* I polymorphism than do healthy control subjects. We studied the Greenland Inuit, a population with a low incidence of ischemic heart disease; polymorphisms of the fibrinogen gene; and their association with plasma fibrinogen level. The group studied had a small age range (30 to 34 years), 97% were smokers, 62 were men, and 71 were women. We observed that in the Inuit, frequencies of the rare alleles of the β gene and of the common alleles of the α gene polymorphisms were lower than those published for other populations (all Caucasian). Accordingly, in the Inuit, these distribution patterns give a higher frequency of alleles that are associated with lower plasma fibrinogen levels. We further observed comparable linkage disequilibrium between α and β gene polymorphisms in Caucasian populations. In Inuit men the rare allele of the *Bcl* I and G/A^{-455} fibrinogen polymorphisms was associated with plasma fibrinogen level comparable with the association described in Caucasian populations. In women, however, we did not find a significant association, supporting the desirability of separate data analysis for men and women of the influence of genetic factors on atherosclerotic disease. In conclusion, in the Inuit the association of fibrinogen polymorphisms with fibrinogen levels is comparable with that in Caucasians, but the genes that are associated with lower fibrinogen levels are more frequent in the Inuit than in Caucasians. (*Arterioscler Thromb Vasc Biol.* 1995;15:856-860.)

Key Words • fibrinogen • Inuit • cardiovascular risk indicators • DNA polymorphism

In several epidemiological studies it has been shown that an increased plasma fibrinogen level is an independent risk indicator for cardiovascular disease.[1-6] The Northwick Park Heart Study showed that an increase in fibrinogen level at 1 SD above the mean predicted an 84% increase in the risk for cardiovascular events within the next 5 years.[1]

The mechanism of the association between fibrinogen and risk has not yet been elucidated. It may be that an increased amount of circulating fibrinogen produces an increased propensity for thrombosis[7] or directly contributes to the development of the atherosclerotic lesion.[8] There are also indications that increased plasma fibrinogen levels reflect the inflammatory condition of the vascular wall. This theory is supported by the results of the ECAT Angina Pectoris Study,[9] which found that increases in both fibrinogen and C-reactive protein (CRP) levels are risk indicators for cardiac events in patients with angina pectoris. The PROCAM Study has recently reported comparable results in a healthy population.[5] Another cardiovascular risk factor that is closely linked to inflammation is smoking, which increases the levels of fibrinogen and other acute-phase reactants.[10,11] It is conceivable that smoking also contributes to risk because of its acute-phase–inducing properties.

Genetic variation may also play a role in determining plasma fibrinogen levels. An association between polymorphisms in the genes for the Aα- and Bβ-fibrinogen chains (α and β genes) and plasma fibrinogen levels has been described.[12,13] Recently, Green et al[14] showed that the association between G/A^{-455} genotypes and fibrinogen levels was observed only in smokers, suggesting that the increase in fibrinogen resulting from a low-grade acute-phase reaction (ie, smoking) might depend on polymorphisms of the β-fibrinogen gene. It has also been reported that the binding of nuclear proteins to DNA fragments is influenced by genotype at the G/A^{-455} and C/T^{-148} polymorphic sites of the β-fibrinogen gene,[15,16] the latter of which is located close to the interleukin-6 responsive element of the promoter. If these findings are combined, they imply involvement of β-fibrinogen gene polymorphisms in the cytokine-stimulated regulation of fibrinogen synthesis.

The association between fibrinogen genotypes and plasma levels has not been confirmed in all studies. There is, however, much diversity in the composition of

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the population samples and in the fibrinogen assays used. One of the variant factors is the number of smokers. Because both the ECTIM Study[17] and Green et al[14] have reported that the association between genotypes and fibrinogen levels is stronger in smokers, part of the reported difference in the relation between fibrinogen polymorphisms and fibrinogen levels might be ascribed to this variation.

A fibrinogen restriction fragment length polymorphism at the 3' end of the α-fibrinogen gene has been described (*Taq* I).[12] No significant correlation between this polymorphism and plasma fibrinogen level has been found, but when the average excess of the G/A^{-455} and *Taq* I inferred haplotype is estimated,[18] the presence of functionally distinct genotype combinations is suggested.[19]

We performed a study of fibrinogen polymorphisms in Greenland Inuit, a population with a low incidence of ischemic heart disease.[20] We determined the allele frequencies of the G/A^{-455}, *Bcl* I, and *Taq* I polymorphisms and calculated the associations between the different polymorphisms. In addition, we estimated the association between genotype and plasma fibrinogen level (by functional and immunologic methods) in men and women.

Methods

Study Populations

One hundred ninety-two Inuit, aged 30 to 34 years and living in Nanortalik (southwestern Greenland) were invited to participate in this study. This group was chosen, because in individuals aged 30 to 34 years, no selection for coronary heart disease will be observed. A complete data set, consisting of blood specimens, a filled-in questionnaire, and anthropometric measurements, was obtained from 133 individuals (62 men and 71 women). Upon medical investigation these 133 Inuit appeared to be healthy and did not show any signs or symptoms of severe atherosclerosis. Characteristics of the population have been described elsewhere in detail.[21] We considered individuals who had never smoked (n=4) and those who had stopped smoking more than 10 years ago (n=2) as "nonsmokers," and current smokers and those who stopped less than 10 years ago as "smokers" (six had stopped smoking between 3 months and 3 years before sampling). Of the smokers, 13% smoked fewer than 5 cigarettes/d, 60% smoked 5 to 14 cigarettes/d, and 27% smoked more than 15 cigarettes/d. The mean duration of smoking was 17.5 (SD, 3.6) years. There was no difference in smoking habits between men and women.

CRP and fibrinogen level data were available for all 133 subjects, but polymorphism analysis could not be performed in all samples due to the poor quality of some DNA samples (see Table 1 for number of analyzed samples). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Fifty-two Danes, comparable for age (30 to 34 years) and sex (28 men and 24 women), served as the control group for plasma fibrinogen and CRP levels. Danes were chosen as the reference group because it was not possible to compose a large enough group of Greenland inhabitants without Inuit ancestry and the life style, plasma lipid and (apo)lipoprotein levels, and apparent lack of ischemic heart disease in the Danish population are comparable with those of the Inuit.[21]

Blood Sampling

Blood was collected in sodium citrate (final concentration, 14 mmol/L) and immediately placed in melting ice. After centrifugation (30 minutes, 2000*g*, 4°C) the plasma was collected and frozen at −70°C. The blood cells were stored at −20°C.

Polymorphism Analysis

Each 50-μL polymerase chain reaction (PCR) contained 100 to 400 ng genomic DNA, 100 ng of each appropriate primer, 10 mmol/L Tris/HCl (pH 9.0), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.01% (wt/vol) gelatin, 0.1% (vol/vol) Triton X-100, 0.02 mmol/L dNTP, and 0.1 U *Taq* polymerase (HT Biotechnology Ltd). The reaction components were incubated at 95°C for 5 minutes, followed by 30 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes in a DNA thermal cycler (Perkin-Elmer Cetus).

The primers have been described previously (G/A^{-455} by Thomas et al[13]; *Bcl* I and *Taq* I by Thomas et al[22]). The PCR product (10 μL) was digested with the appropriate restriction enzyme. These digestion products were separated by electrophoresis on a 2% agarose gel in 44 mmol/L Tris/borate–1 mmol/L EDTA containing 0.5 μg/mL ethidium bromide and visualized under UV light. The alleles with the restriction site and the noncleavable alleles were designated *B1* and *B2* for the *Bcl* I polymorphism, G^{-455} and A^{-455} for the G/A^{-455} polymorphism, and *T1* and *T2* for the *Taq* I polymorphism, respectively.

Plasma Protein Measurements

Fibrinogen activity levels were measured with the modified Clauss assay.[23] The within-day and between-day coefficients of variation (CVs) were 3.2% and 4.9%, respectively. Fibrinogen antigen levels were measured nephelometrically by using rabbit polyclonal anti-human fibrinogen (Dako) antibodies. The within-day and between-day CVs were 1.7% and 4.2%, respectively. Normal plasma (Nycomed Pharma) was used to calibrate the fibrinogen assays. The ratio of the two fibrinogen assays and its 99% confidence interval (CI) were calculated. Seven samples were outside this range and omitted. CRP levels were measured with an enzyme immunoassay using rabbit antibodies against human CRP (Dako) as capture and tagging antibodies. The within-day and between-day CVs were 2.9% and 6.2%, respectively. CRP standard serum (Behringwerke) was used as the calibrator.

Statistical Analysis

Deviations in genotype frequency in the Inuit samples from that expected for a population in Hardy-Weinberg equilibrium were analyzed by the χ^2 test. Genotype frequencies in the Inuit and published frequencies were compared by a χ^2 test. Standardized disequilibrium statistics were calculated as described by Chakravarti et al.[24] Allele frequencies in the Inuit were determined by gene counting, and 95% CIs for the allele frequencies were calculated from sample allele frequencies[25] on the basis of an approximation to binomial and normal distributions when n is large.

With an ANCOVA, adjusted fibrinogen levels for each genotype were estimated and the significance of genotypes in determining plasma fibrinogen levels was estimated, with BMI, waist-to-hip ratio, and CRP levels as covariates. A multiple linear regression model was used to assess the amount of variance in plasma fibrinogen level that could be explained by BMI, CRP, smoking status, and genotype in men and women separately.

Statistical analysis was performed using the SOLO and LOTUS123 computer programs. Statistical significance was set at $P<.05$.

Results

Genetic Distribution of Inuit and Caucasian Populations

In the Inuit population allele distributions of the *Bcl* I, G/A^{-455}, and *Taq* I polymorphisms were in Hardy-Weinberg equilibrium, as expected for a general population. However, a different genetic background was

TABLE 1. Frequency of Rare Fibrinogen Alleles

	Bβ Fibrinogen						Aα Fibrinogen		
	B2 Allele of *Bcl* I RFLP			A^{-455} Allele of G/A^{-455} RFLP			T2 Allele of *Taq* I RFLP		
	n*	Frequency†	95% CI‡	n*	Frequency†	95% CI‡	n*	Frequency†	95% CI‡
Study									
London[12]	91	.25§	0.19-0.32				91	.28§	0.22-0.35
Norway[28]	118	.17§	0.13-0.23				118	.27§	0.22-0.33
London[27]	53	.16§	0.10-0.23				53	.26§	0.18-0.36
UK									
All[13]				292	.19§	0.16-0.22			
Smokers[13]				120	.20§	0.15-0.26			
Nonsmokers[13]				172	.19§	0.15-0.24			
Edinburgh[28]	126	.10	0.07-0.15				126	.25§	0.14-0.23
Sweden									
All[14]				86	.25§	0.20-0.31			
Smokers[14]				57	.20§	0.13-0.27			
Nonsmokers[14]				29	.34§	0.22-0.46			
UK and France[17]				648	.21§	0.19-0.23			
UK[22]	293	.15	0.12-0.18				276	.27§	0.24-0.31
US[29]	50	.20							
Inuit (this study)	126	.12	0.09-0.17	131	.11	0.08-0.15	121	.47	0.41-0.53

RFLP indicates restriction fragment length polymorphism; CI, confidence interval.
*Number of individuals evaluated.
†Frequency of the rare allele.
‡95% Confidence interval of the rare allele frequency.
§Frequency is significantly different from that of the Inuit ($P<.05$ by χ^2 test).

indicated for the Inuit when compared with Caucasian populations, because the frequencies of the rare alleles of the G/A^{-455} and *Bcl* I polymorphisms and of the common allele of the *Taq* I polymorphism were significantly lower when compared with most, but not all, Caucasian populations (Table 1).[12,13,22,26-29]

Allelic Associations

In the Inuit there was strong linkage disequilibrium (Δ) between the two polymorphisms of the β-fibrinogen gene, giving a strong association between the *B1* and G/A^{-455} alleles, comparable with that observed in Caucasian populations (Table 2). The Δ between polymorphisms of the α and β genes in the Inuit was also significant but somewhat weaker. Reports on linkage disequilibrium between α and β genes in Caucasian populations vary, but most groups studied have been small.[22,29] In the ECTIM Study[17] (F. Cambien et al, unpublished observations, 1995) of 668 healthy individuals, there was linkage disequilibrium between the polymorphism of the α gene (*Taq* I) and those of the β gene: *Taq* I–*Bcl* I Δ=−.37; *Taq* I–G/A^{-455} Δ=−.53; and *Bcl* I–G/A^{-455} Δ=.96, with $P<.001$ for all and a significant association between the *T2* allele on one side and *B1* and G^{-455} alleles on the other.

Genetic Contributions to Plasma Fibrinogen Levels

The acute-phase markers fibrinogen and CRP were both higher (2.81 g/L in the functional assay, 2.81 g/L for fibrinogen antigen, and 2.9 mg/L for CRP) in the Inuit than in the Danish control group (2.30 g/L in the functional assay, 2.19 g/L for fibrinogen antigen, and <1.5 mg/L for CRP). Adjustment for the acute-phase state by adding CRP to the ANCOVA still gave a comparable difference between these populations (results not shown).

If all Inuit are studied as a single group, there is no significant association in the ANCOVA between any of the three fibrinogen polymorphisms and plasma fibrinogen level. However, when the group is divided by sex, only in men did we observe a significantly higher plasma fibrinogen level in those with the G/A^{-455} genotype than with the G/G^{-455} genotype. In women, there was a similar trend, but this was not significant (Table 3). Stratification by α- and β-fibrinogen haplotype did not reveal any more informative genotype combination (data not shown).

In the Inuit, BMI, CRP, smoking status, and genotype together accounted for 25% (in men) and 35% (in women) of the variation in plasma fibrinogen level. Removal of genotype from the regression model reduced the amount of variance explained, to 16% in men and 26% in women, suggesting that in men and women the fibrinogen polymorphism genotypes account for 9% of the variation in fibrinogen level after adjustment for covariates. Removal of other covariates from the regression equation for men and women suggested that CRP

TABLE 2. Allelic Association Between the *Taq* I Polymorphism of the α-Fibrinogen Gene and the G/A^{-455} and *Bcl* I Polymorphisms of the β-Fibrinogen Gene in Inuit and Caucasian Populations

	Inuit	Caucasian
Bcl I–G/A^{-455}	Δ=0.91, χ^2=221, $P<.001$	Δ=0.85, χ^2=322, $P<.001$[22]
G/A^{-455}–*Taq* I	Δ=−0.32, χ^2=24, $P<.001$	Δ=−0.09, χ^2=3, $P>.1$[22]
Bcl I–*Taq* I	Δ=−0.29, χ^2=22, $P<.001$	Δ=0.01, χ^2=1, $P>.1$[22] Δ=0.01, $P>.1$[29]

Δ indicates linkage disequilibrium coefficient.

TABLE 3. Plasma Fibrinogen Levels (by Functional and Immunologic Methods) in Inuit With Different Genotypes for G/A^{-455}, *Bcl* I, and *Taq* I Fibrinogen Polymorphisms

		Whole Group			Men			Women		
		n*	Mean	SEM	n*	Mean	SEM	n*	Mean	SEM
Functional assay										
G/A^{-455}	G/G^{-455}	93	2.69	0.05	44	2.58	0.07	49	2.75	0.06
	G/A^{-455}	26	2.86	0.10	2	2.93†	0.13	14	2.95	0.11
	A/A^{-455}	0			0			0		
Bcl I	*B1B1*	101	2.70	0.05	46	2.63	0.06	55	2.70	0.05
	B1B2	22	2.85	0.11	10	2.94	0.14	12	2.96	0.12
	B2B2	0			0			0		
Taq I	*T1T1*	25	2.79	0.10	3	2.77	0.14	12	2.77	0.12
	T1T2	71	2.72	0.06	7	2.66	0.10	44	2.78	0.06
	T2T2	17	2.59	0.13	8	2.45	0.17	9	2.78	0.14
Immunologic assay										
G/A^{-455}	G/G^{-455}	93	2.78	0.06	44	2.63	0.08	49	2.86	0.08
	G/A^{-455}	26	2.86	0.12	12	2.98†	0.14	14	2.92	0.15
	A/A^{-455}	0			0			0		
Bcl I	*B1B1*	101	2.79	0.06	46	2.69	0.08	55	2.84	0.08
	B1B2	22	2.86	0.13	10	2.99	0.18	12	2.92	0.16
	B2B2	0			0			0		
Taq I	*T1T1*	25	2.79	0.13	13	2.77	0.17	12	2.79	0.16
	T1T2	71	2.76	0.08	27	2.71	0.12	44	2.80	0.09
	T2T2	17	2.90	0.15	8	2.69	0.21	9	3.15	0.19

Plasma fibrinogen levels are mean±SEM and are in grams per liter.
*Number of individuals evaluated.
†Levels are significantly different from G/G^{-455} ($P<.05$ by ANOVA).

levels accounted for 7% and 19%, respectively, of the variation in plasma fibrinogen level and BMI accounted for 9% and 2%, respectively.

Discussion

The polymorphisms for α- and β-fibrinogen genes were studied in the Inuit, a population with a low incidence of ischemic heart disease.[20] We observed different allele frequencies in the polymorphisms of the fibrinogen genes, with lower frequencies of the rare A^{-455} and *B2* alleles of the β-fibrinogen gene polymorphisms and higher frequencies of the rare *T2* allele of the α-fibrinogen gene polymorphism in the Inuit compared with Caucasian populations. In this and other studies, the A^{-455}, *B2*, and *T1* alleles are associated with higher fibrinogen levels, suggesting that their lower frequency among the Inuit may partially explain their lower incidence of ischemic heart disease.

The Inuit are a population with very few genetic influences from other populations. This isolation has resulted in some genetic differences in blood groups (ABO, Rh, and MNS blood group systems), the HLA system, and erythrocyte enzymes (for review, see Reference 30). It may, therefore, also be possible that the fibrinogen gene locus has developed differently than in Caucasians and has a different allelic distribution. To study a possible genetic difference in the fibrinogen genes in the Inuit, we assessed the linkage disequilibrium between polymorphisms. The association between *Taq* I and the polymorphisms of the β-fibrinogen gene was weaker than that between the polymorphisms of the β gene but comparable in the Inuit and in one study of Caucasians (ECTIM Study[17] [F. Cambien et al, unpublished observations, 1995]). In other studies of Caucasians,[22,29] no linkage disequilibrium was detected between the α- and β-fibrinogen polymorphisms. One reason for this apparent discrepancy could be the smaller number of subjects in these studies (n=276 and n=50, respectively).

In the total Inuit group no significant relation could be found between fibrinogen levels and genotypes of the β-fibrinogen genes. However, we observed increased fibrinogen levels in Inuit men with the G/A^{-455} genotype when we compare them with men with the G/G^{-455} genotype. In women, no significant associations were found, although the trend was similar. This difference in regulation of fibrinogen levels in men and women has also been reported recently by Humphries et al,[31] who suggest an allele-specific effect of hormones on transcription. Because the percentage of smokers in the Inuit group was approximately 97%, our observation that there is a correlation between genotype and fibrinogen level in the Inuit might corroborate the theory of Green et al,[14] ie, that low-grade stimulation of fibrinogen synthesis, eg, smoking, is expressed more strongly in men with the A^{-455} allele. Direct involvement of β-fibrinogen polymorphisms in the regulation of fibrinogen expression is suggested by the differential binding of nuclear proteins to DNA with G^{-455} or A^{-455} (F. Green et al, unpublished data, 1993). This study also suggests that fibrinogen levels are regulated differently in men and women.

Our results may also help to explain the inconsistency that is found in the literature about the relation between fibrinogen levels and fibrinogen polymorphisms.[12-14,17,26,27] The reported studies vary in a number of factors that are known to affect plasma fibrinogen levels, eg, sex ratio and the fraction and definition of smokers. Furthermore, these studies are also inconsistent in their adjustment for sex, BMI, smoking status, acute-phase status, and age. The importance of controlling for such factors that affect plasma fibrinogen levels is clearly illustrated by the present

study, wherein we have been able to show a sex difference in the association between genetic polymorphisms and plasma fibrinogen levels. It has been stressed before that data for men and women should not be heedlessly combined in atherosclerosis research,[32] a statement that seems to be supported by the results of our study.

The roles of the *B2* and A^{-455} alleles of the *Bcl* I and G/A^{-455} β-fibrinogen polymorphisms in the regulation of fibrinogen level under conditions that induce low-grade inflammation also merit further investigation.

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