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# HLA-B27 AND RELATED GENES IN THE AETIOPATHOGENESIS OF ANKYLOSING SPONDYLITIS

by

#### **COLLEEN M. HIGGINS**

A thesis submitted for the degree of PhD of the University of London

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### **ABSTRACT**

It is well established that there is a very strong association between the MHC class I transplantation antigen HLA-B27 and the inflammatory disease ankylosing spondylitis (AS). It is unclear if HLA-B27 is directly involved in the development of AS or if it is linkage disequilibrium with another gene and HLA-B27 is a marker for this true disease gene. Restriction fragment length polymorphism (RFLP) analysis was used to examine whether a particular variant of the HLA-B27 gene is associated with the disease in English and Polish populations.

Using a probe from the 5' region of a HLA-B27 gene, a 14Kbp PvuII fragment was found that was more common in English HLA-B27+AS+ than HLA-B27+AS-subjects (p<0.01) suggesting an involvement for this fragment in disease development.

A PvuII fragment of 8.9Kbp was identified in more English HLA-B27+AS<sup>-</sup> than HLA-B27+AS<sup>+</sup> subjects (p<0.01). While a trend could be seen for the Polish group, the number of HLA-B27 positive healthy individuals was too small for statistical analysis. This fragment is thought to be the same as the 9.2Kbp PvuII RFLP reported by McDaniel et al (1987) to be more frequent in HLA-B27 positive patients than in HLA-B27 positive healthy controls. Differences in hybridization patterns were observed in this study compared with those observed by McDaniel et al (1987) with supposedly similar probes. For example, in this study the 8.9Kbp PvuII fragment was not detected with probes derived from the 3' region of the HLA-B27 gene. While the different hybridization patterns may explain the conflicting results, it is also possible that population differences exist. Analysis of the available tissue types showed an association between the 8.9KbpPvuII RFLPwith HLA-A3/A9, confirming the finding of Ahearn et al (1989).

In addition, no disease associated RFLP was identified using probes derived from the 3' region of the HLA-B27 locus as well as the restriction enzymes *EcoR1* and *HindIII*.

The possibility that another gene is involved in the development of AS was also considered. Due to its proximity to the HLA-B locus, the tumour necrosis factor-α (TNF-α) gene was thought to be a possible candidate for such a gene. In order to determine the level of polymorphism of the human TNF-α gene, genomic DNA from healthy individuals were digested with 26 different restriction enzymes and hybridized with a probe for the TNF-α gene. No RFLP was detected with any of the enzymes used. In addition, a small study of English and Polish patients and controls also showed that no disease associated *PvuII*, *EcoR1* of *HindIII* RFLP with the TNF-α gene. This suggests that no gross structural changes have occurred within the TNF-α locus that gives rise to AS.

To examine differences in the HLA-B27 nucleotide sequence that are not revealed as RFLPs, the HLA-B27 gene of the HLA-B\*2705 subtype was cloned from an AS patient heterozygous for this allele. This ensured that a HLA-B27 gene of disease haplotype was isolated. Comparisons with published nucleotide sequences of other HLA-B27 genes isolated from healthy individuals and AS patients did not reveal any differences in the predicted amino acid sequence. This suggests that alterations in the HLA-B27 antigen do not play a role in the development of AS. The nucleotide sequences of the promoter, class I regulatory element and interferon responsive sequence of this gene were identical to those of other HLA-B27 genes, indicating that the expression of this gene is normal.

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PCR? does Rfil pick of many Changes However, alterations in the sequences of potential binding sites for trans-acting factors may have some

importance in the expression of this gene.

It is possible that the expression of the HLA-B27 gene in AS patients is not regulated normally. In order to investigate this, EBV immortalized B lymphoblastoid cell lines were established from 2 HLA-B27 positive patients, 1 HLA-B27 negative patient and 1 HLA-B27 positive healthy individual. Attempts were made to study the regulation of endogenous HLA-B27 expression by IFN-a, IFN- $\gamma$  and TNF- $\alpha$  in these cells. This was analyzed at the surface protein level by staining the cells with anti-class I antibody and at the RNA level by Northern hybridization using probes from the 3'UT region of the 'normal' HLA-B27 gene as well as an oligonucleotide probe thought to be specific for HLA-B27. Each cell line responded differently to treatment and no conclusions about the regulation of HLA-B27 expression in AS patients by cytokines could be reached. In particular, the cell line established from the HLA-B27 positive healthy individual did not express class I genes at the mRNA level whereas the other cell lines expressed quite high level of class I mRNA and protein. To study the control of HLA-B27 expression more directly, a construct was made with the reporter CAT gene driven by the promoter of the HLA-B27 gene that had been isolated previously from an AS patient. While this construct expressed low levels of CAT enzyme in HeLa cells, it was of more interest to study and compare the control of expression by cytokines in the EBV cell lines. Attempts were made to set up conditions for transfections of these cell lines by electroporation and establishing stable transfectants by selecting for G418 resistance.

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#### **ABBREVIATIONS**

AGPT aminoglycosyl 3' phosphotransferase

amp ampicillin bp base pair

CAT chloramphenicol acetyl yransferase

cDNA complemenatry DNA

dATP deoxyribosyladenine 5' triphosphate
dCTP deoxyribosylcytosine 5' triphosphate
dGTP deoxyribosylguanine 5' triphosphate

DNA deoxyribonucleic acid

DTT dithiothreitol

dTTP deoxyribosylthymine 5' triphosphate

EBV Epstein Barr virus

EDTA ethylenediamine tetraacetic acid

FCS fetal calf serum
G418 Geneticin

HBS Hepes buffered saline

HLA human leukocyte antigen

hrs - hours

IFN interferon

IPTG isopropyl-β-D-thiogalactopyranoside

Kbp Kilobase pair

LB Luria Bertani medium

mA milliamps μFd microfarads μg micrograms

MHC major histocompatibility complex

 $\begin{array}{cc} \text{mins} & \text{minutes} \\ \mu l & \text{microlitres} \\ m l & \text{millilitres} \end{array}$ 

mRNA messenger RNA

pB27-3'FR probe from the 3'FR region of the HLA-B27 gene
pB27-3'UT probe from the 3'UT region of the HLA-B27 gene
pB27-5' probe from the 5' region of the HLA-B27 gene

pB27-oligo oligonucleotide probe for HLA-B27

PBS phosphate buffered saline

RNA ribonucleic acid
RNase ribonuclease

SDS sodium dodecyl sulphate
SSC standard sodium citrate

SSPE standard saline phosphate EDTA buffer

TLC thin layer chromatography
TNF tumour necrosis factor

Tris tris(hydroxymethyl)aminomethane

V volts

XGAL 5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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# **CHAPTER 1**

# GENERAL INTRODUCTION



#### 1.1. CLINICAL BACKGROUND

Ankylosing spondylitis (AS) is a chronic inflammatory disorder strongly but not absolutely associated with possession of the transplantation antigen HLA-B27 and characterized by inflammation of the sacro-iliac joints and the spine. The first symptoms are usually lower backache and morning stiffness which is relieved with activity, but occasionally pain appears in the peripheral joints or less often as acute anterior uveitis. The first radiographic changes are seen in both sacro-iliac joints and, after several years, there may be complete bony ankylosis (figure 1a). In patients with severe disease, the entire spine becomes increasingly painful and stiff resulting in a stooped posture with limited movement of the lumbar spine and neck (figure 1b).

One of the characteristic pathological features of spondyloarthritis is an enthesopathy (Moll,1983). This is an inflammatory lesion that destroys the enthesis i.e, the attachment of ligament or tendon to bone. The damage at the bone surface is repaired, resulting in the formation of an irregular bony spur at the fibrous/bony interface. Enthesopathy along the vertebral column results in the formation of bony bridges between the vertebrae called syndesmophytes (figure 1c), eventually producing a completely fused, 'bamboo spine'.

Pain is often experienced at other sites. In particular, hip and shoulder joint involvement occurs at some stage in about one third of patients (Arnett et al. 1977; Khan,1984) while acute anterior uveitis (AAU) is the most common extra-skeletal manifestation, occurring in about 25% of patients at some point during the progress of the disease (Khan,1984; Mehra et al,1983). AAU is more common in HLA-B27 positive patients than in those who are HLA-B27 negative and is usually unilateral, with painful attacks subsiding within a few weeks only to recur at a later date.

Other complications that can be experienced with AS include (Khan,1988):

(a) cardiovascular involvement; the risk of aortic valve disease increases with disease duration and in the presence of peripheral joint arthritis; (b) pulmonary involvement; pain is due to enthesopathy of the costo-costal regions but patients may also develop pulmonary fibrosis; (c) neurological involvement; this may arise as a result of compression of the sciatic nerve or because the increasing rigidity of the spine can result in fracture and chord damage; (d) amyloidosis: this rarely occurs but may lead to renal failure

A strong association between AS and the transplantation antigen HLA-B27 has been observed (Brewerton *et al*,1973; Schlosstein *et al*,1973) with over 90% of Caucasian patients in the United Kingdom carrying this antigen compared with about 8% of the general population. The frequency of this antigen in other populations is discussed further in section 1.3.2. Recent data indicate that only 1-10% of HLA-B27 positive individuals in the general population are likely to develop AS compared to 20-30% of HLA-B27 positive first degree relatives of AS patients (Van der Linden *et al*,1985). This suggests that other genetic factors operate in the disease. The disease affects more men than women with a ratio of around 7:3, and men also experience more severe disease symptoms (Khan *et al*,1988).

Ankylosing spondylitis is considered to be the prototype of a group of disorders referred to as the *spondyloarthropathies*. These disorders include psoriatic arthritis, Reiter's syndrome and other forms of reactive arthritis due to infection with enteric organisms such as *Yersinia*, *Shigella*, *Salmonella*, and the arthritis of chronic inflammatory bowel disease i.e., ulcerative colitis and Crohn's disease. These diseases are grouped together as they share many features *viz*: negative tests for rheumatoid factor; absence of rheumatoid nodules; asymmetrical peripheral arthritis; radiologic evidence of sacroiliitis; related ocular, genital and gastrointestinal manifestations; a tendency to cluster in families and an association of some degree with HLA-B27 (table 1).

Ankylosing spondylitis may occur in association with any of these other disorders and is then sometimes referred to as 'secondary' AS, but most AS patients have no evidence of these associated diseases and are classified as having 'primary' AS (Calin *et al*,1984; Khan *et al*,1984). The differences between these are summarized in table 1.

Several hypotheses have been suggested to explain the development of the heterogeneous disorder, ankylosing spondylitis. Although many studies have proposed *Klebsiella* organisms as the infecting agent in AS, it is unclear how AS develops from such an infection. One hypothesis suggests that the HLA-B27 molecule shares an antigenic epitope with the putative infecting agent, *Klebsiella* and that this molecular mimicry causes an autoimmune response against the host's HLA-B27 antigen (Ebringer *et al*, 1983). The possibility that the HLA-B27 molecule is in some way altered by the infecting agent has also been considered (Geczy *et al*, 1983). While the role of the HLA-B27 molecule in the development of AS is not yet understood, many of the observations made suggest that AS is an autoimmune disease caused by autoimmune recognition of the HLA-B27 antigen. Mechanisms for the development of autoimmunity in general are therefore discussed in section 1.2 and autoimmunity with respect to AS is considered further in section 1.3.

#### 1.2. AUTOIMMUNITY

#### 1.2.1. AUTOIMMUNITY AND AUTOIMMUNE DISEASE

Autoimmunity can be defined as specific humoral or cell-mediated immunity to constituents of the body's own tissues. If tissue damage results from the recognition of self-antigens by the body's immune system this is referred to as autoimmune disease. While autoimmunity reflects the loss of immunologic tolerance, it is now clear that lymphocytes capable of reacting with self-antigens are normally present and, in fact, may serve an essential function in the healthy immune system. It has been suggested that the presence of a large variety of autoantibodies in the blood may be necessary in order to remove effete cells and molecules from the body (Shoenfeld,1987). Recognition of self is certainly essential for the recognition of foreign antigens on the surface of cells since this can only occur if they are presented to the immune system in the context of self-antigens of the major histocompatibility complex (MHC); namely the class I and class II histocompatibility antigens.

Recognition of antigen binding sites by the anti-idiotypic networks of the immune response also involves recognition of self.

Since self-reacting lymphocytes and autoantibodies are normally present, it is believed that autoimmune disease may only be an exaggeration of events that normally occur all the time in the body. At present, it is suggested that autoimmune disease is the result of a combination of immunologic, genetic and environmental factors such that autoimmune disease may develop in an individual of the right immunogenetic background exposed to an exogenous disease trigger which may be viral, bacterial or parasitic in nature (Shoenfeld,1984). Various mechanisms have been proposed to explain the development of autoimmune diseases.

#### 1.2.2. AUTOIMMUNE DISEASE MECHANISMS

#### a) Genetic predisposition

In all the autoimmune diseases studied, there is evidence for some degree of genetic involvement. While it is unlikely that a single gene is responsible for all these diseases, the MHC gene complex has been shown to have some association with quite a number (table 2). For example, 59% of patients with multiple sclerosis have the class II tissue antigen HLA-DR2 compared with 26% of controls (Strominger,1986) and individuals who are positive for HLA-DR2 have a relative risk for developing MS of 4.8 (Roitt,1984). The most striking association is that between AS and HLA-B27; over 90% of AS patients in the UK carry HLA-B27 compared with 8% of the normal population (Brewerton et al,1973) and this association has been shown to exist in all the racial groups studied (Khan,1988).

Since antigens, including autoantigens, are only recognized by the immune system in the context of MHC molecules, it has been proposed that aberrant expression of the MHC may result in the development of autoimmune disease (Bottazzo et al,1983; Hanafusa et al,1983). In other words, if expression was induced in cells that do not normally express these molecules, then these cell surface markers would become potential autoantigens. Indeed, about 80% of patients with autoimmune thyroid diseases were shown to strongly express class II HLA-DR molecules on thyroid tissue compared with none of the controls (Hanafusa et al,1983).

Particular allelic forms of MHC molecules become associated with autoimmune diseases such as HLA-DR4 with rheumatoid arthritis. These alleles may bind autoantibodies or autoreactive T cells more effectively than others or their expression may be more easily induced (Shoenfeld *et al*,1987). However, it is difficult to suggest that the induction of MHC expression is essential for the triggering of disease since class I antigens are already present on most nucleated cells and class II antigens are produced regularly in response to infection. Induction may, however, be necessary for autosensitization i.e., the initial induction of MHC expression may not cause an autoimmune response but it may prime the immune system so that an enhanced secondary response will occur upon challenge at a later date.

#### b) Molecular mimicry between microbial and host antigens

Molecular mimicry refers to the similarities that have been observed between epitopes on the surface of host cells and those expressed on infectious micro-organisms. This similarity is thought to provide a mechanism by which autoimmune disease can occur. An antigenic determinant of an infecting micro-organism may generate an immune response which cross reacts with a host antigen, leading to autoimmune tissue damage.

For example, monoclonal anti-DNA antibodies derived by the fusion of lymphocytes from systemic lupus erythamatosus (SLE) patients to myeloma cells have been shown to bind to *Mycobacteria* (Shoenfeld *et al*,1986). Since this binding is inhibited by DNA and other polynucleotides, the antigenic determinants present in the mycobacterial cell wall are thought to be antigenically similar to determinants of DNA molecules. Conversely, monoclonal anti-*Mycobacterium tuberculosis* antibodies can recognize DNA and appear to behave as antinuclear antibodies (Shoenfeld *et al*,1986).

Attempts have been made to identify the common features between the host antigen and the antigenic determinant of the infectious agent in such diseases (Oldstone et al,1987; Schwimmbeck et al,1987). Recent studies have shown that particular proteins of the infecting organism may share a degree of amino acid homology with host proteins (table 3). For example, the papilloma virus E2 protein shows amino acid homology with the insulin receptor, suggesting a mechanism for the development for type 1 diabetes (Oldstone,1987). Other examples of amino acid homology between infectious agents and self antigens are given in table 3.

It is possible that the aetiology of AS also involves immunological cross-reactivity as antibodies to *Klebsiella* have been shown to cross-react with HLA-B27 (Ebringer *et al*,1983; Geczy *et al*,1983). Sequence analysis has revealed 6 amino acids that are identical between the hypervariable domain of the HLA-B27 antigen and *K.pneumoniae* nitrogenase (Schwimmbeck *et al*,1987) (table 3). The suggestion that ankylosing spondylitis is the result of cross-reactivity in the immune response to these proteins is discussed further in section 1.3.3.

Although the probability of a 6 amino acid sequence being identical in two unrelated proteins is very small, it is not necessarily biologically significant. For example, the Epstein Barr Virus (EBV) BBLF protein also shares a 6 amino acid identity with the conserved region of HLA-B27 but the two proteins do not induce cross-reactive immune responses (Oldstone, 1987). Moreover, a search for antibodies to EBV in a group of HLA-B27 positive patients was unsuccessful. For the homology to have any relevance to disease development, it must therefore occur within an immunogenic domain of the host protein.

The degree of homology will also contribute to the level of cross-reactivity between host and microbial proteins. It can be seen in table 3 that the acetylcholine receptor α chain sequence is similar to that of the polyoma virus middle T antigen and yet these peptides do not cross-react. The less homologous herpes simplex virus (HSV) glycoprotein D sequence on the other hand induces strong immunological cross-reactivity (Dyrberg *et al*,1986).

For molecular mimicry to result in autoimmunity, the host antigen and the microbial marker must be recognized as both similar and different. For the cross-reactivity to occur, similar epitopes

must be recognized, yet if they are too similar immunological tolerance will result and an immune response to the microbe, and consequently the host antigen, will not be initiated. The host antigen can act as a triggering agent for the immune response long after the infectious organism has been cleared from the body. Self-antigens that may normally be concealed would become exposed as a result of tissue injury, thereby becoming potential targets for other autoantibodies. Chronic disease would therefore result from the continuous exposure of new self antigens so that autoimmune disease can arise long after the initial infection.

#### c) Altered self

The altered self hypothesis proposes that the self-antigen is altered in some way so that it appears foreign to the immune system thus causing an autoimmune response. For example, self antigen may be altered by the proteolytic enzymes released during inflammation (Shoenfeld *et al*, 1986).

For example impaired galactosyl transferase activity in B cells from rheumatoid arthritis patients results in fewer IgG oligosaccharide moieties terminating in galactose (Parekh et al,1985; Axford et al,1987). This truncation exposes N-acetylglucosamine residues that are normally concealed (Parekh et al,1985) making IgG a potential target for autoimmunity.

Certain evidence indicates that the HLA-B27 antigen from AS patients may also be altered. Geczy et al (1983) suggested that a modifying factor produced by various strains of enteric bacteria alters the HLA-B27 protein so that it is immunologically different from that of HLA-B27 positive healthy individuals. This modification of HLA-B27 as a possible cause of AS will be discussed in more detail in section 1.3.3.

#### d) Polyclonal activation

It has been suggested that synthesis of autoantibodies is the result of polyclonal activation of B lymphocytes upon infection (Shoenfeld *et al*,1987). When a microbial infection results in the polyclonal activation of B lymphocytes, antibodies a produced with a broad range of specificities some of which may act as autoantibodies. However, it is not understood how a polyclonal activator can induce the production of specific autoantibodies as described in particular autoimmune diseases. Nevertheless, it has been shown that normal lymphocytes that have been stimulated with either peptidoglycan or lipopolysaccharide, will specifically secrete rheumatoid factor rather than antibodies against red blood cells and DNA (Shoenfeld *et al*,1987).

#### e) The anti-idiotypic network

The anti-idiotypic network was proposed as a contributing element to the regulation of the immune system (Jerne, 1974). A foreign antigen will induce a set of antibodies or idiotypes, which in turn induces anti-antibodies or anti-idiotypes. This becomes relevant to autoimmunity when the anti-idiotype is specific for the antigen binding site of the idiotype: the anti-idiotype may then mimic the structure of the original antigen (figure 2). For example, host antibodies directed against a virus may have the same shape as the receptor to which the virus binds in order to enter the cell so that the anti-viral antibodies (idiotype) may mimic the structure of the receptor (self-antigen). Anti-idiotypic

antibodies which are believed to be normally present may therefore recognize the receptor or selfantigen (figure 2). In this way, autoantibodies can be generated spontaneously.

Whilst autoantibodies and autoreactive T lymphocytes are normally produced and appear to be necessary for health, the development of autoimmune disease seems to require a number of factors such as the right genetic and humoral background coming together in a single individual.

# 1.3. AETIOLOGY AND PATHOGENESIS OF ANKYLOSING SPONDYLITIS

# 1.3.1. ABERRANT HUMORAL AND CELLULAR IMMUNITY

Many changes in humoral and cellular immunity have been reported in AS patients: some of these are indicative of inflammation, as they occur in other inflammatory diseases, while others appear specific for the spondyloarthropathies.

#### a) Humoral immunity

Patients experiencing inflammatory episodes of AS are known to show general signs of inflammation as seen in other rheumatic diseases i.e., elevation of acute phase reactants such as C-reactive protein (CRP) and serum amyloid-associated protein, a decrease in serum prealbumin levels and an increased erythrocyte sedimentation rate (ESR) (Khan *et al*,1984).

Clinically active AS is usually associated with an elevation of serum IgA levels (Cowling et al,1980a). Since IgA is the major immunoglobulin produced by the gastrointestinal mucosa, it has been suggested that this may localize the triggering of antigen or pathogen to the gastrointestinal tract (Cowling et al,1980a). Furthermore, anti-Klebsiella IgA molecules have only been identified in patients with active AS and this elevation correlates with increased CRP (Cowling et al,1980b). Regular measurement of IgA is considered a useful indicator of disease activity and those patients whose IgA levels do not decrease with treatment appear to suffer more severe symptoms with resulting ankylosis of the axial skeleton (Khan,1988).

Antibodies to peptidoglycan have also been shown to be elevated in some AS and Reiter's syndrome patients while no association with rheumatoid factor or antinuclear antibodies has been found (Khan,1984). Although *Klebsiella* antigens have been shown to stimulate the production of anti-DNA antibodies by normal PBMC, attempts to detect anti-DNA antibodies in the sera of AS patients were unsuccessful (Isenberg *et al*,1987).

While the serum complement levels are considered to be normal in AS and related diseases (Khan et al,1988), some studies have reported the presence of C3 breakdown products (Meri et al,1988) implying that the complement system has been activated. Studies indicate that activation of

complement resulted in higher levels of C3 breakdown products in HLA-B27 positive sera than in HLA-B27 negative sera and suggested that HLA-B27 positive individuals have an elevated responsiveness to complement which may contribute to the development of AS (Meri et al, 1988).

While circulating immune complexes have been reported in the sera of AS patients with active disease (Espinoza et al,1982; Deicher et al,1983), the levels are not as high as those seen in sera from Reiter's syndrome and reactive arthritis patients (Duquesnoy et al,1983). The level of IgA within the immune complexes was found to be significantly increased in AS patients (Deicher et al,1983). The presence of circulating immune complexes indicates that an immune response may be part of the AS process, although nothing is known about the antigens. However, tissue damage resulting from the deposition of immune complexes occurs only rarely in AS (Bailey et al,1980) suggesting that these complexes may not contribute to disease development (Deicher et al,1983).

#### b) Cellular immunity

Attempts to associate particular T cell populations with AS have so far been contradictory, with normal, increased and decreased percentages of T cells being reported (Fan et al,1977; Christiansen et al,1978; Byrom et al,1979; Nilsson et al,1980; Veys et al,1983). For example, Kinsella et al (1985a) showed the number of circulating natural killer (NK) cells, as well as their cytotoxic activity, to be significantly increased in AS patients, whereas the helper and suppressor / cytotoxic T cell ratio levels were normal. Although it is known that tumour and virus-infected cells are targets for NK cells, there is no evidence to suggest that AS is precipitated by a viral infection. Kinsella et al (1985b) suggest that elevated NK cytotoxicity in AS may be involved in the immune response to the proposed triggering bacterial infection. The association between the HLA-B27 molecule and AS as well as that between the HLA-B locus and the regulation of cytotoxic activity by NK cells (Heberman et al,1981; McDevitt,1980) would support the role of cell-mediated immunity via NK cells in the pathogenesis of AS.

To date, attempts to show aberrant *in vitro* cell mediated immunity in AS patients have been inconsistent. Some *in vitro* studies have shown that cells from HLA-B27 positive AS patients (HLA-B27+ AS+) and HLA-B27 positive healthy (HLA-B27+ AS-) individuals have a diminished mitogenic response to phytohaemagglutinin (Christiansen *et al*,1978; Richter *et al*,1983) and pokeweed mitogen (Richter *et al*,1983) and an impaired mixed lymphocyte reaction, compared with HLA-B27 negative healthy control (HLA-B27- AS-) cells. The *in vitro* cell mediated response to the proposed triggering bacteria, in particular *Yersinia* and *Klebsiella*, have been reported variously as impaired, normal or enhanced (Leino*et al*,1983; Kinsella *et al*,1984). For example, PBMC from HLA-B27+ AS+ patients have been reported to respond to a greater degree to *Klebsiella* K43 cell membranes in a leukocyte migration inhibition assay than do HLA-B27+ and HLA-B27- controls (Gross *et al*,1983).

From the studies of T cell function, Kinsella *et al* (1985a,b) suggest that cell mediated immunity in AS is probably normal in the majority of patients. Nevertheless, Sheldon *et al* (1985) noted that the proliferative responses of lymphocytes to certain enteric bacterial antigens differed between patients with the various HLA-B27-associated disorders. The lymphocytes from AS patients gave a lower response to *Y.enterocolitica* and *K.pneumoniae* than cells from Reiter's syndrome and

reactive arthritis patients, and the response to *Streptococcus faecalis* was significantly less than in normal controls. Consequently, it was proposed that HLA-B27 positive individuals who show a reduced response to bacterial infection, ie: hypo-responders, develop AS while the hyper-responders develop Reiter's syndrome and reactive arthritis (Sheldon *et al.*1985)

#### c) Inflammation mediated injury

While an aberrant immune response has been suggested as the mechanism for the development of AS (Ebringer *et al*,1985a; McGuigan *et al*,1985; Oldstone,1987), it is possible that an enhanced inflammatory reactivity may be involved (Rosenbaum,1981). The elevated production of inflammatory mediators may contribute to the inflammatory symptoms seen in patients with HLA-B27 associated diseases, including AS. In support of this is the finding by Repo *et al* (1988) that if the monocytes from HLA-B27+ patients and healthy subjects as well as those from HLA-B27- patients were previously exposed to the mitogen, *E.coli* lipopolysaccharide, they secreted significantly more tumour necrosis factor-α (TNF-α) than did cells from HLA-B27 negative healthy controls. The increased TNF-α production was associated, therefore, with the presence of disease as well as with HLA-B27. Of the many effects attributed to this molecule, TNF-α has been shown to stimulate chemotaxis (Ming *et al*,1987), antibody-dependent cellular cytotoxicity (Shalaby *et al*,1985) and production of free radicals (Klebanoff *et al*,1986) by polymorphonuclear cells. Furthermore, secretion of interleukin-1 (IL-1) was also shown to be been elevated in HLA-B27 positive monocytes (Repo *et al*,1988). It is possible, therefore, that enhanced production of TNF-α and IL-1 may render a HLA-B27 positive subject to more susceptible to inflammation-mediated injury.

This is supported by the finding that polymorphonuclear leukocytes (PMN) from HLA-B27<sup>+</sup> AS<sup>+</sup> and HLA-B27<sup>+</sup> AS<sup>-</sup> subjects have an increased responsiveness to chemotactic factors compared with HLA-B27<sup>-</sup> AS<sup>-</sup> subjects (Pease *et al*,1984). PMN from HLA-B27<sup>+</sup> AS<sup>+</sup>, HLA-B27<sup>-</sup> AS<sup>+</sup> and HLA-B27<sup>+</sup> AS<sup>-</sup> subjects were shown to migrate significantly further towards zymosan activated serum than those from HLA-B27 negative subjects suggesting that AS and presence of HLA-B27 are both associated with increased chemotaxis *in vitro* (Pease *et al*,1984). Furthermore, zymosan activated sera from HLA-B27 positive subjects were shown to stimulate PMN migration more than the sera from HLA-B27 negative subjects (Repo *et al*,1983). *In vivo* studies using the skin-window technique demonstrated significantly higher numbers of PMNs in the skin chambers applied to HLA-B27 positive than to HLA-B27 negative subjects (Koivuranta *et al*,1984).

PMN are known to play a role in acute and chronic inflammatory reactions. These cells migrate from the circulation to the site of tissue injury in response to soluble mediators of inflammation such as C5a. Cells will bind C5a to the cell surface and move along a concentration gradient of attractant - chemotaxis. Cells also show increased random movement in the attractant in the absence of a gradient - chemokinesis. In this highly chemotactic environment, PMN thus accumulate in excess at the site of inflammation to release lysosomal enzymes and oxygen-derived free radicals such as superoxide anions, hydrogen peroxide and hydroxyl radicals (Clark,1983). These exert their toxic effects nonspecifically. The elevated chemotactic responsiveness has been suggested to be an inherent feature of HLA-B27 positive cells ((Pease et al,1984). Since HLA-B27- AS+ cells have also been

shown to exhibit increased motility it is suggestive that either the disease itself may produce this change or that the increased motility reflects a predisposition to AS which shows incomplete linkage to HLA-B27 (Pease et al,1984). Leirsalo et al (1980) also showed a correlation with disease severity and suggested that HLA-B27 is a marker for both severe disease and increased cell motility and that increased motility may be the cause of more severe disease. However, this is not supported by the fact that polymorphs are not always obvious within the primary lesion of AS i.e, the enthesopathy (Ball,1971).

#### 1.3.2. ASSOCIATION WITH HLA-B27

As noted previously, a strong association between AS and the HLA-B27 antigen has been observed such that greater than 90% of AS patients of European extraction are positive for HLA-B27 compared with 8% of the general population (Brewerton *et al*,1973; Schlosstein *et al*,1973). The relative risk for individuals carrying this antigen is greater than 70.0 (Tiwari *et al*,1985).

Two hypotheses have been suggested to explain the remarkable association of HLA-B27 with AS, namely the 'one gene' and the 'two gene' hypotheses.

#### a) The one gene hypothesis

The one gene hypothesis proposes that the HLA-B27 gene product is responsible for the development of AS. Observations favouring such a direct role include the following:

- 1) HLA-B27 has shown the strongest disease association of all other HLA-B locus alleles and alleles of the HLA-A, C, DR loci that have been studied (table 2) (Khan, 1988).
- 2) The association between HLA-B27 and AS has been established in every population studied (Khan *et al*,1984) (table 4). However the degree of association varies considerably; for example, 79-100% of Caucasian patients are positive for HLA-B27 compared with 57% in American blacks (Khan *et al*,1985).

The prevalence of AS within a particular racial group appears to correlate with the frequency of HLA-B27 within that population (table 4) (Khan *et al*,1985). For example, in African blacks of unmixed ancestry, HLA-B27 is virtually absent as is AS (Khan,1985). On the other hand, the incidence of HLA-B27 in North American Indians is relatively high, especially among the Haida (table 4). The prevalence of spondyloarthropathies is also high with up to 20% of patients showing evidence of sacroiliitis (Khan *et al*,1985).

3) No family has been reported in which AS dissociates from HLA-B27. In families with multiple cases of AS, the disease invariably segregates with HLA-B27 unless there are members with psoriasis or chronic inflammatory bowel disease (Woodrow et al,1985; Khan,1987). In addition, it is unusual to find a family with two or more first-degree relatives affected with HLA-B27 negative primary AS in the absence of psoriasis or inflammatory bowel disease in the family (Woodrow et al,1985).

If the one gene hypothesis is correct, this would imply that the HLA-B27 antigen in patients is in some way different from that of normal individuals. At least 8 different subtypes of HLA-B27 have now been recognized using cytotoxic T lymphocytes, monoclonal antibodies and isoelectric focusing (e.g., Grumet et al,1982; Breur-Vriesendorp et al, 1986; Choo et al, 1986a,b). Although these have been shown to be present at different levels in different populations, no single HLA-B27 epitope appears to be disease-related (Karr et al,1982; Breur-Vriesendorp et al, 1987), implying that a public antigenic determinant shared by the subtypes is involved rather than a private epitope. The subtypes of HLA-B27 will be discussed further in chapter 4.

#### b) The two gene hypothesis

The two gene hypothesis proposes that another gene, or 'AS gene', predisposes to the development of AS and that HLA-B27 is merely a linked marker. These genes are thought to be in linkage disequilibrium i.e., the frequency of these genes occurring together is greater than would be expected by chance, as indicated by the products of their individual frequencies. This can come about either because there is some evolutionary pressure for these genes to remain together or because insufficient time has elapsed for them to become randomly distributed throughout the population. For example, the class I alleles, HLA-A1 and HLA-B8 are known to be in linkage disequilibrium: their individual gene frequencies are 16% and 10% respectively, so the expected frequency of finding these two alleles together would be 1.6%, yet the observed frequency is much higher at 8.8% (Roitt,1984). The alleles HLA-A3 and HLA-B7 are also in linkage disequilibrium since their observed paired gene frequency is 2.8% compared with the expected value of 1.3%. Also, HLA-B8 and HLA-DR3 as well as HLA-B7 and HLA-DR2 are in linkage disequilibrium so that the extended haplotypes HLA-A1, B8, DR3 and HLA-DR2 are relatively common (Roitt,1984).

Congenital adrenal hyperplasia is one example of a disease initially shown to be linked to an HLA allele (HLA-B47) which was later proven to be a marker for the true disease susceptibility gene, encoding 21-hydroxylase (21-OHase) deficiency (White *et al*, 1984). The HLA-B47 allele is in linkage disequilibrium with other HLA alleles so that the disease haplotype HLA-A3, B47, DR7 (Dupont *et al*, 1984) is tightly linked to a 21-OHase gene (White *et al*, 1985).

Evidence in support of the two gene hypothesis as an explanation for the remarkable association between HLA-B27 and AS is summarized below:

1) Although the relative risk for AS is extremely high, only about 20% of HLA-B27 positive individuals develop the disease (Tiwari *et al*,1985). In addition, for most populations studied, the association between AS and HLA-B27 is not absolute (table 4). Clearly, individuals who do not carry HLA-B27 can develop AS.

The distribution of HLA alleles in HLA-B27<sup>-</sup> AS<sup>+</sup> individuals has been studied with conflicting results (Van den Berg-Loonan,1977; Khan *et al*,1980; Arnett,1984). Arnett *et al* (1984) found that 62% of white patients with Reiter's syndrome, sacro-iliitis and spondylitis had either HLA-B27, Bw22 or B40 which belong to the HLA-B7 cross-reactive group (HLA-B7-CREG) of antigens that also includes HLA-Bw42 and HLA-B27 (Khan *et al*,1985). Another study has shown the HLA-Bw35-CREG antigens to be more frequent in HLA-B27 negative patients (Wagener *et al*,1983) while two

others indicated a higher incidence of HLA-Bw16 in white HLA-B27 negative patients compared with controls (Khan et al,1980; Van den Berg-Loonan et al,1977). The antigen HLA-Bw16 is known to be associated with psoriasis and psoriatic arthritis, including spondylitis (Svejgaard et al,1977; Espinoza et al, 1978) and with spondylitis in Crohn's disease (Van den Berg-Loonan et al,1977). It is possible, therefore, that the genes for susceptibility to psoriasis and inflammatory bowel disease may be important in the development of spondylitis in HLA-B27 negative individuals even if these symptoms are never observed (Wright,1978; Woodrow et al,1978)

2) If an 'AS gene' does exist, it would be expected that HLA-B27- AS+ and HLA-B27+ AS+ individuals would have clinically the same disease (Wright, 1978; Woodrow et al, 1978). However, comparison of these 2 groups has shown that differences do exist (table 5). For example, HLA-B27 positive patients have a much higher incidence of acute anterior uveitis (Khan et al, 1981). HLA-B27 negative patients show a much later age of onset (Khan et al, 1978; Woodrow, 1978; Van den Berg-Loonan et al, 1977) and multiple cases do not tend to appear in families (Hammoudeh et al, 1983). At present, the HLA data for HLA-B27 negative patients is conflicting but it is clear that AS is a clinically and genetically heterogeneous disease.

#### 1.3.3. A POSSIBLE ROLE FOR *KLEBSIELLA*

It has become generally accepted that the etiology of AS is likely to be multi-factorial. Discordance of identical twins for AS, with regard to both presence and severity of symptoms (Eastmond *et al*,1977)), points to environmental factor(s) being important, in addition to genetic predisposing factors. Since other HLA-B27 associated diseases such as Reiter's syndrome and reactive arthritis are known to follow bacterial infections, many studies have attempted to associate such an infection with AS.

Current hypotheses attempting to explain the involvement of bacteria in the pathogenesis of AS fall into two major groups:

- (1) molecular mimicry, whereby a surface molecule of the infecting agent mimics the host's HLA-B27 antigen so that it becomes an autoantigen
- (2) modification of the HLA-B27 molecule by a protein encoded by a plasmid harboured by the infecting agent.

#### a) Molecular mimicry

When compared with controls, AS patients were found to have an increased recovery of faecal *Klebsiella*, particularly during active episodes of disease (Ebringer *et al*,1977; Eastmond *et al*,1980; Kuberski *et al*,1983). In addition, this increased faecal recovery correlated with elevated erythrocyte sedimentation rates suggesting a correlation between infection by *Klebsiella* and inflammation in AS (Cowling *et al*,1980a).

Ebringer and coworkers have suggested that an antigenic determinant of *Klebsiella* mimics the HLA-B27 molecule causing the formation of antibacterial antibodies that can bind to the partially cross-

reacting class I antigen, thus inducing an inflammatory autoimmune disease. This hypothesis is supported by the following observations:

- (1) Human tissue typing sera specific for HLA-B27 as well as rabbit antisera raised to HLA-B27 positive lymphocytes from both AS patients and healthy controls showed increased binding activity for *Klebsiella* extracts in haemagglutination and radiobinding assays (Ebringer *et al*,1976; Avakian *et al*,1980; Welsh *et al*,1980). Conversely, rabbit antisera to *Klebsiella* were found to be more cytotoxic for HLA-B27+ AS+ lymphocytes than for HLA-B27+ AS- cells when compared with non-immune sera (Welsh *et al*,1980). In addition, binding of rabbit anti-*Klebsiella* serum to radio-labelled *Klebsiella* extracts was inhibited to a greater degree by human sera specific for HLA-B27 than by non-HLA-B27 human antisera (Avakian *et al*,1980).
- (2) Monoclonal antibodies specific for the M1 and M2 epitopes of HLA-B27 were found to recognize bacterial antigens. The M1 monoclonal antibodies recognized a 16,000 MW antigen present in the cell envelope of *Yersinia enterolotica* type 9 and *Klebsiella pneumoniae* K21 and K43 while the monoclonal antibodies recognizing M2 reacted with an antigen of 20,000 MW from *Shigella flexneri* type 2a (van Bohemen *et al*,1984).
- (3) AS patients exhibit a specific elevation of serum IgA antibodies to *Klebsiella* antigens (Trull *et al*,1983). Since it is known that IgA is synthesized primarily in the gastrointestinal tract, the high faecal recovery and elevated IgA levels indicate that the bacterial antigenic trigger may act across this surface (Cowling *et al*,1980b).
- (4) Protein sequence analysis has shown that the 6 amino acids Gln-Thr-Asp-Arg-Glu-Asp appear between residues 72-77 of the hypervariable domain of HLA-B27.1 and residues 188-193 of *K.pneumoniae* nitrogenase (Schwimmbeck *et al*,1987) (table 3). Sera from HLA-B27+ AS+ and HLA-B27+ Reiter's syndrome patients reacted with a synthetic peptide including this 6 amino acid region from the subtype HLA-B27.1 (Schwimmbeck *et al*,1987) as well as a peptide derived from *K.pneumoniae* nitrogenase. Moreover, antibodies to the HLA-B27.1 hypervariable domain have been shown to recognize *Yersinia*, a bacterium strongly implicated in some cases of Reiter's syndrome and, to a lesser degree, in AS (Chen *et al*,1987). However, these studies have not been confirmed (Tsuchiya *et al*,1989).

Ebringer et al (1985b) have suggested that the course of AS is as follows: an infection, which may be subclinical, occurs in the gut giving rise to antibody production, serum IgA levels in particular being elevated. The lymph nodes that drain the colon and rectum are proposed as the sites of antibody production since these lie close to the affected tissues, namely the sacro-iliac joints and lumbar spine. Since the HLA-B27 molecule cross-reacts immunologically with the infecting organisms, the antibodies directed against these bacteria will also recognize the self-antigen, causing activation of complement and thereby bringing about inflammation. Free radicals and hydrolytic enzymes released during inflammation may cause tissue damage at different sites to that of the original infection. Circulating antibodies may then bind self-antigens at other sites such as the uveal tract, resulting in anterior uveitis. Chronic disease would be the result of recurrent episodes of infection, each causing inflammation.

#### b) Plasmid Hypothesis

It has been postulated that the cross reactive cellular determinant might be part of the HLA-B27 molecule modified by the addition of some bacterial component and that this alteration might initiate AS (Arnason *et al*,1978; Geczy *et al*,1980). In other words, this hypothesis supports the altered-self mechanism for the development of ankylosing spondylitis.

The following observations provide evidence in favour of the modification of HLA-B27 or a related molecule :

- (1) Rabbit antisera to *Klebsiella pneumoniae* strain K43 BTS 1 specifically lyse HLA-B27<sup>+</sup> AS<sup>+</sup> lymphocytes in a complement dependent <sup>51</sup>Cr- release assay but do not lyse HLA-B27<sup>+</sup> AS<sup>-</sup>, HLA-B27<sup>-</sup> AS<sup>-</sup>, HLA-B27<sup>-</sup> AS<sup>+</sup> cells (Geczy *et al*,1983; Prendergast *et al*,1983). This cytotoxic activity was absorbed by clinical isolates of the following bacteria; *Salmonella typhimurium* strains BTS 69 and BTS 111, *S.newport* BTS 52, *Shigella boydii* BTS 20, *Shigella flexneri* BTS 29, *E.coli* BTS 11 and *Campylobacter jejuni* BTS 13 (Prendergast *et al*,1983) but not by the non-cross-reactive *Klebsiella* strain F77 (Geczy *et al*,1983). HLA-B27<sup>+</sup> AS<sup>+</sup> cells remain susceptible to lysis after 20 generations in culture suggesting that the determinant recognized by these antisera is permanently expressed and, therefore, is genetically determined.
- (2) The culture supernatants of *K.pneumoniae* K43 contain a factor which, when incubated with HLA-B27<sup>+</sup> AS<sup>-</sup> cells, renders these cells susceptible to lysis by specific antisera i.e., they behave serologically like HLA-B27<sup>+</sup> AS<sup>+</sup> cells. This phenomenon is transient (Geczy *et al*,1980). In other words, the HLA-B27<sup>+</sup> AS<sup>+</sup> cells were somehow changed to appear similar to HLA-B27<sup>+</sup> AS<sup>+</sup> lymphocytes. This phenomenon was transient, implying that some component or modifying factor in the bacterial culture medium attaches reversibly to the HLA-B27 molecule on the cell surface (Geczy *et al*,1983).
- (3) The cell culture supernatants obtained from HLA-B27<sup>+</sup> AS<sup>+</sup> lymphoblastoid cell lines contain a factor which is similar to that isolated from that of *Klebsiella* K43 (Orban *et al* 1983). Peripheral blood lymphocytes (PBMC) and EBV immortalized lymphoblastoid cell lines from HLA-B27<sup>+</sup> AS<sup>-</sup> and HLA-B27<sup>-</sup> AS<sup>-</sup> subjects were exposed to the supernatants of HLA-B27<sup>+</sup> AS<sup>+</sup>, HLA-B27<sup>+</sup> AS<sup>-</sup> and HLA-B27<sup>-</sup> AS<sup>-</sup>cell cultures in order to assay modifying activity. Only HLA-B27<sup>+</sup> AS<sup>-</sup> cells pretreated with the culture filtrate of HLA-B27<sup>+</sup> AS<sup>+</sup> cell cultures were recognized by anti-Klebsiella K43 serum in a <sup>51</sup>Cr release cytotoxicity assay (Orban *et al*,1983).
- (4) The cross-reactive determinant is seen only in the context of HLA-B27 (Geczy et al,1986a). CTL were raised against HLA-B27+ AS- lymphocytes modified by incubation with the PBMC from an HLA-identical sibling suffering from AS (i.e., B27+ AS+). These specifically lysed HLA-B27+ AS+ but not HLA-B27+ AS-, HLA-B27- AS+, or HLA-B27- AS-PBMC. This cytotoxic activity could be blocked if the modified HLA-B27+ AS+ cells were treated with monomorphic anti-class I or anti-HLA-B27 sera but not with a control against HLA-B44, which was also expressed on the target cell surface. This would indicate that the CTL recognize a disease-associated structure only in association with HLA-B27 (Geczy et al,1986a).

Geczy and coworkers have suggested that the ability of *Klebsiella* and other enteric bacteria to modify HLA-B27+AS- cells *in vivo* is due to a plasmid(s) approximately 1.86x10<sup>7</sup> D (Sullivan *et* 

al,1983; Cameron et al,1983). This is supported by the finding that Klebsiella isolates cured of plasmid(s) with acridine orange lost the ability to synthesize the modifying factor (MF) (Cameron et al,1983). In addition, transfer of the plasmid(s) to non-cross-reactive E.coli resulted in the recipient strain being able to synthesize MF (Cameron et al,1983).

The activity of anti-Klebsiella K43 specific antibodies can be absorbed by a variety of Gram negative and positive faecal bacteria isolated from patients with AS. If the modifying activity is encoded by a plasmid, it is possible that this genetic material is passed between faecal bacteria and may explain the failure to consistently identify one species of bacteria as the triggering infectious agent (Prendergast et al, 1984).

Thus the persistence of the *Klebsiella*-cross-reactive marker on HLA-B27+AS+ cells is thought by Geczy and colleagues to be due to the modification of HLA-B27+AS- cells *in vivo* by a plasmid(s). The plasmid product(s) appears to be responsible for the *in vitro* modification of HLA-B27+ AS- cells and this modification is seen only in the continuous presence of modifying factor. However, it is not clear if it is the HLA-B27 molecule itself that binds the product(s) or whether it merely potentiates the binding to an associated receptor. Nevertheless, Geczy and coworkers have proposed that the expression of prokaryotic genes by human cells may be relevant to the aetiology of the HLA-B27 associated arthropathies as well as to other MHC associated diseases thought to be triggered by bacterial infections.

# c) Both the molecular mimicry and plasmid hypotheses have not been independently confirmed

The studies of Geczy's and Ebringer's groups remain controversial, as attempts by others to confirm their findings are contradictory. For example, reports confirming the findings of Geczy and colleagues have come from groups working in very close collaboration (Archer et al,1985; Van Rood et al,1985; Geczy et al,1986) but not as yet from an independent study. Attempts by other groups using the  $^{51}$ Cr-release assay (Beaulieu et al,1983; Kinsella et al,1986; Singh et al,1986; Cameron et al,1987) as well as bacterial isolates (Beaulieu et al,1983; Singh et al,1986; Cameron et al,1987) and antisera (Singh et al,1986) provided by Geczy have failed to reproduce this group's findings. Other assays have been used such as ELISA (Cameron et al,1987) and dye exclusion (Kinsella et al,1986) without success.

In addition, attempts to confirm modifying factor activity as seen by Geczy *et al* (1985) have been made by Georgopoulos and Pain (as reported by Kinsella,1985b) without success even though, again, bacterial isolates and EBV transformed cell lines from Geczy were included. The bacteria from Geczy's group were shown to carry plasmids, but no *in vitro* lymphocytoxicity could be detected. However, another attempt to corroborate the role of plasmids in the aetiology of AS has shown that a plasmid of 3.5Kbp found in the cross-reactive strain *Klebsiella* K43-BTS 1 was also prepared from the enteric isolates of patients with AS (Winrow *et al*,1988). The bacterium was shown to have two plasmids of this size: one encoding tetracycline resistance (BTS1.Tet) and the other amplification by chloramphenicol (BTS1.CAP). Using BTS1.Tet cDNA as a probe, hybridization experiments indicated weak homology with HLA-B27+AS+ lymphocyte DNA but not between patient DNA and BTS1.CAP

nor between BTS1.CAP and plasmid DNA isolated from the non-cross-reactive bacteria, F77 (Winrow et al.,1988). Although this is suggestive of the involvement of plasmids, the finding of weak homology between human DNA and a Klebsiella sequence actually supports the notion of molecular mimicry as the mechanism for development of AS.

On the basis of amino acid homology between *Klebsiella* and HLA-B27, Viitanen *et al* (1988) investigated the existence of homology between *Yersinia enterocolitica* and patient DNA. No hybridization was seen with *Yersinia* total DNA by the HLA-B27 gene isolated from a normal individual nor between genomic DNA prepared from AS patients and *Yersinia* DNA fractions. Although DNA sequences from Gram negative bacteria do not appear to be homologous with human sequences, it does not exclude the possibility that molecular mimicry exists between bacterial proteins and the HLA-B27 antigen. The cross-reactive epitope may be a conformational determinant that cannot be detected by DNA hybridization experiments.

Although many studies have tried to ascertain a role for *Klebsiella* in the development of AS, the association of the disease with HLA-B27 remains undisputed. With this in mind, attempts have been made to determine if the HLA-B27 gene is in some way different in patients suffering from ankylosing spondylitis.

### 1.4. PROJECT AIMS

The very strong association between ankylosing spondylitis and HLA-B27 supports the notion that only one gene, the HLA-B27 gene, is directly involved in disease development. This implies that the HLA-B27 gene from individuals with AS might be different from that of healthy individuals. Using probes derived from a normal HLA-B27 gene, this possibility has been investigated using restriction fragment length polymorphism (RFLP) analysis and a search has been made for a fragment(s) that may identify patients from normal individuals.

It has also been suggested that other genes within the major histocompatibility complex (MHC), linked to the HLA-B27 gene but distinct from it, may be primarily responsible for determining susceptibility to AS. Discordance for the disease in identical twins lends support for this two gene hypothesis and one candidate for this 'AS gene' is that encoding tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). The level of polymorphism of this gene within a normal population was assessed by RFLP analysise. Association between TNF- $\alpha$  and AS has been sought.

In order to investigate the possiblity of a direct role for the HLA-B27 locus, the gene from an ankylosing spondylitis patient heterozygous for this allele was cloned from a  $\lambda$ gt10 library and sequenced. Comparisons with published sequences have been made to identify any differences that may possibly result in disease development.

While the sequence of the HLA-B27 allele from patients may not be different from that of the healthy population it is possible that the regulation of expression of this locus may be. An investigation has been initiated using EBV-transformed cell lines from HLA-B27+AS+, HLA-B27-AS+ and HLA-B27+AS- individuals. The effect of the cytokines, IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$ , on the expression of HLA-B27 by these cell lines has been analyzed at the protein level by using an anti-class I antibody as well as at the mRNA level by Northern hybridization.

## 1.5. FIGURES AND TABLES

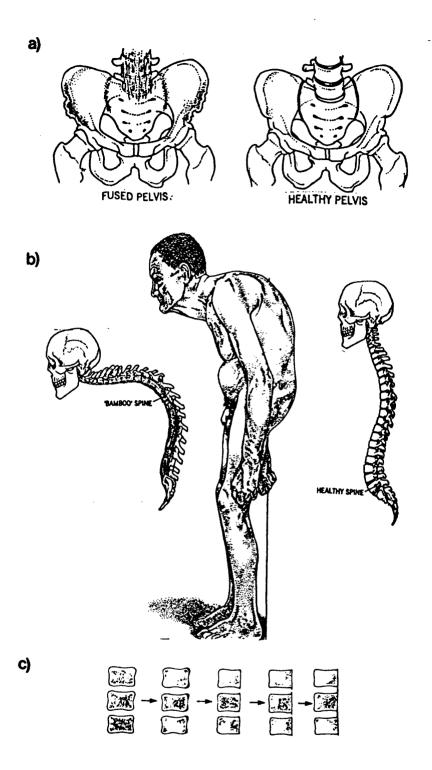


Figure 1: a) Comparison of fused sacro-iliac joints from an AS patient (left) and a healthy individual (right). Sacro-iliac joints become fused as AS progresses.

b) The stooped posture seen in AS patients with severe disease. As the disease progresses, syndesmophytes form between the vetebrae resulting in the classic 'bamboo spine'.

c) Formation of syndesmophytes between vertebrae. Source: The Independent, 8th November, 1988.

Table 1: Comparison of clinical features in primary and secondary ankylosing spondylitis

	Primary AS	Spondylitis with IBD	Psoriatic spondylitis	Spondylitis with Reiter's syndrome	Spondylitis with Reactive arthritis
Age at onset (yr)	15-40	more variable	more variable	18-40	more variable
Sex distribution	M > F	M = F	M > F	M > F	M = F
HLA-B27*	> 90%	Approx. 50%	Approx. 50%	Approx. 90%	Approx. 85%
Urethritis		•	ı	+++	+
Prostatitis	+	•	1	++	+
Conjunctivitis	-/+	-/+	+	+++	+
Acute anterior uveitis	++	++	+	+++	+
Mucosal involvement	•	+		+++	,
Spinal involvement	+++	+	+	+	+

IBD = chronic inflammatory bowel disease

\* = in whites

Source: Khan et al ,1988; adapted from Khan,1984

Table 2: Antigen association data for some HLA associated diseases.

Source: Thomson (1986)

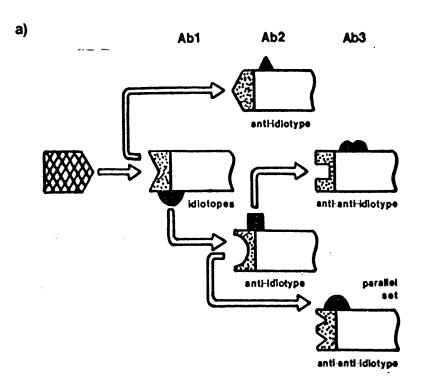
	Free	quen	cy (°	<sub>o</sub> )		
Discase	HL	<b>A</b>	Pati- ents	Con- trols	tive	Attribu table risk (δ)
Hodgkin's disease	Al		40	32.0	1.4	0.12
Idiopathic haemo-						
chromatosis	<b>A</b> 3		76	28.2	8.2	0.67
Behçet's disease	<b>B</b> 5		41	10.1	6.3	0.34
Congenital adrenal						
hyperplasia	B47		9	0.6	15.4	0.08
Ankylosing spondylitis	B27	,	90	9.4	87.4	0.89
Reiter's disease	B27		79	9.4	37.0	0.77
Acute anterior uveitis	B27		52	9.4	10.4	0.47
Subacute thyroiditis	B35		70	14.6	13.7	0.65
Psoriasis vulgaris	Cw	6	87	33.1	13.3	0.81
Dermatitis herpetiformis	D I	OR3	85	26.3	10.8	0.72
Coeliac disease	DI	DR3	79	26.3	15.4	0.80
Sicca syndrome	D I	DR3	78	26.3	9.7	0.70
Idiopathic Addison's						
disease	D I	OR3	69	26.3	6.3	0.58
Graves' disease	DI	DR3	56	26.3	3.7	0.42
Insulin-dependent	DI	OR3	56	28.2	3.3	0.39
diabetes	D I	DR4	75	32.2	6.4	0.63
	DI	DR2	10	30.5	0.2	
SLE	DΙ	DR3	70	28.2	5.8	0.58
Multiple sclerosis	DI	DR2	59	25.8	4.1	0.45
Optic neuritis	DΙ	DR2	46	25.8	2.4	0.27
Rheumatoid arthritis	D I	DR4	50	19.4	4.2	0.38
IgA nephropathy	D١	DR4	49	19.5	4.0	0.37
Hydralazine-induced SLE	DI	DR4	73	32.7	5.6	0.60
Hashimoto's thyroiditis	DΙ	DR5	19	6.9	3.2	0.13
Pernicious anaemia	DΙ	DRS	25	5.8	5.4	0.20
Pauciarticular onset						
juvenile rheumatoid						
arthritis	DI	DR5	50	16.2	5.2	0.40

Table 3: (a) Sequence similarities between microbial proteins and human host proteins

(b) Sequence similarities between acetycholine receptor  $\alpha$  chain and the non-cross reactive polyoma virus middle T antigen and the cross-reactive herpes simplex glycoprotein

(a) PROTEINRESIDUEHuman cytomagalovirus IE279Human lymphocyte antigen DR70Polio virus VP270Acetylcholine receptor176Papilloma virus E276Insulin receptor76Rabies virus glycoprotein147Insulin receptor764Klebsiella pneumoniae nitrogenase186Human lymphocyte antigen B2770Adenovirus 12 E1B384A-gliadin206Human immunodeficiency virus p24160Human lgG constant region466Measles virus P313Corticotropin18Myelin basic protein61(b) PROTEIN61	SEQUENCE  Pro - Asp - Pro - Leu - Gly - Arg - Pro - Asp - Glu - Asp Val - Thr - Glu - Leu - Gly - Arg - Pro - Asp - Ala - Glu  Ser - Thr - Thr - Lys - Glu - Ser - Leu - Lys - Asp - Ser  Thr - Val - Ile - Lys - Glu - Ser - Leu - Lys - Asp - Ser  Val - Tyr - Gly - Leu - Glu - Ser - Leu - Lys - Asp - Ser  Val - Tyr - Gly - Leu - Glu - Ser - Leu - Lys - Asp - Leu  Thr - Lys - Glu - Ser - Leu - Val - Ile - Ile - Ser  Asn - Lys - Glu - Ser - Leu - Val - Ile - Ile - Ser  Asn - Lys - Glu - Ser - Leu - Val - Ile - Ile - Ser  Asn - Lys - Glu - Ser - Leu - Val - Ile - Ile - Ser  Glu - Thr - Asp - Arg - Glu - Asp - Leu  Leu - Arg - Gln - Thr - Thr - Pro - Ser - Gln - Gln - Gly  Gly - Val - Glu - Thr - Thr - Thr - Pro - Ser  Gly - Val - Glu - Thr - Thr - Thr - Pro - Ser  Gly - Val - Glu - Thr - Thr - Thr - Pro - Ser  Gly - Val - Glu - Thr - Thr - Thr - Pro - Ser  Gly - Val - Glu - Thr - Thr - Thr - Thr - Pro - Ser  Gly - Val - Glu - Thr - Thr - Thr - Thr - Glu - Cys - Lys  Leu - Glu - Cys - Ile - Arg - Ala - Leu - Lys  Leu - Glu - Cys - Ile - Arg - Asn - Leu - Gly - Gln - Glu  Glu - Ile - Ser - Asp - Asn - Leu - Gly - Gln - Glu  Glu - Ile - Ser - Phe - Lys - Leu - Gly - Gln - Glu  SEQUENCE
Acetylcholine receptor α chain	Pro - Glu - Ser - Asp - Gln - Pro - Asp - Leu
Polyoma virus middle T antigen	Pro - Glu - Ser - Asp - Gln - Asp - Gln - Leu
Herpes simplex virus glycoprotein	Pro - Asn - Ala - Thr - Gln - Pro - Glu - Leu

Source: Oldstone, 1987



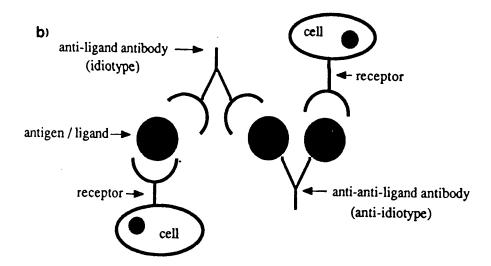


Figure 2: a) Formation of autoantibodies via the anti-idiotypic network. Anti-idiotypic antibodies (Ab2) that recognize the antigen binding site of the idiotypic antibody (Ab1) may mimic the antigen. Source: Roitt *et al* (1985)

b) Anti-idiotypic autoantibodies that mimic the structure of the original antigen are relevant to autoimmunity as it may be possible for these autoantibodies to bind the antigen receptor i.e., the self antigen.

Table 4: HLA-B27 frequency in caucasoid, mongoloid and negroid populations

Population Caucasoid Eurocaucasoids (whites) Indians & Pakistanis Iranians Arabs Jews Mongoloid Chinese Hong Kong Taiwan Singapore	number patients 2022 130 25 32 31 196 77 75	<b>B27(%)</b> 79-100 83-100 92 81 81 81 99 99 95	number controls 16162 456 400 355 456 102 297 238 208	4-13 2-8 3 3 3 3 4 4 4
Philipino Thai North American Indians Haida Navajo Bella Coola Pima Zuni Hopi Metizo <sup>a</sup> South American Indians <sup>b</sup> African blacks American blacks	17 71 17 5 3 14 14 67	86 100 100 69-81 57	222 138 120 129 400 1404 440 259 798 13330	5-8 50 36 255 113 9 9 0 0 1 24

a: basically mongoloid (Asiatic origin) but have had a continuous admixture of European (primarily Spanish) and to a small extent negroid genes since the 16th century
b: of unmixed ancestory
c: from Congo and Zambia; of unmixed ancestory
Source: Khan et al ,1988 previously adapted from Khan,1984

Table 5: Comparison of clinical features of B27 positive and B27 negative patients with primary ankylosing spondylitis.

	B27 (+)	B27 (-)
HLA antigens	all races	increased in non-whites
	B27	increased B7 in blacks
		increased Bw16 (Bw38) in whites
		(? inflammatory bowel disease or psoriasis genes)
Age of onset (yr)	1540	18-50
Familial aggregation	++	+
Acute anterior uveitis	<b>+</b> +	<b>+ +</b>
Source: Khan,M et al (1988)		

## CHAPTER 2

# RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF THE HLA-B27 GENE

### 2.1. INTRODUCTION

# 2.1.1. STRUCTURE OF THE HUMAN MAJOR HISTOCOMPATIBILTY COMPLEX

#### a) Chromosomal localization and the division of the major histocompatibility complex into regions

The human major histocompatibility complex (MHC), also known as the HLA system, has a length of 4200 Kb (Ragoussis *et al*,1989) and is found on the short arm of chromosome 6 between 6p21.31 and 6p21.33 (Spring *et al*,1985; Ziegler *et al*,1985). The MHC is divided into three regions known as the class I, class II and class III regions with lengths of 2000Kb, 1000Kb and 1200Kb respectively (Ragoussis *et al*,1989). Each is made up of a number of genes and the organization of these genes and their individual structures have been determined from molecular cloning and chromosome walking experiments while pulse field gel electrophoresis and related techniques have allowed a full physical map of the HLA complex to be determined (e.g., Ragoussis *et al*,1989; Sargent *et al*,1989a; Spies *et al*,1989) (figure 3).

#### b) The class II region

The class II region encodes a polymorphic set of cell surface glycoproteins that are expressed only on macrophages, B cells and activated T cells and are thought to be involved in the regulation of the immune response (Kaufman et al,1984). Each glycoprotein is a heterodimer consisting of an A chain of approximately 33 KD and a B chain of about 28 KD (Robson et al,1984). The three subregions, DR, DQ, DX and DP, contain genes for both the A and B chains whereas two additional subregions, DN and DO, encode only an A chain and a B chain respectively. Pulse field gel electrophoresis has allowed precise linkage of this region. The order of these genes is illustrated in figure 3.

#### c) The class III region

Conventional linkage analysis positioned the class III region between the class II and class I regions (Robson *et al*,1984) within a distance of 1200 Kbp (Ragoussis *et al*,1989) (figure 3). The class III region comprises the genes for the second complement component C2, for factor B and genes encoding two forms of the fourth complement component, C4A and C4B (Chaplin *et al*,1983; Carroll *et al*,1984). In addition, two steroid 21-hydroxylase genes (21-OHase A and B) have also been localized to a 6 Kb region flanking the 3' end of each of the two C4 genes (Carroll *et al*,1985).

In addition to the class III genes, many other genes have recently been mapped to the region between the class I and class III regions (figure 3):

1) The genes encoding TNF-α and the TNF-β occupy a position between the class III region and the HLA-B locus of the class I region (Spies *et al*,1986; Carroll *et al*,1987; Ragoussis *et al*,1988). The TNF-α gene will be discussed in more detail in chapter 3.

- 2) Two genes encoding the heat shock protein HSP70 have been mapped to the region between the TNF genes and the class III region and of these, the HSP70-1 gene is known to be expressed (Sargent et al,1989b)
- 3) Levi-Strauss *et al* (1989) detected the RD gene by searching for sequences conserved between species. This gene is situated within the class III region between Bf and C4A and its product has an unusual periodic structure with a core of acidic/basic amino acid dipeptide repeats.
- 4) By searching for CpG rich regions within the MHC, Sargent *et al* (1989a) localized 12 novel genes to the class III/TNF region. One of these was positioned about 10 Kb centromeric to the TNF-α gene and shown to be the human homologue of the murine B144 gene expressed specifically in B cells and macrophages. The other genes, numbered G1 to G11, appear to be ubiquitously expressed although their function is as yet unknown.
- 5) Spies et al (1989) also searched for CpG islands and identified five 'HLA-B associated transcripts' or BAT genes within the TNF region. It is thought that the BAT-2, BAT-3 and BAT-4 genes correspond respectively to the G2, G3 and G5 genes detected by Sargent et al (1989a). The BAT-1 gene was positioned between TNF-b and HLA-B while the genes BAT-2 to BAT-5 were localized on the centromeric side of B144 (Spies et al,1989). Like the G genes of Sargent et al, the BAT genes are ubiquitously expressed.

#### d) The class I region

The human class I region has been mapped to the telomeric side of the class III region and Southern blotting analysis indicates that in the EBV cell line, LCL 721, this multi-gene family contains 17 loci (Orr et al,1982). Of these, six are known to encode proteins and the best characterized of these are HLA-A, HLA-B and HLA-C. These loci encode glycoprotein chains of approximately 44 KD which are associated non-covalently at the cell surface with β2-microglobulin. The order of these class I loci from the centromere is HLA-B, C, A (Robson et al,1984) (figures 3,4) and linkage studies have shown that the distance between the HLA-B locus and the C locus is 130 Kb (Pontarotti et al,1988). Since overlapping clones have not yet been isolated from between HLA-C and A the map for this subregion is as yet only tentative but the present estimate of the distance between these two genes is 1200 Kb with the total length of the class I region estimated at 2000 Kb (Ragoussis et al,1989). The structure and function of these class I antigens will be discussed in more detail in sections 2.1.3 and 2.1.4.

Recently, functional class I genes in addition to the HLA-A, B,C genes have been identified (Carroll et al,1987; Srivastava et al,1987; Koller et al,1989). HLA-E is contained within a 6.2 Kb Hind III fragment and has been positioned at 650 Kb telomeric to HLA-C (Carroll et al,1987). The clone, RS5, is considered to be an allele of this gene (Carroll et al,1987; Srivastava et al,1987). Ragoussis et al (1989) identified a novel class I gene cda12 and found it to be on the centromeric side of HLA-A (figure 3). Two further genes, now designated HLA-G and F, are found on Hind III fragments of 6.0 and 5.4 Kb respectively and have been positioned to the telomeric side of HLA-A (Koller et al,1987) (figure 4).

The majority of HLA- class I-related sequences in the genome of LCL721 do not seem to encode expressed class I antigens (Koller *et al*,1987). Sequence analysis has shown five of these sequences to be pseudogenes and all of these map to the region around HLA-A or telomeric of this locus (Koller *et al*,1989) (figure 4).

HLA deletion mutants have been used to map class I loci to the telomeric side of the HLA-A gene which may be the human equivalent of the mouse Qa and Tla loci (Orr et al, 1983a). These murine genes encode class I molecules which are less polymorphic than the classical class I antigens, H-2D, K and L, and are expressed in a tissue specific rather than ubiquitous manner (Guillemot et al, 1984). However, the function of these molecules is unknown. The HLA-E, F and G genes have been suggested as the human equivalent of the murine Qa and Tla genes, although expression and functional studies have yet to confirm such speculation (Koller et al, 1987).

#### 2.1.2. CLASS I GENES

#### a) The structure of class I genes

Comparisons of class I sequences has shown the overall structure of class I genes and their products to be highly conserved within a species as well as between species (Guillemot *et al*,1988). Class I genes encode glycoprotein heavy chains with a relative molecular mass ( $M_{\Gamma}$ ) of 44,000 (Ploegh *et al*,1981) and cloning and sequencing analysis has shown that each exon within class I genes encodes a separate domain of these proteins (Malissen *et al*,1982) (figure 5). The first exon encodes the leader peptide and the first, second, third polypeptide domains are coded for by exons 2, 3 and 4 respectively. Exon 5 encodes the transmembrane region and the cytoplasmic domain is encoded by exons 6 and 7 as well as a short exon 8 that is contiguous with the 3' untranslated region. This structure appears to be typical for HLA-A and C loci whereas in all HLA-B genes sequenced so far, a termination codon is found at the end of exon 7 so that the eighth exon is untranslated and their products are two amino acids shorter at the carboxyl terminus (Gussow *et al*,1987).

#### b) Polymorphism of class I genes

The class I heavy chain molecules are highly polymorphic. About 49 alleles of the B locus and 23 of the A locus are known (Klein,1987) while about 8 alleles of the HLA-C locus have been identified so far (Roitt *et al*, 1985) (table 6). The recently identified HLA-E locus appears to have 3 allelic forms (Koller *et al*,1988; Srivastava *et al*,1987; Mizuno *et al*,1988).

Sequence comparisons have revealed that a high degree of homology exists between alleles as well as between loci. Comparison of allelic sequences shows that greater homology exists in the 3' half than the 5' half (Weiss et al,1985; Ways et al,1985). Indeed, locus specific probes have been derived from the appropriate 3' regions (Koller et al,1984). In contrast, when alleles of different loci were compared, the sequences did not show greater homology towards one end but rather the homology was relatively constant throughout with a higher degree of divergence overall (Weiss et al,1985) (table 7).

The 5' exons, in particular exons 2 and 3, show the most polymorphic variation. This is not surprising since these exons encode the hypervariable  $\alpha 1$  and  $\alpha 2$  domains which form the antigen binding site. This site is discussed in more detail in section 2.1.3.

#### 2.1.3. CLASS I ANTIGENS

#### a) The three dimensional structure of membrane bound class I molecules

Class I molecules are integral membrane proteins composed of two polypeptide chains (Cresswell *et al*,1973); a heavy chain with a relative mass of 44,000 that spans the membrane and is associated non-covalently with an invariant subunit,  $\beta_2$ -microglobulin, of  $M_r$  12,000.  $\beta_2$ -microglobulin is encoded by a gene on chromosome 15 (Orr *et al*, 1983b).

The extracellular portion of the class I heavy chain is divided into three domains,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  (Ploegh *et al*, 1981), each about 90 amino acids long (Malissen *et al*,1982). The  $\alpha 3$  domain and the  $\beta 2$ -microglobulin molecule interact non-covalently, as do the  $\alpha 1$  and  $\alpha 2$  domains. A 26 amino acid hydrophobic region anchors the molecule into the cell membrane and there is a cytoplasmic segment of 30 amino acids. The three-dimensional structure for the HLA-A2 molecule has been determined (Bjorkman *et al*,1987a,b) and is considered to be the same for all class I molecules (figure 6).

#### The a3 and \( \beta\_2\)-microglobulin domains

The  $\alpha 3$  and  $\beta 2$ -microglobulin domains, which are proximal to the membrane, are relatively conserved and show amino acid sequence homology as well as similar tertiary structure to immunoglobulin constant regions (Bjorkman *et al*,1987a).

Both the  $\alpha$ 3 and  $\beta$ 2-microglobulin domains are composed of two antiparallel  $\beta$ -pleated sheets, one made up of four and the other of three  $\beta$ -strands, which are connected to each other by a disulphide bond (figure 6a). In  $\alpha$ 3 this bond occurs between cysteine (Cys)-203 and Cys-259 while in  $\beta$ 2-microglobulin the bond is between Cys-25 and Cys-80 (Bjorkman *et al*,1987a).

#### The $\alpha 1$ and $\alpha 2$ domains

The  $\alpha 1$  and  $\alpha 2$  domains are held away from the cell membrane and are thought to form an antigen binding site enabling foreign antigens to be presented to cytotoxic T lymphocytes. These domains are highly polymorphic and show only weak sequence homology to each other (Parham *et al*,1988). Their 3-dimensional structures, on the other hand, are nearly identical and, in contrast to  $\alpha 3$  and  $\beta_2$ -microglobulin, are not similar to immunoglobulin constant or variable domains (Bjorkman *et al*,1987a) (figure 6b).

The  $\alpha 1$  and  $\alpha 2$  domains interact with each other to form an antigen binding site. The amino terminals of these domains each form 4  $\beta$ -pleated strands which come together to make up a single antiparallel  $\beta$ -sheet with 8 strands that serves as the floor of the antigen binding site. The carboxyl terminals form  $\alpha$ -helices which sit on top of the  $\beta$ -sheet (figure 6b) and are connected by a disulphide bond between residue 101 in the N-terminal  $\beta$ -strand and residue 164 in the  $\alpha$ -helix of  $\alpha 2$ . A deep groove exists between the two  $\alpha$ -helices and the amino acid side chains from the central  $\beta$ -strands of

the  $\alpha 1$ - $\alpha 2$   $\beta$ -sheet form the sides of this groove. The function of this structure as an antigen binding site is supported by the occupation of this site by an unknown molecule when the HLA-A2 molecule was crystalized (Bjorkman *et al*,1987a).

#### b) Localization of polymorphic residues within class I antigens

In order that the class I molecule can bind a great variety of processed antigens, the amino acid residues that take part in this interaction must exhibit a high degree of polymorphism. Most of the highly polymorphic residues are found in the  $\alpha 1$  and  $\alpha 2$  domains; some of these residues are positioned in the  $\beta$ -sheet (the floor of the antigen binding site) so that they point up between the ahelical regions. Polymorphism at these sites may affect the interaction with antigen rather than interactions with T cell receptors. Residues located on the sides of the helices facing into the binding site are also thought to bind foreign antigens while those amino acids positioned on the top face of the helices with their side chains pointing outwards probably interact directly with the T cell receptor (figure 7).

#### c) Polymorphic variation between class I alleles

The number of allelic forms of class I genes seems to increase as more sensitive detection techniques become available. For example, on the basis of reactivity with alloantisera, the antigen HLA-B40 has been subdivided into the two subtypes HLA-Bw60 and HLA-Bw61 (Ways et al,1987). Using monoclonal antibodies, a third variant has been identified, namely HLA-B40\* (Ways et al,1987). This might suggest the HLA is even more polymorphic than is presently believed. Comparisons of MHC haplotypes in laboratory strains of mice with those of wild type strains has led to a similar conclusion for this species (Klein et al,1981).

Extensive comparison of amino acid sequences of class I molecules has shown that the overall homology between the HLA-A and HLA-B loci is 86% (Gussow *et al*,1987) and such comparisons have revealed some residues that define locus specificity. Of the 274 amino acids in the polymorphic domains α1 and α2, 183 residues are conserved between all the HLA-A, B and C alleles (Parham *et al*,1988). To put this another way, 91 positions are polymorphic. Only six positions are found to be locus specific in these extracellular domains: Val-52, Glu-183 and Glu-268 are HLA-C specific; HLA-A locus specific residues are Met-138, Met-189 and a HLA-B specific Arg is found at position 239. However, at no position are all the loci different i.e., two loci will have the same residue while the third will be different. Comparison of the transmembrane regions has revealed eight, three and five residues specific for HLA-A, -B and -C respectively (Gussow *et al*,1987). The cytoplasmic domain comprises five C locus and three A locus specific residues whereas the B locus proteins show fewer specific residues in this region. The transmembrane and cytoplasmic domains display a high degree of locus specificity. Indeed allelic forms of class I genes are more homologous in these regions with locus specific probes being derived from this portion of the gene as well as the 3' untranslated region (Gussow *et al*,1987).

Comparison of HLA-B alleles with HLA-A alleles has also shown that the arrangement of variation is slightly different; for the A locus alleles, the amount of variation is similar between the  $\alpha 1$ 

and  $\alpha 2$  domains whereas for the B alleles the variation in the  $\alpha 1$  domain is much greater (Ways *et al*,1985). Figure 8 compares the HLA-B molecules for which sequences are known. The expected high level of polymorphism within the  $\alpha 1$  and  $\alpha 2$  domains can be seen clearly with less variation occurring in the  $\alpha 3$  region. The residues that are specific to the HLA-B locus are also indicated.

#### d) Conserved features of class I molecules

#### Glycosylation

Glycosylation sites are highly conserved. Human class I antigens have a carbohydrate moiety attached at Asn 86 (Nathenson *et al*,1974) which extends away from the polypeptide structure and is positioned in a loop connecting  $\alpha 1$  and  $\alpha 2$  (Bjorkman *et al*,1987a). Glycosylation at this position, however, does not appear to be necessary for the association of the HLA heavy chain with  $\beta_2$ -microglobulin nor for cell-surface expression (Ploegh *et al*,1981; Santos-Aguado *et al*,1987).

#### Conserved amino acids

Many residues within the antigen binding site are conserved. For example, ten residues that point into the antigenic recognition site are completely conserved across 22 human sequences (Cowan et al,1987; Gussow et al,1987). This might imply that these residues interact with a constant component of processed antigens. Every 4th or 5th residue is conserved within the  $\alpha$ 1 helix so that a completely conserved face on the top of the helix exists that is not part of the antigen-recognition site. A similar pattern to that of  $\alpha$ 1 is observed for the  $\alpha$ 2 helix Since these side chains are found on the top of the class I molecule, it is unclear whether they form part of the T cell recognition site or whether they help stabilize the protein structure (Bjorkman et al,1987b).

#### e) Secreted form of class I molecules

While class I molecules of the major histocompatibility complex are generally considered to be typical integral membrane glycoproteins (Ploegh *et al*,1981), lipid-associated and water soluble forms have been demonstrated (Krangel,1986). The lipid soluble molecules are the result of the natural shedding process of membrane bound antigens from the cell surface. The water soluble molecules, on the other hand, arise from an alternative RNA splicing mechanism that deletes exon five, which encodes the transmembrane hydrophobic region (Krangel,1986). Both forms of secreted class I molecules have been demonstrated *in vitro* in the culture supernatants of lymphoblastoid cell lines and peripheral blood lymphocytes (Krangel,1986) as well as *in vivo* in human plasma (Krangel,1987). The function of secreted class I molecules is unknown.

#### 2.1.4. FUNCTION OF CLASS I MOLECULES

#### a) Presentation of foreign antigens to cytotoxic T lymphocytes

It is generally accepted that the primary function of class I molecules is the presentation of foreign antigens, particularly viral peptides, to cytotoxic T lymphocytes. However, CD8+ T cells can

only recognize these antigens when they are associated with the same MHC haplotype as that expressed by the T cell. This is known as MHC restriction (Zinkernagel et al,1974). The antigens are processed and bound within the antigen peptide groove for presentation to the T cell receptor. The formation of such a complex is supported by the fact that 'electron dense' material remained bound to the putative antigen binding site throughout the purification of the class I molecule HLA-A2 as described by Bjorkman et al, (1987). Furthermore, peptides added exogenously to target cells bind to class I molecules and can trigger cytotoxicity by T cells (Hosken et al,1989). Moreover, Chen et al, (1989) demonstrated direct binding of an influenza matrix peptide to HLA-A2 and of an influenza nucleoprotein peptide to HLA-B37. Therefore, it is likely that peptides bind directly to MHC class I proteins.

#### b) Possible function for polymorphism

The genes encoding the major histocompatibility complex class I molecules are the most polymorphic loci known (Klein, 1987). Reasons for this high level of polymorphism remain obscure although studies with inbred mice have shown that certain class I molecules are better at presenting certain antigens than others. This implies polymorphism within the major histocompatibility complex allows for a greater chance of survival due to the larger number of antigens that can be presented to the immune system. In the mouse, for example, H-2Kk is known to be associated with a very high immune response to poxvirus a weak response to lymphocytic choriomeningitis virus. In contrast, presentation of the lymphocytic choriomeningitis virus in the context of H-2Dk yields a good immune response whilst there is no measurable response to poxvirus in the context of H-2KK (Zinkernagel, 1979). In addition, the influenza matrix peptide and nucleoprotein peptide have been shown to induce influenza specific CTL by binding to HLA-A2 and HLA-B37 respectively (Gotch et al, 1987a,b; Townsend et al, 1986). Other HLA alleles that cannot present these antigens to CTL bind these viral peptides at lower affinities (Chen et al, 1989). If an organism possessed only one type of class I molecule the number of foreign antigens to which the body could mount a protective immune response would be much reduced, rendering the organism more susceptible to a lethal infection. Polymorphism therefore reduces the risk of a population or an individual being a nonresponder to, for example a highly pathogenic organism.

This idea has been challenged (Klein, 1987) with the suggestion that many studies attempting to support this hypothesis have been performed under very artificial conditions and that when natural parasites are used to expose the hosts in a more natural way rather than with adjuvants, the MHC appears to have little effect on the resistance or susceptibility, even in inbred strains. In this report, Klein speculated that it is negative selection that affects the MHC molecules rather than positive selection. In other words, the mutations that occur within the MHC loci cause the antigens to become non-functional but they probably do not make them better at presenting certain types of pathogens to the immune system.

## 2.1.5. MECHANISMS FOR THE GENERATION OF POLYMORPHISM IN CLASS I GENES

Comparisons of sequences from different species has shown that the class I molecules of human, mouse, rabbit and rat are related since these sequences share many amino acid residues throughout the length of the class I polypeptide. This implies that the basic structure of class I genes has remained unchanged since the origins of placental mammals. Klein *et al.*, (1986) proposed that an ancestral class I gene was duplicated a number of times, and these duplicated genes diversified to give rise to the modern HLA class I alleles. Three different hypotheses have been suggested to explain the subsequent diversification of the MHC class I alleles: gene conversion; exon shuffling and genetic drift.

#### a) Gene conversion

Gene conversion is a non-reciprocal interaction whereby one gene interacts with another so that part or all of the nucleotide sequence of the second gene becomes identical to that of the first. This interaction can occur between related genes located anywhere in the genome such as alleles on homologous chromosomes, tandem copies of a gene on the same chromosome or tandem genes from sister chromatids. It can also take place during mitosis or meiosis (Baltimore, 1981).

Nonreciprocal gene conversion between non-allelic genes can introduce sequence homogeneity in linked genes and can generate extensive divergence and polymorphism in allelic genes (Parham et al,1988). This means that alleles from different loci, for example alleles from the HLA-A and the HLA-B loci, can undergo a gene conversion event whereby the A allele may donate sequences to the B allele so that B becomes more like A and less like other B alleles. Many studies have presented evidence in favour of gene conversion involving short nucleotide stretches as the means by which polymorphism is generated in class I molecules. For example, Weiss et al, (1983) showed that the mutant murine H-2Kb gene, H-2Kbm1, resulted from a gene conversion event. Comparison of the H-2Kb and H-2Kbm1 sequences revealed seven nucleotide changes within the H-2Kbm1 gene which result in three amino acid substitutions. These nucleotide changes are clustered in a 13-nucleotide region of the H-2K gene and are identical to that of the H-2Ld gene in the same region suggesting that H-2Ld may have donated these nucleotides to the H-2Kb gene. Therefore, non-allelic gene conversion is a likely explanation for the origin of the Kbm1 mutant. This gene conversion event occurred between non-allelic genes and between two different haplotypes implying that gene conversion can occur between different chromosomes in a heterozygous individual.

For human MHC sequences, the role of gene conversion in the generation of sequence divergence has been questioned. For this mechanism to have contributed significantly to the generation of polymorphism, the alleles from different loci would be expected to be more similar than the alleles from the same locus. Parham *et al*, (1988) have suggested that extensive sequence comparisons do not support this. Since residues that show apparent locus specificity exist in the polymorphic domains, it is thought that the exchange of sequences between the HLA-A, B and C loci has not been a major contributor to diversity at these genes. However, gene conversion does appear to have resulted in the shared residues 62-65 between HLA-A2 and HLA-Bw58 (Ways *et al*,1985) as well as residues 63-67 between HLA-B51 and HLA-B8 (Hayashi *et al*,1989).

#### b) Exon shuffling

Exon shuffling brings together the exons of different genes through intron-mediated recombination to create new genes. The parent genes can either code for proteins of a different function or for different domains of a related protein. Holmes *et al* (1985) showed by peptide mapping that the antigen HLA-Aw69 is a hybrid of HLA-A2 and Aw68. This was confirmed by gene sequence analysis which showed the region encoding the al domain is identical to the homologous region of HLA-Aw68 and the a2 and a3 coding regions of HLA-Aw69 share identity with these regions from HLA-A2.

Gene conversion differs from exon shuffling in that the former requires preexisting homology, while the recombination of exons may occur with little homology by illegitimate recombination events occurring in intervening sequences. Furthermore, if gene conversion was the event responsible for generating HLA-Aw69, at least 1 Kb of DNA would have been exchanged. This is much larger than the few nucleotides that have been previously shown to be involved. From this, it was proposed that a reciprocal intra-allelic recombination event was responsible and is localized to an 86 bp region at the exon 2 / intron 2 boundary (Holmes *et al*,1985). Estimates of the mutation rate for MHC genes suggest this recombination event took place more than 330,000 years ago.

Other examples that can be explained through an exon shuffling mechanism *in vivo* are found in the following sequences: HLA-Bw42 is identical to HLA-B7 in the a1 domain and to B8 in the a2 domain while HLA-Bw41 is identical to Bw60 in the a1 domain and has an a2 domain that is identical to B8 but for two exceptions, namely Leu-95 to Trp and Ser-97 to Arg (Parham *et al*,1988).

Parham et al (1988) have suggested that the exon shuffling mechanism may be responsible for intra-locus diversification but that gene conversion can occur between alleles of different loci.

#### c) Genetic drift or the trans-species hypothesis

The hypotheses for the generation of MHC polymorphism described above are post-species in nature since they are based on the assumption that the present day species evolved from a few individuals and therefore from a limited number of MHC alleles. In contrast to this, the trans-species model suggests that modern day species developed from a large number of individuals and therefore a correspondingly large number of alleles which were the ancestral forms of the major alleles now found in the human population. In other words, the alleles are much older than the species and the main HLA alleles existed in the primates long before humans became separate. If the alleles have existed for so long, it is conceivable that the high level of polymorphism found between the present day alleles arose from random point mutation (Klein,1987).

Evidence for the trans-species hypothesis comes from the comparison of human and chimpanzee (chLA) class I sequences (Lawlor et al,1988). Such comparisons did not reveal any species specific residues that could distinguish human alleles from those of the chimpanzee. In fact, certain human alleles seem to be more like chimpanzee alleles than other human alleles. This sequence similarity indicates that much of the present polymorphism in HLA and chLA class I alleles existed before divergence of the two species 3.7-7.7 million years ago and hence the differences between HLA-A and B alleles were present before the evolution of modern man (Lawlor et al,1988). This apparent

stability of the class I polymorphism argues against the necessity to generate a lot of polymorphism quickly by mechanisms such as gene conversion (Klein,1987)

The degree of sequence divergence between two duplicated genes is believed to be an indication of the time that has elapsed from the duplication event. Alleles that diversify only by point mutation evolve slowly whereas gene conversion allows rapid accumulation of variability to occur. While it is possible that all the ancestral forms of the modern alleles were already present at the time of species separation, it is difficult to explain the clustered variation as seen in class I molecules since polymorphism arising by random point mutation would be expected to occur at equivalent frequency along the entire length of the gene. Further, alleles at a single class I locus differ from each other by only a few residues yet a very large number of alleles exist. While gene conversion assumes that a relatively short time has elapsed since the class I loci were duplicated, this mechanism does explain how clustered variation involving a few residues could arise. Exon shuffling explains how particular alleles arose but is unlikely to be a general mechanism for the generation of MHC polymorphism.

# 2.1.6. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

#### a) Definition of RFLP

Polymorphism of the class I molecules can be detected serologically using alloantisera or monoclonal antibodies, or by reactions based on cell-mediated immunity such as allograft reaction, cell-mediated lymphocytoxicity or the mixed lymphocyte reaction. Biochemical techniques such as gel electrophoresis, peptide mapping and amino acid sequencing can be used to analyze class I polymorphism at the protein level while the gene encoding such proteins can be analyzed directly by sequencing and mapping with restriction enzymes.

Some of the allelic variations may result in the generation of restriction fragment length polymorphisms (RFLPs) which may be associated with a disease and thus be useful as a diagnostic marker. The term 'RFLP' refers to the differences in the sizes of fragments resulting from the digestion of DNA with restriction endonucleases. DNA restriction enzymes recognize specific sequences in DNA and catalyze endonucleolytic cleavage, yielding fragments of defined length which can then be separated according to molecular size by gel electrophoresis. Thus, fragments encoding specific sequences, and any variations thereof that effect their size, can be detected by Southern blot hybridization to a radiolabelled probe (Southern,1975). The different sized fragments are called RFLPs and these can arise as a result of either the creation or loss of a restriction enzyme recognition site or by the insertion or deletion of DNA. Such differences are of interest as the detected mutation may be associated with a disease by either being the direct cause itself or being linked to another gene that is. Genetic diseases such as sickle cell anaemia, cystic fibrosis and Huntington's chorea can be detected by RFLP analysis.

#### b) RFLPs of HLA-B27 and ankylosing spondylitis

Many studies suggest that the HLA-B27 molecule itself is directly involved in the development of ankylosing spondylitis. This implies that the gene encoding this antigen is in some way different in patients compared with that from healthy individuals. RFLP analysis can be used to identify any genetic rearrangement of the HLA-B27 locus that may have occurred or detect polymorphic fragments that are present or absent in patients compared with controls. Initial attempts to study HLA polymorphism using Southern hybridization and RFLP analysis were difficult as the DNA probes used showed a high level of cross-reactivity with sequences of other class I loci. The analyses have been made easier with the use of locus specific probes.

Using such probes, RFLPs have been reported for the HLA-B27 allele and attempts have been made to correlate these with AS. Trapani *et al*, (1985a,b) detected a 3.5 Kb *Taq* 1 fragment that segregated with HLA-B27. However, this fragment did not correlate with the presence of AS. Four *Taq* 1 RFLPs of 3.5 Kb, 2.5 Kb, 3.8 Kb and 4.0/8.0 Kb have been detected with a B locus specific probe derived from intron 7 of the HLA-B7 gene (Ness *et al*,1987). Of these, the 3.5 Kb fragment is the same as that seen by Trapani *et al*, (1985a,b). Although it is not yet statistically significant, the 2.5 Kb fragment was observed in normal individuals but rarely appeared in AS patients.

Probes from the 5' flanking region of the HLA-B gene also detect *Taq* 1 RFLPs. Comparison of AS patients with normal individuals did not reveal any differences in their hybridization pattern, although only three patients were analyzed (Weiss *et al*,1988).

An RFLP that appears to correlate with disease status has been identified using a full length HLA-B7 cDNA probe (McDaniel et al,1987). A Pvu II fragment of 9.2 Kb was found to be present in 73% of B27 positive AS patients compared with only 23% of controls. These differences are stastically significant. In family studies, this fragment segregated independently of HLA-B27 suggesting that this class I HLA 9.2 Kb PvuII fragment is in linkage disequilibrium with a second HLA allele which is also required for disease development.

The DNA located upstream and downstream of the HLA-B27 gene is polymorphic but no RFLP within these regions is significantly increased in AS patients compared with controls. If the 9.2 Kb Pvu II fragment is in linkage disequilibrium with a disease gene, this gene does not appear to be present within these regions.

## 2.1.7. AIM

In order to assess the association of the 9.2 Kb Pvu II fragment with ankylosing spondylitis, RFLP analysis was performed. Genomic DNA from peripheral blood lymphocytes of B27 positive ankylosing spondylitis patients as well as B27 positive and negative healthy controls was studied. Probes derived from the 3' untranslated and the 3' flanking regions of an HLA-B27 gene from a normal individual were used to search for disease associated RFLPs with the enzymes Pvu II, Eco R1 and HindIII. Two Caucasian populations were analyzed, namely English and Polish, in order to eliminate any influence that the choice of population may have.

#### 2.2. RESULTS

Using probes derived from a HLA-B27 gene and cDNA of normal haplotype (Weiss *et al*,1985; Szots *et al*,1986), genomic DNA from English and Polish AS patients and healthy individuals were digested with the restriction enzymes *Pvu* II, *Eco* R1 and *Hin* dIII and analyzed for any RFLP that might associate with the disease.

# 2.2.1. RFLP ANALYSIS OF Pvu II DIGESTED HUMAN DNA

#### a) Hybridization with the full length HLA-B27 cDNA probe

Blots of PvuII digested genomic DNA show at low stringency between 18-20 bands when hybridized to a full length HLA-B27 cDNA probe: the majority of these bands are derived from either MHC class I genes which have some sequence homology with HLA-B27. As shown in figure 9 increasing the stringency of hybridization causes many of these bands to disappear partly or completely due to limited sequence homology with this probe. While the hybridization pattern is complex, very little polymorphic variation is observed between individuals.

#### b) Hybridization with the pB27-5' probe

Since the 5' sequence shows a greater inter-allelic sequence homology as discussed previously, hybridization of the pB27-5' probe resulted in a complex hybridization pattern (figure 10). The pattern seen following hybridization with pB27-5' was similar to that seen with the full length cDNA probe except that no fragments below 2.6 Kbp were observed. The frequencies at which each band appears in each group studied was determined in order to find fragments that correlate with the presence of the disease (table 8).

A Pvu II fragment of 14 Kbp that hybridized with pB27-5' was more common in English HLA-B27+ AS patients than in HLA-B27+ healthy individuals (table 8). The occurrence of this RFLP was significantly elevated in this patient group with 82% being carriers compared with 29% of the HLA-B27+AS- control subjects (p<0.01). This fragment, however, was absent from the HLA-B27-AS+ individual suggesting that the presence of this fragment increases the risk of developing AS in English HLA-B27+ individuals. This was not observed for the Polish population.

In contrast, a *Pvu* II fragment of 8.9 Kbp occurred more frequently in English HLA-B27<sup>+</sup> healthy individuals than in HLA-B27<sup>+</sup> patients (table 8). This result is statistically significant with p < 0.01. This would appear to be a negative correlation, the absence of this fragment being associated with increased risk of disease development in HLA-B27<sup>+</sup> individuals. A similar observation was made for the Polish group with the 8.9 Kbp fragment appearing over 3 times as often in the HLA-B27 positive controls than in the patient group: while a trend can be seen for this group, the number of controls is too small for statistical analysis. This fragment was also observed in the only HLA-B27 negative patient DNA studied (figure 10).

The 4.5 Kbp (p<0.01) and 3.7 Kbp (p<0.001) Pvu II RFLPs were significantly more common in HLA-B27 negative (70% and 67% respectively) than HLA-B27 positive (36% and 23% respectively) individuals (table 8). This suggests that HLA-B27 is not contained within Pvu II fragments of these sizes. Further, the 3.7 Kbp Pvu II fragment is also more frequent in healthy subjects (64%) than AS patients (16%) (p<0.001) indicating that this fragment is not related in any way to the disease.

When the incidence of uveitis was considered among English patients, it was found that the 3.7 Kbp Pvu II fragment was significantly more frequent in those patients with uveitis (36%) than in those without (5%) (table 9). The 8.9 Kbp Pvu II fragment was present to the same extent in patients with and without uveitis. The incidence of uveitis among the Polish patients was unknown.

#### c) Hybridization with pB27-3'UT

Hybridization of *Pvu* II digested genomic DNA with probes prepared from the 3'UT region of the HLA-B27 locus resulted in a less complex pattern of hybridization (figure 11). This is in agreement with the belief that probes derived from the 3' region of class I genes show locus specificity under high stringency hybridization conditions (Koller *et al.*,1984).

The 3'UT probe detected a different pattern of hybridizing *PvuII* fragments to that detected by the 5' region probe. None of the bands appear to be shared between the two probes. In particular, the 8.9 Kbp fragment detected with the full length and 5' cDNA probes does not hybridize with the 3'UT probe.

Hybridization at low stringency with the pB27-3'UT probe revealed fragments of the sizes indicated in figure 11a. Of these only the 550 bp hybridization signal remained intense following high stringency washing. The fragments of 1080 bp, 1500 bp, 1800 bp, 2400 bp, 2800 bp, and 3400 bp remained bound by the probe at high stringency (figure 11b) but the intensities of these bands were much reduced indicating that these are homologous sequences rather than HLA-B locus 3'UT sequences.

No fragments that hybridized with the pB27-3'UT probe were found to be significantly elevated in any of the groups studied from either the English or Polish populations (table 10).

#### d) Hybridization with pB27-3'FR;

The pB27-3FR probe detected yet another set of hybridizing *Pvu* II fragments not shared with any of the other probes. At low stringency, fragments of 8.0 Kbp, 3.5 Kbp, 2.9 Kbp, 2.6 Kbp, 2.4 Kbp, 1.5 Kbp, and 1.2 Kbp were detected (figure 12a). Increasing the stringency removed the 8.0 Kbp and 2.6 Kbp fragments (figure 12b).

The incidence of the 2.4 Kbp band was found to be significantly increased in English HLA-B27 negative healthy individuals (84%) when compared with HLA-B27 positive subjects irrespective of their disease status (51%) (p<0.001) (table 11). This fragment is clearly not a marker for the disease but appears to be associated with the absence of the HLA-B27 allele.

No fragments hybridizing to the pB27-3FR probe was more common in either the patient or control groups in the Polish population.

# 2.2.3. RFLP ANALYSIS OF *Eco* R1 AND *Hin* d III DIGESTED HUMAN DNA

#### a) Analysis of EcoR1 digested DNA

Hybridization at low stringency of *Eco* R1 digested patient and control DNA with probes specific for the 3' region of the HLA-B27 locus revealed bands of the sizes indicated in figure 13. Of these, only the bands of 7.9 Kbp, 7.6 Kbp, 6.9 Kbp and 6.6 Kbp remained bound by the probes following high stringency washing. The 6.9 Kbp and 6.5 Kbp fragments contain entire coding sequences of HLA-B genes while the 7.9 and 7.3 Kbp bands encode HLA-C alleles (Gussow *et al*, 1987).

As these cross hybridizing alleles are contained within such fragment sizes, hybridization with the pB27-3'UT and pB27-3'FR probes were expected to give the same hybridization patterns. This was indeed observed (data not shown). Although these bands show a low level of polymorphism, none are associated with AS.

#### b) Analysis of Hin dIII digested DNA

Hybridization of *Hin* dIII digested patient and control DNA with the full length HLA-B27 cDNA (figure 15) and pB27-3FR (figure 14) resulted in a complex pattern of fragments. None of the polymorphic bands appeared more frequently in any of the patient and control groups studied.

#### 2.3. DISCUSSION

RFLP studies using the restriction endonucleases *PvuII*, *EcoR1* and *HindIII* were performed to search for differences that will distinguish the HLA-B27 locus of AS patients from that of normal individuals in English and Polish populations. Although the enzymes *EcoR1* and *HindIII* showed polymorphic patterns of hybridization with 3' region and full length cDNA probes, no fragments appeared to be associated with the presence or absence of disease. As McDaniel *et al* (1987) had previously shown an association between the presence of a 9.2 Kbp *PvuII* RFLP with AS, more extensive analysis of *PvuII* digested genomic DNA was carried out using full length cDNA as well as 5' and 3' region probes.

Hybridization of *PvuII* digested genomic DNA with a full length and 5' region class I cDNA probes resulted in complex patterns of fragments which was identical to that obtained by Orr *et al* (1983b). Since the 5' region of class I genes display a high level of polymorphism, it was expected that any probe encompassing this region would cross hybridize with allelic forms of other class I genes. In contrast, the 3' region is less polymorphic and sequences derived from this region are believed to show locus specificity (Koller *et al*,1984). In agreement with this, the hybridization patterns detected using the pB27-3'UT and pB27-3'FR probes were less complicated than that seen with the 5' region probe.

Using these probes, several polymorphic bands were observed at a different frequency between AS patients and healthy individuals. A 14 Kbp Pvu II fragment which hybridized with the pB27-5' probe appeared to be disease related since it was significantly more common in HLA-B27<sup>+</sup> AS<sup>+</sup> patients than HLA-B27<sup>+</sup> healthy controls. However, the intensity of this hybridizing fragment was quite low indicating that it is a sequence that is merely related to the 5' region of the HLA-B locus. This fragment was absent from the HLA-B27<sup>-</sup>AS patient, suggesting that it may be in linkage disequilibrium with the HLA-B27 allele and the risk of developing AS is increased in HLA-B27<sup>+</sup> individuals only.

A Pvu II fragment of 8.9 Kbp detected using the full length and 5' region cDNA probes was increased in HLA-B27 positive healthy individuals when compared with HLA-B27 positive patients. This was found for both English and Polish populations. This 8.9 Kbp Pvu II band therefore appears to be a marker for those HLA-B27 positive individuals who will not develop AS. Since this 8.9 Kbp Pvu II fragment was also observed in the HLA-B27- AS+ individual, it cannot be HLA-B27. As this 8.9 Kbp fragment was not detected with either the 3'UT or the 3'FR probes, it is neither homologous nor contiguous with the 3'UT sequences of the HLA-B locus. It may, however, be positioned to the 5' side of these probes or it may be from another gene.

While a similar correlation was observed for the 8.9 Kbp Pvu II RFLP in both English and Polish populations, this fragment occurs at a different frequency within the English AS patient group (28%) compared with Polish patients (15%). Other RFLPs were also observed at different frequencies within each population, however, it is unlikely that the presence or absence of any of these fragments is indicative of a disease difference but rather reflects a population difference.

McDaniel et al, (1987) reported a 9.2 Kb Pvu II DNA fragment that appeared to confer increased risk for AS in HLA-B27 positive individuals. While no Pvu II fragment of 9.2 Kbp was

observed to cross-hybridize with any of the probes used, it is however, very possible that the 8.9 Kbp Pvu II fragment described above is identical to the 9.2 Kbp fragment described by McDaniel et al. This fragment was found by McDaniel and coworkers to hybridize to both a full length B7 cDNA probe and the supposedly HLA-B locus specific probe pHLA-1.1 under high stringency conditions. The hybridization pattern observed by McDaniel and coworkers using the full length cDNA probe was similar to that presented here and in other studies (Orr et al,1983; Ahearn et al,1989). However, hybridization with the pHLA-1.1 probe as reported by this group differs markedly from the pattern observed with pB27-3 UT shown here as well as the from the hybridization patterns obtained by others (Orr et al,1983b; Ahearn et al,1989).

The pHLA-1.1 probe is believed to be derived from the 3'UT region of a HLA-B gene since it shows a high level of sequence homology with the equivalent region of the HLA-B7 mRNA (Ploegh et al,1980; Koller et al,1984). The Pvu II fragments bound by this probe as observed by McDaniel et al are not consistent with those that bind pB27-3'UT. In particular, the 4.2 and 5.2 Kbp bands claimed by McDaniel's group to hybridize with pHLA-1.1 at high stringency do not hybridize with pB27-3'UT even under low stringency conditions. Furthermore, hybridization of the 550 bp Pvu II fragment which represents self hybridization of the probe was not observed by McDaniel's group, making it impossible to gauge the relative intensities of the bands they reported. Hybridization with pB27-3'UT at high stringency resulted in a very strong signal from the 550 bp band and very weak intensities of the remaining fragments. Even if these blots were over exposed, a pattern similar to that seen by McDaniel would not be observed.

The identity of the 3'UT region probe used here was confirmed by sequencing, yet it does not have a similar hybridization pattern to pHLA-1.1. The differences in sequence between the pHLA-1.1 and pB27-3'UT probes could not be sufficient to explain the different hybridization patterns. Since pHLA-1.1 is a cDNA clone, it represents an expressed gene. It is likely to be a HLA-B locus sequence since it displays a high level of homology to the HLA-B7 mRNA and is therefore unlikely to have been rearranged. While the probes used here have been well characterized, the identity of the pHLA-1.1 probe was not confirmed by McDaniel *et al* (1987). Indeed, the hybridization pattern observed by other groups using the pHLA-1.1 probe do not agree with that seen McDaniel *et al* (Orr *et al*,1983b; Ahearn *et al*,1989). These patterns are, however, consistent with that seen with pB27-3'UT. It is possible therefore that pHLA-1.1 as used by McDaniel and coworkers is not a HLA-B locus specific probe.

Several studies using different populations have been performed attempting to confirm the original association of the 9.2 Kbp Pvu II RFLP with AS (McDaniel et al,1987). In contrast to the negative correlation reported here for English and Polish populations, Weiss and coworkers (1988) reported that the 9.2 Kbp Pvu II fragment was present in all the German individuals analyzed when probed with a PstI fragment derived from the 3' end of the HLA-B27 cDNA clone pB1. The probe used by this group contains more coding sequence than pB27-3'UT, therefore, while pB27-3'UT does not detect this fragment, it is possible that the probe used by Weiss et al (1988) does. Two other American studies including subjects from Baltimore (Ahearn et al,1989) and Minnesota/Texas (Durand et al,1988) have been studied in addition to that from Alabama analyzed by McDaniel and coworkers. No

association could be found between the 9.2 Kbp Pvu II fragment and the presence of AS in subjects in these additional studies.

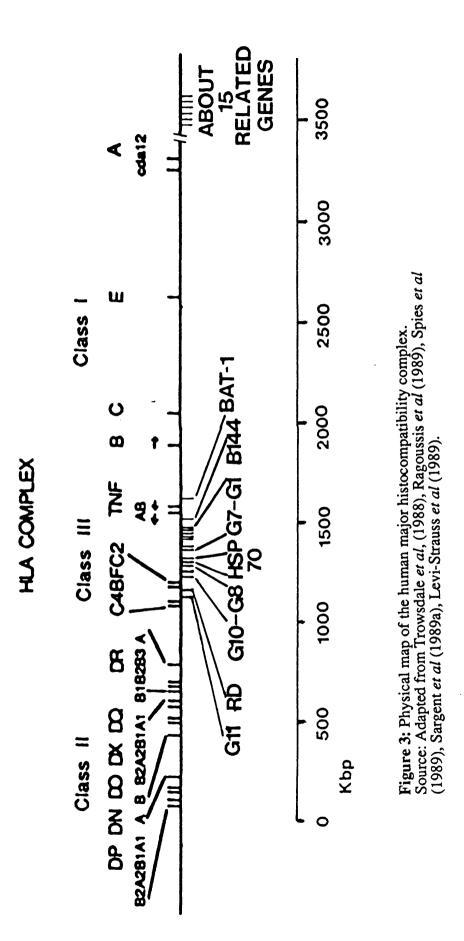
Since this 9.2 Kbp Pvu II fragment is clearly not derived from a HLA-B27 sequence, it is possible that these sequences are in linkage disequilibrium. Aheam et al (1989) analyzed their subjects' tissue types and were able to show linkage of this fragment with the HLA-A3 and HLA-A9 alleles. In addition, reanalysis by this group of the results presented by McDaniel et al were consistent with this finding. For the subjects whose tissue types were known in this study, those who carried the 8.9 Kbp Pvu II RFLP were also positive for either HLA-A3 or A9. There were some individuals who had this fragment but whose tissue type was unknown. In addition, one individual did not have this fragment but was positive for HLA-A3/A9. While Durand et al (1988) have been able to confirm this linkage in their subjects from Minnesota/Texas, more extensive tissue typing would need to be carried out to confirm such linkage for the English and Polish populations studied here. Since a negative correlation has been observed between the presence of this fragment and AS, such a correlation would therefore appear to exist between HLA-A3/A9 and AS in English and Polish populations.

It is possible that the inconsistencies in these studies is due to differences in the populations studied. For example, it is possible that the entire German population have a particular haplotype carried on the 8.9 Kbp Pvu II fragment that is not as common in HLA-B27 positive AS patients living in the United Kingdom or Poland. In addition, this particular haplotype may be uncommon in HLA-B27+AS- individuals in Alabama. It is difficult to evaluate the population differences since the hybridization patterns of Weiss et al have not been published and the identity of the probes used by McDaniel et al is questionable.

Association of this *Pvu* II RFLP with the HLA-A3/A9 alleles suggests that class I genes other than HLA-B27 may also be markers for AS. It is possible that individuals of a particular extended haplotype will be at greatest risk of developing AS. More extensive tissue typing would need to be done in order to determine if this is so.

Using a full length HLA-B27 cDNA and region specific probes, no confirmation of the association of a 9.2 Kbp *Pvu* II RFLP with AS has been made. Indeed, a negative correlation has been observed between a 8.9 Kbp *Pvu* II RFLP and this disease.

## 2.4. FIGURES AND TABLES



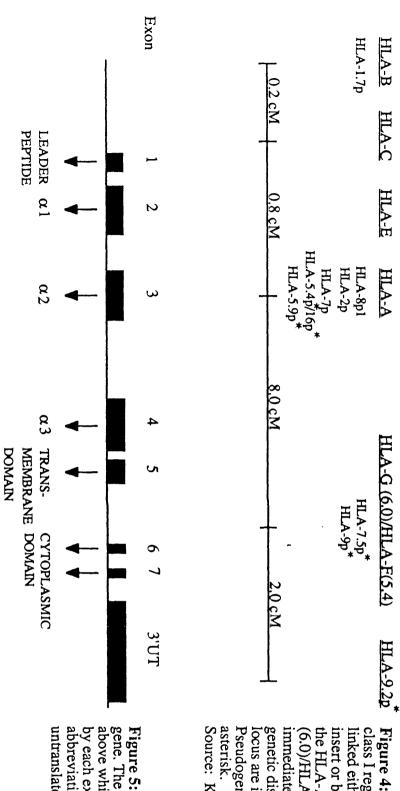


Figure 4: Physical map of the HLA class I region. HLA class I loci linked either within the same cosmid insert or by overlapping cosmids to the HLA-A, HLA-B or HLA-G (6.0)/HLA-F (5.4) genes are indicated immediately below each locus. The genetic distances between each marker locus are indicated in centimorgans (cM). Pseudogenes are indicated with an asterisk.

Source: Koller et al, (1989)

Figure 5: Structure of a human class I gene. The exon number is indicated above while the protein domain encoded by each exon is indicated below. The abbreviation 3'UT indicates the 3' untranslated region.

Table 6: Distinct antigenic specificities detected serologically at each HLA class I locus. Source: Roitt et al (1985).

HI	LA-B	HLA-C	HLA-A
Bw4	Bw47	Cw1	A1
B5	Bw48	Cw2	A2
Bw6	B49	Cw3	A3
B7	Bw50	Cw4	A9
B8	B51	Cw5	A10
B12	Bw52	Cw6	A11
B13	Bw53	Cw7	Aw19
B14	Bw54	Cw8	A23
B15	Bw55		A24
B16	Bw56		A25
B17	Bw57		A26
B18	Bw58	-	A28
B21	Bw59		A29
Bw22	Bw60		A30
B27	Bw61		A31
B35	Bw62		A32
B37	Bw63		Aw33
B38	Bw64		Aw34
B39	Bw65		Aw36
B40	Bw67		Aw43
Bw41	Bw70		- Aw66
Bw42	Bw71		Aw68
B44	Bw72		Aw69
Bw46			

Table 7: Sequence comparison of class I genes

LE	LENGTH				% DIVER	RGENCE	
HLA-B27	В7	A 2	B27/B7	B27/A2	A2/A3	H-2Kb/H-2Kd	H-2Kb/H-2Ld
517	507	526	8.1	10.2	10.6	3.9	25.
73	73	73	10.9	15.0	4.1	7.8	10.9
129	123	129	9.3	12.4	10.9	3.7	24
270	270	270	5.2	10.7	3.0	10.3	11.1
241	245	240	5.4	9.5	3.3	4.3	13.5
276	276	276	6.1	8.3	4.7	11.2	12.7
575	575	599	1.0	15.8	7.7	4.0	9.7
276	276	276	0.4	10.1	4.0	6.1	4.0
92	92	97	1.1	20.6	3.1	4.7	5.5
117	117	117	0.8	14.5	2.6	5.8	13.3
442	442	436	2.2	14.5	5.0	3.9	5.0
33	33	33	0	12.2	3.0	6.0	0
106	106	142	1.9	16.9	2.1	2.3	4.6
48	48	48	0	12.5	2.1	2.6	10.2
182	182	169	2.7	23	4.7	1.8	9.0
424	422	405	21.8	z	0.5	5.0	11.6
209	209	160	1.9	귎	5.5	4.0	z
	424	424 422 209 209		422	422 405 21.8 209 160 1.9	422 405 21.8 209 160 1.9	422 405 21.8 nd 209 160 1.9 nd

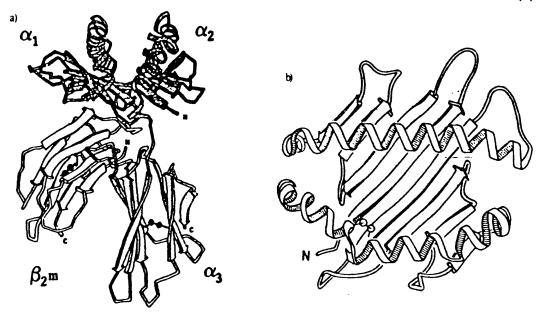


Figure 6: a) Schematic representation of the side view of the HLA-A2 class I molecule.

b) The top view of the HLA-A2 molecule as seen by the T cell receptor Source: Bjorkman et al (1987a)

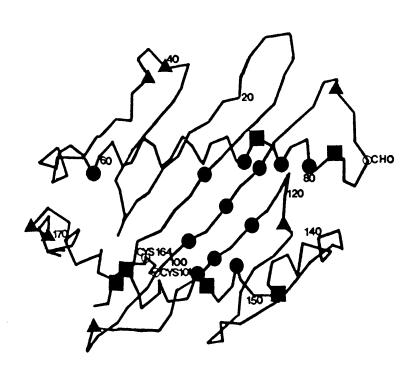


Figure 7: Mutations at the residues indicated have been shown to effect antigen binding (a), T cell receptor binding (a), or have no effect due to position outside recognition site (a).

Source: Bjorkman et al (1987a).

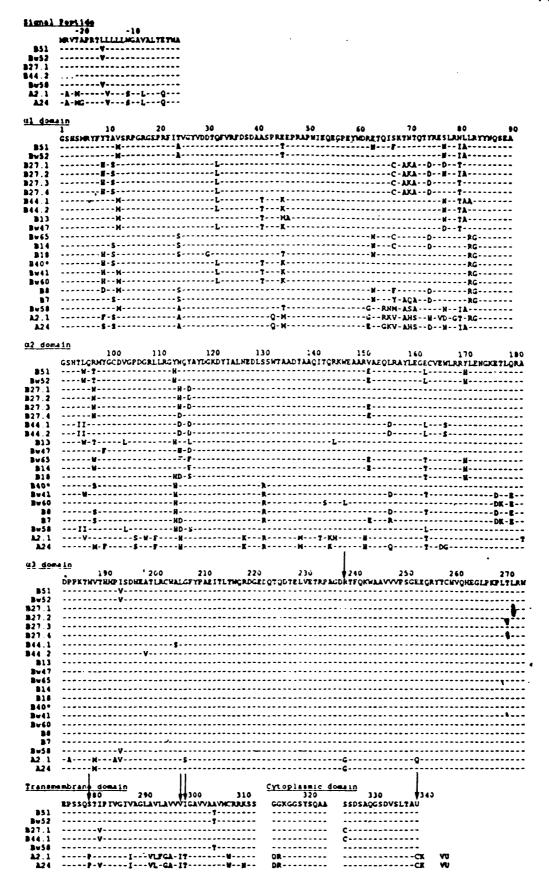


Figure 8: Sequence comparison of HLA-B antigens. The HLA-B specific residues as discussed in the text are indicated with arrows. Source: Parham et al (1988)

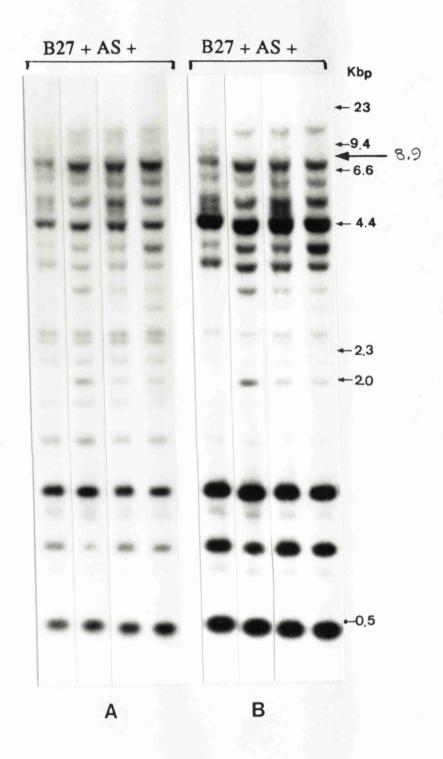


Figure 9: Autoradiographs of Pvu II digested genomic DNA from 4 HLA-B27+AS+ Polish subjects hybridized with the full length HLA-B27 cDNA probe. DNA was electrophoresed through 0.8% agarose TBE gels at 45V for 17 hours and transferred to Hybond-N. Following hybridization, the blot was washed with (a) 2xSSC/0.5% SDS and (b) 0.1xSSC at  $65^{\circ}C$  for 1 hour and exposed against X-ray film for (a) 3 days and (b) 7 days. The position of the 8.9 Kbp RFLP is indicated with an arrow. The sizes of the  $\lambda$  *Hin*dIII markers are indicated on the right.

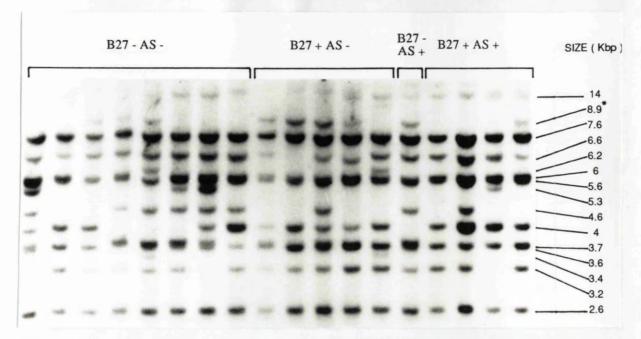


Figure 10: Autoradiograph of *Pvu* II digested genomic DNA from 8 HLA-B27<sup>-</sup>AS<sup>-</sup>, 5 HLA-B27<sup>+</sup>AS<sup>-</sup>, 1 HLA-B27<sup>-</sup>AS<sup>+</sup> and 4 HLA-B27<sup>+</sup>AS<sup>+</sup> English subjects hybridized with the pB27-5' probe. DNA was electrophoresed through 0.8% TAE agarose at 60V for 17 hours before Southern transfer. The Southern blot was washed at 0.1xSSC/0.5% SDS 65<sup>0</sup>C following hybridization and exposed for 2 days. The sizes of each hybridizing fragment are indicated on the right hand side. The 8.9 Kbp *PvuII* RFLP is indicated with an asterisk.

			ENGLISH			POLISH	
	SIZE (Kbp)	B27+ AS+ ( n = 34 )	B27+ AS- ( n = 7 )	B27- AS- ( n = 25 )	B27+ AS+ ( n = 20 )	B27+ AS- ( n = 4 )	B27- AS- ( n = 9 )
100	14	82	29	72	70	75	78
1	8.9*	28	86	35	15	50	44
S. 100 TO	7.6	100	100	100	100	100	100
	6.6	100	100	96	100	100	100
	6.2	12	43	8	10	0	0
	6	36	29	68	15	25	22
	5.6	76	71	76	85	100	100
	5.3	30	0	39	30	25	44
-	4.6	38	14	70	35	50	44
	4	94	100	76	95	75	78
	3.7	16	57	67	55	50	33
-	3.6	94	86	76	75	100	67
	3.4	9	14	14	0	0	0
	3.2	91	86	81	95	100	89
	2.6	100	100	100	100	100	100

**Table 8:** Frequencies at which *Pvu* II fragments hybridizing to the pB27-5' probe appeared in each group within both English and Polish populations. The 8.9Kbp fragment is indicated with an asterisk.

Table 9: Frequencies at which each pB27-5' hybridizing fragment appears in those English patients with and without uveitis. The abbreviation NS indicates that the difference between the two groups in not statistically significant

FRAGMENT SIZE (Kbp)	% WITH UVEITIS (n=14)	% WITHOUT UVEITIS (n=20)	p VALUE
14.0	64	<b>93</b>	NS
8.9	<b>- 36</b>	28	NS
7.6	100	100	NS
6.6	100	100	NS
6.2	18	22	NS
6.0	22	10	NS
5.6	91	93	NS
5.3	18	36	NS
4.6	46	39	NS
4.0	82	90	NS
3.7	36	5	p<0.05
3.6	82	100	NS
3.4	0	15	NS
3.2	100	90	NS
2.6	100	100	NS

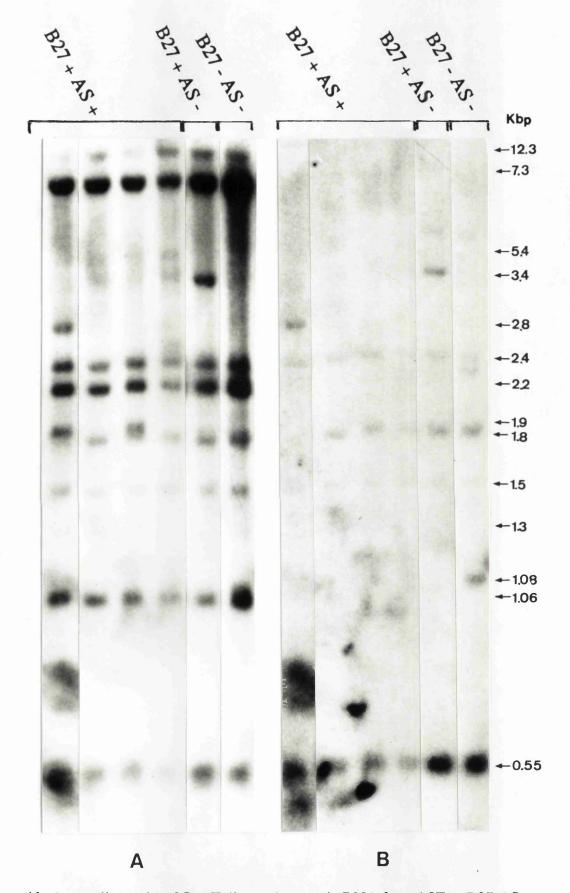


Figure 11: Autoradiographs of *Pvu* II digested genomic DNA from 1 HLA-B27-AS-, 1 HLA-B27+AS- and 4 HLA-B27+AS+ English subjects hybridized with the pB27-3'UT probe. DNA was electrophoresed through 0.8% TBE agarose at 45V for 17 hours before Southern transfer. The Southern blot was washed at (a) 2xSSC/0.5% SDS and (b) 0.1xSSC/0.5% SDS 65<sup>0</sup>C for 1 hour each following hybridization and exposed for (a) 5 days and (b) 10 days. The sizes of each hybridizing fragment are indicated on the right.

nts hybridizing to the pB27-3'UT probe appeared in each group in each population. The Table 10: Freq asterisks indica

FRAGMENT SIZE (Kbp)		ENGLISH			POLISH	
` •	HLA-B27+	HLA-B27+	HLA-B27-	HLA-B27+	HLA-B27+	HLA-B27-
	(n=24)	(n=7)			(n=4)	(n=7)
2.3	100	100	100	100	100	100
.3	100	100	100	100	100	100
4.	17	0	4.8	9	25	0
*4.	25	53	14	11	0	0
*∞:	∞	14	9.5	9	25	14
*4.	100	100	100	100	100	100
2.	100	100	100	100	100	100
6:	33	14	19	28	0	0
*&:	92	98	95	83	100	100
.5*	100	100	100	100	100	100
1.3	100	100	100	100	100	100
*80	∞	29	19	11	0	0
.02	100	100	100	100	100	100
.55*	100	100	100	901	100	100

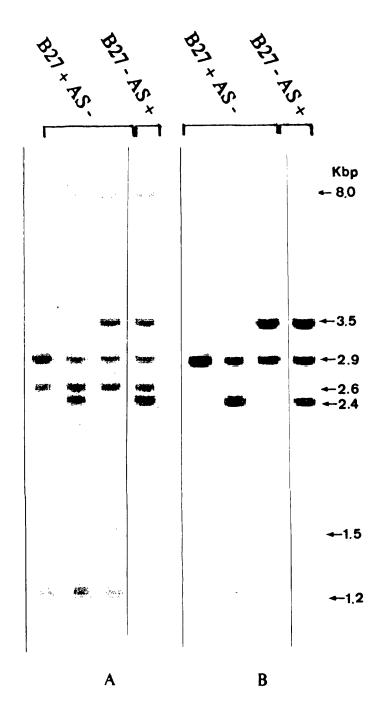


Figure 12: Autoradiographs of *Pvu* II digested genomic DNA from 1 HLA-B27<sup>-</sup>AS<sup>+</sup> and 3 HLA-B27<sup>+</sup>AS<sup>-</sup> English subjects hybridized with the pB27-3<sup>-</sup>FR probe. DNA was electrophoresed through 0.8% TAE agarose at 60V for 17 hours before Southern transfer. The Southern blot was washed at (a) 2xSSC/0.5% SDS and (b) 0.1xSSC/0.5% SDS 65<sup>0</sup>C for 1 hour each following hybridization and exposed (a) overnight and (b) for 5 days. The sizes of each hybridizing fragment are indicated on the right.

Table 11: Frequencies at which Pvu II fragments hybridizing to the pB27-3'FR probe appeared in each group within both English and Polish populations. The asterisks indicate the fragment remaining after high stringency washing. The statistical significance of each fragment is indicated either as a p value or as not significant (NS).

	PVALUE	NS	SN	SN	SN	SN	SZ	SN
	HLA-B27- AS- (n=9)	100	0	. 68	<b>1</b> 0	29	78	100
POLISH	HLA-B27+ AS- (n=4)	100	0	100	100	25	75	75
	HLA-B27+ AS+ (n=20)	100	10	901	100	09	100	100
	p VALUE	NS	NS	SN	NS	p<0.01	NS	SN
	HLA-B27- AS- (n=25),	100	11	88	9/	84	88	88
ENGLISH	HLA-B27+ AS- (n=7)	100	14.3	98	100	29	100	100
	HLA-B27+ AS+ (n=27)	100	4	95	95	<b>S</b> 6	100	100
FRAGMENT	SIZE (NOP)	8.0	3.5*	2.9*	2.6	2.4*	1.5*	1.2*

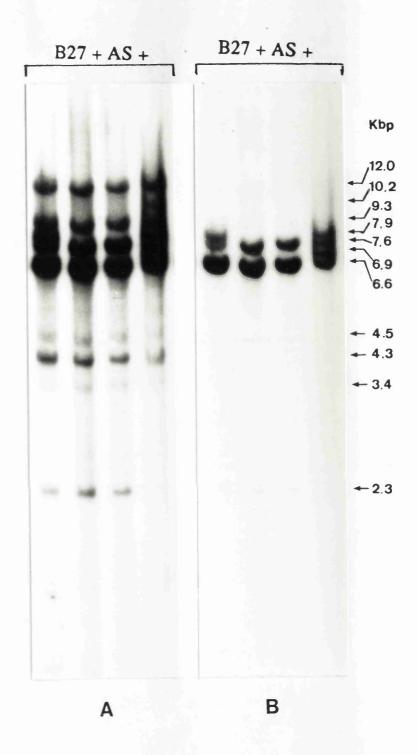


Figure 13: Autoradiographs of *Eco*R1 digested genomic DNA from 4 HLA-B27+AS+ Polish subjects hybridized with pB27-3'FR. DNA was electrophoresed through 0.8% TAE agarose at 60V for 17 hours before Southern transfer. The Southern blot was washed at (a) 2xSSC/0.5% SDS and (b) 0.1xSSC/0.5% SDS 65<sup>0</sup>C for 1 hour each following hybridization and exposed for (a) 5 days and (b) 7 days. The sizes of each hybridizing fragment are indicated on the right.

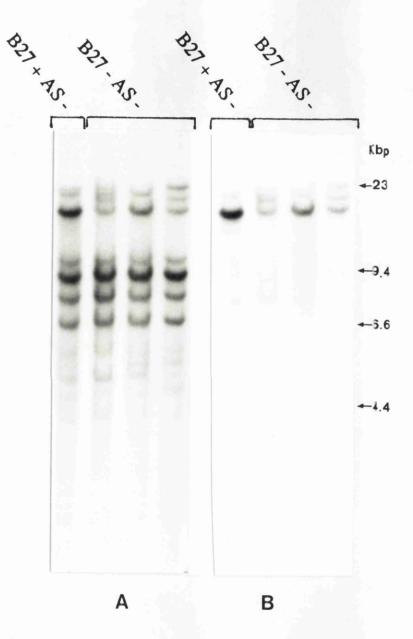
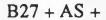


Figure 14: Autoradiographs of *Hin*dIII digested genomic DNA from 1 HLA-B27+AS<sup>-</sup> and 3 HLA-B27-AS<sup>-</sup> Polish subjects hybridized with pB27-3FR. DNA was electrophoresed through 0.8% TAE agarose at 60V for 17 hours before Southern transfer. The Southern blot was washed at (a) 2xSSC/0.5% SDS and (b) 0.1xSSC/0.5% SDS 65<sup>0</sup>C for 1 hour each following hybridization and exposed for (a) 5 days and (b) 7 days. The sizes of the  $\lambda$  *Hin*dIII markers are indicated on the right.



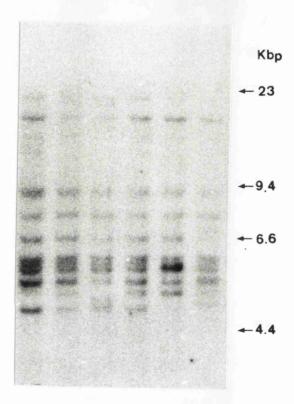


Figure 15: Autoradiograph of *Hind*III digested genomic DNA from 6 HLA-B27+AS+ English subjects hybridized with the full length HLA-B27 cDNA probe. DNA was electrophoresed through 0.8% TAE agarose at 60V for 17 hours before Southern transfer. The Southern blot was washed at 0.1xSSC/0.5% SDS 65 $^{\circ}$ C for 1 hour following hybridization and exposed for 5 days. The sizes of the  $\lambda$  *Hin*dIII markers are indicated on the right.

## CHAPTER 3

## RESTRICTION FRAGMENT

## LENGTH POLYMORPHISM

**ANALYSIS** 

OF THE

TNF-α GENE

### 3.1. INTRODUCTION

#### 3.1.1. TNF- $\alpha$ AS A CYTOKINE

Cytokines are non-antibody proteins released by activated non-lymphocytic cells such as macrophages, keratinocytes, fibroblasts and transformed cell lines. These molecules have a role in the communication between cells during viral infection, inflammation, immunity, and haematopoiesis and are similar to the lymphokines that are produced by mitogen or antigen activated lymphocytes since they also affect the growth and mobility of leukocytes (Taniguchi,1988). The cytokines include interleukins, interferons, lymphotoxin (TNF- $\beta$ ) and cachectin or tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ).

#### 3.1.2. STRUCTURE AND SECRETION OF TNF- $\alpha$

#### a) General structure of TNF-α

The sequence encoding human TNF- $\alpha$  is a single copy gene comprised of four exons and contained within a 2.9 Kb Eco R1 fragment (Nedwin et al,1985; Shirai et al,1986). The lengths of the primary transcript and the mature mRNA are 2762 and 1672 bases respectively (Pennica et al,1984) and encode an unglycosylated 157 amino acid polypeptide of relative molecular mass (Mr) 17,350 (Jones et al,1989). The amino acid sequence is highly conserved between species, hence the lack of species specificity (Kioussis et al,1987), and also shows homology with TNF- $\beta$  (Pennica et al,1984). The active form of the TNF- $\alpha$  protein is thought to be trimeric and binds to a glycoprotein receptor of Mr 75 K (Jones et al,1989).

#### b) Chromosomal localization of TNF-α

Both the human and mouse TNF- $\alpha$  and TNF- $\beta$  genes are tandemly arranged within a region of about 7 Kb and separated by about 1.2 Kb (Nedospasov *et al*,1986) (figure 16). Using specific probes, these genes have been localized to the MHC by *in situ* hybridization to metaphase chromosomes (Spies *et al*,1986) and Southern hybridization of human-murine somatic cell hybrids (Nedwin *et al*,1985).

The TNF genes have been linked to the class III region and the HLA-B locus by pulse field gel electrophoresis (Inoko et al,1987; Carroll et al,1987; Ragoussis et al,1988). From these studies, the distance from TNF to the class III region is estimated to be 300-400 Kb while the distance between TNF and the HLA-B locus is about 210 Kb (Spies et al, 1989).

Pulse field gel electrophoresis was also used to determine the orientation of the two TNF genes within the human MHC (Carroll *et al*,1987). A probe for the 5' end of TNF- $\beta$  was linked to the HLA-B locus whereas a probe for the 3' end of the TNF- $\alpha$  gene was linked to the class III region. Therefore the order of these genes is TNF- $\alpha$  (3' $\rightarrow$ 5'), TNF- $\beta$  (3' $\rightarrow$ 5') and HLA-B (5' $\rightarrow$ 3') (figure 15).

#### c) Comparison with TNF-β

The TNF- $\alpha$  and TNF- $\beta$  genes share 35% homology in the putative promoter region, but little homology exists in the first three exons and the introns (Nedwin *et al*,1985). The overall amino acid sequence homology of the secreted forms of TNF- $\alpha$  and TNF- $\beta$  is 30% (Pennica *et al*,1984). The highest degree of nucleotide sequence homology (56%) exists within the fourth exon (Nedwin *et al*,1985). The biological activities of these molecules reside within the region encoded by this exon which correlates with TNF- $\alpha$  and TNF- $\beta$  sharing many of their activities (Kriegler *et al*,1988).

While these molecules have similar biological activities, they are produced by different cell types and have distinct induction kinetics (Nedwin *et al*,1985). TNF- $\alpha$  mRNA can be detected within 2 hours of mitogenic stimulation with the product being secreted from monocytes after about 4-24 hours whereas TNF- $\beta$  mRNA is detectable in T cells only after 8 hours and secretion of the protein after 24-48 hours (Nedwin *et al*,1985).

#### 3.1.3. BIOLOGICAL ACTIVITIES OF TNF-α

TNF- $\alpha$  and TNF- $\beta$  are structurally similar polypeptides that share a common receptor as well as many biological activities (Aggarwal *et al*,1985) but which are produced by different cells, TNF- $\alpha$  is expressed by activated macrophages whilst mitogen activated T lymphocytes produce TNF- $\beta$  (Old,1985; Beutler *et al*,1985). TNF- $\alpha$  has been shown to have a wide range of activities and has been implicated in the pathogenesis of cachexia, rheumatoid arthritis and inflammatory tissue destruction.

#### a) Stimulation of collagenase synthesis by TNF- $\alpha$

TNF- $\alpha$  may play a significant role in the pathogenesis of inflammatory diseases of joints and other tissues. Bertolini *et al*, (1986) showed that purified recombinant TNF- $\alpha$  initiates bone resorption by activated osteoclasts, as assayed by the release of incorporated <sup>45</sup>Ca. Incorporation of tritiated proline into collagenase digestible protein showed that TNF- $\alpha$  also stimulated the production of collagenase, thus inhibiting the synthesis of new bone collagen. This is further supported by the finding of decreased levels of alkaline phosphatase, a marker enzyme for the formation of new bone, in osteoblasts treated with TNF- $\alpha$  (Bertolini *et al*,1986).

TNF-α also enhance the production of collagenase and prostaglandin E<sub>2</sub> by isolated human dermal fibroblasts and rheumatoid synovial cells (Dayer *et al*,1985) as well as the degradation of articular cartilage *in vitro* (Saklavata,1986). Collagenase disrupts the extracellular collagen matrix in inflamed tissues while prostaglandin E<sub>2</sub> induces bone resorption by osteoclasts during inflammation as well as the production of intracellular proteases (Dayer *et al*,1985). Induction of the synthesis of these molecules by TNF-α suggests a role for this cytokine in the development of rheumatoid arthritis and other inflammatory joint diseases that had been previously attributed to interleukin-1 (IL-1) (Dayer *et al*,1981; Mizel *et al*, 1981).

Dermal fibroblasts and synovial cells have different sensitivities to both TNF- $\alpha$  and IL-1 (Dayer et al,1985) suggesting that TNF- $\alpha$  and IL-1 may influence different tissues. In addition, although IL-1 and TNF- $\alpha$  are both produced by activated macrophages, the type of stimuli that initiate their

production are different. For example, LPS treatment stimulates higher levels of TNF- $\alpha$  production than do concanavalin A or phorbol ester. In contrast, IL-1 is produced in equivalent amounts following each of these stimuli (Dayer *et al*,1985). It is possible that the different responses of target tissues to different stimuli enhance the variability of the immune system further when defending against infectious organisms.

#### b) Induction of class I expression by TNF-α

Recombinant human TNF-α, like the IFNs and IL-1, has been shown to induce the expression of HLA-A, B and C antigens in some cell types. For example, elevated surface expression of class I molecules has been reported in untransformed human vascular endothelial cells and dermal fibroblasts *in vitro* (Collins *et al*,1986). This elevation correlates with an increase in the steady state levels of class I mRNA, suggesting that TNF-α enhances the rate of transcription (Guillemot *et al*,1988). Induction of class I expression by TNF-α and other cytokines will be discussed in more detail in chapter 5.

#### c) Activation of polymorphonuclear cells by TNF-α

Polymorphonuclear cells (PMN) are phagocytic cells involved in the removal of infectious agents from the body. TNF- $\alpha$  activates phagocytosis and cytotoxicity by these cells as well as chemotaxis and the production of free radicals (Ming *et al*,1987; Shalaby *et al*,1985; Klebanoff *et al*,1986). The infectious agent attaches to the surface of the PMN via non-specific receptors or receptors for opsonins such as IgG and C3b (Klebanoff *et al*,1986). Activation of phagocytosis by TNF- $\alpha$  appears to occur as a result of an increase in the surface expression of the C3b receptor. This surface marker is an adherence promoting glycoprotein and has been reported to be involved in the TNF- $\alpha$  mediated stimulation of PMN adherence to endothelial cells.

TNF- $\alpha$  was reported by Ming *et al*, (1987) to be a potent chemoattractant for PMN and monocytes but not for other motile cells such as natural killer cells. This effect occurs at low TNF- $\alpha$  concentrations (<1 U/ml), suggesting that TNF- $\alpha$  may act as a chemoattractant *in vivo* for PMN and monocytes in response to infection.

TNF- $\alpha$  activation of PMN induces release of the lysosomal enzymes myeloperoxidase,  $\beta$ glucuronidase and lysozyme as well as the release of oxygen-derived metabolites. These metabolites
have been implicated in the killing of microbial agents and as a cause of inflammation-mediated tissue
injury. After phagocytosis, the metabolic activity of PMN increases rapidly, resulting in increased
production of toxic H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>-· TNF- $\alpha$  has been shown to directly enhance the production of
H<sub>2</sub>O<sub>2</sub> by PMN through stimulation of this respiratory burst (Klebanoff *et al.*,1986).

#### 3.1.4. TNF- $\alpha$ AND INFLAMMATION

Cytokines are important in the acute inflammatory response initiated by infection or trauma. Inflammatory diseases of skin, gastrointestinal tract, joints, muscle, and the central nervous system all involve infiltration of tissues by monocytes and macrophages. These cells produce both IL-1 and TNF- $\alpha$  in response to a number of stimuli such as endotoxin, muramyl dipeptides, lectins and immune

complexes and, once induced, these cytokines can be distributed through the circulation to a large number of sites. It has been suggested that the production of these cytokines during inflammation could contribute to tissue destruction (Rosenbaum,1981). Indeed, as IFN- $\gamma$  and IL-1/TNF- $\alpha$  have opposing effects on the synthesis of new bone and the level of collagenase, the decrease in IFN- $\gamma$  production in rheumatoid arthritis patients and increases in IL-1 and TNF- $\alpha$  could contribute to the loss of bone and cartilage (Hopkins *et al*,1988; Buchan *et al*,1988).

The chemotactic response of PMN has been shown to be elevated in HLA-B27 positive individuals, implicating these cells in the development of ankylosing spondylitis by an inflammation mediated process (Pease *et al*,1984; Repo *et al*,1983). The role of PMN in AS is further supported by the fact that the chemotactic response of these cells is elevated by TNF-α, which is itself elevated in HLA-B27 positive individuals as well as HLA-B27 negative patients. Stimulated monocytes from both HLA-B27 positive and HLA-B27 negative patients suffering from the HLA-B27 associated *Yersinia* reactive arthritis as well as those from healthy HLA-B27 positive controls secrete significantly more TNF-α than do cells of HLA-B27 negative controls. Therefore, increased TNF-α secretion appears to be associated with both reactive arthritis and the presence of HLA-B27 (Repo *et al*,1988) and may cause inflammation mediated tissue injury through stimulation of PMN activity.

TNF-α stimulates such functions of PMN as chemotaxis, antibody-dependent cellular cytotoxicity, phagocytosis, production of free radicals and adherence to endothelial cells. TNF-α and IL-1 are also known to induce production of one another (Dinarello *et al*,1986) such that continued stimulation of one by the other could lead to chronic inflammation and inflammation-mediated injury. In agreement with this is the positive correlation between TNF-α and IL-1 levels in patients and HLA-B27 positive controls (Repo *et al*,1988). Therefore, the enhanced TNF levels in HLA-B27 positive individuals in response to infection may induce the production of IL-1, which in turn could stimulate further production of TNF-α. Both of these cytokines can enhance the activities of PMN so that continual activation of one by the other can lead to continual stimulation of PMN and possibly result in tissue injury. It is feasible that similar tissue damage may develop in HLA-B27 negative individuals who overexpress TNF-α.

# 3.1.5. POSSIBLE INVOLVEMENT OF TNF- $\alpha$ IN THE PATHOGENESIS OF AUTOIMMUNE DISEASE

The linkage of the TNF- $\alpha$  to the HLA-B locus may be relevant to the development of HLA associated diseases. As discussed previously, it is not known whether HLA-B27 is directly involved in the pathogenesis of ankylosing spondylitis and related diseases or whether it is a marker for the true disease gene with which it is in linkage disequilibrium. Stimulation by TNF- $\alpha$  of the production of collagenase in human synovial cells (Dayer *et al*,1985) as well as the resorption of bone and proteoglycan in cartilage (Bertolini *et al*,1986; Saklavata,1986) suggests the possibility that joint damage as seen in AS may be caused by abnormal expression of TNF- $\alpha$ . In other words, it is possible that the TNF- $\alpha$  gene is the 'AS gene'. In support of this, significantly more TNF- $\alpha$  was found to be

secreted by activated monocytes from HLA-B27 positive and HLA-B27 negative reactive arthritis patients as well as those from healthy HLA-B27 positive controls compared with cells from HLA-B27 negative controls (Repo *et al*,1988). Therefore, the HLA association of many diseases could, in fact, be due to linkage with an altered TNF-α gene.

TNF-α has been shown to be directly involved in the development of autoimmune lupus nephritis of (NZWxNZB) F<sub>1</sub> mice (Jacob *et al*,1988). These mice have reduced levels of TNF-α and the direct involvement of this cytokine in the pathogenesis of this disease is supported by the fact that replacement therapy causes a delay in the onset of disease symptoms. The reduced TNF-α level correlates directly with an RFLP in the TNF-α gene. Using a probe for the fourth exon of the mouse TNF-α, the parental strains of the (NZWxNZB) F<sub>1</sub> mouse were analyzed for an RFLP that might be linked to a mutation accounting for the development of lupus nephritis (Jacob *et al*,1988). NZB mice develop autoimmune haemolytic anaemia and mild glomerulonephritis while the NZW mice are phenotypically normal yet the majority of female (NZWxNZB) F<sub>1</sub> mice develop severe glomerulonephritis (Jacob *et al*,1988). Both the NZW and NZB mice were shown to have a non-polymorphic *Bam* H1 / *Hinc* II fragment of 4.3 Kb. The NZB mouse also had a 1.2 Kb polymorphic band whereas the NZW strain had a polymorphic 2.7 Kb fragment which is inherited by the F<sub>1</sub> mice. The development of nephritis by these F<sub>1</sub> mice therefore correlates with the inheritance of an altered TNF-α gene from the NZW parent strain.

#### 3.1.7. AIM

In order to establish whether the TNF- $\alpha$  gene is involved in the development of ankylosing spondylitis, the degree of polymorphism of the TNF- $\alpha$  gene has been studied in normal individuals using a number of different restriction enzymes.

A small study comparing ankylosing spondylitis patients with normal controls has also been performed. Genomic DNA from patients and controls were digested with *PvuII*, *Eco*R1 and *Hin*dIII and hybridized with a probe for the human TNF-α gene to search for a RFLP that associates with AS.

### 3.2 RESULTS

Since the association of AS with HLA-B27 is not absolute, it is possible that another gene linked to the HLA-B27 locus may also be involved in disease development. The physical proximity of the TNF-α to the HLA-B locus (Inoko *et al*,1987; Carroll *et al*,1987; Ragoussis *et al*,1988) has aroused interest in this gene as a possible candidate for involvement in the development of AS.

It was necessary to determine the degree of polymorphism of the TNF- $\alpha$  gene within the general population before attempting to search for a disease associated RFLP of this gene. A human TNF- $\alpha$  genomic clone had been previously isolated from a human acute lymphocytic leukaemia cell line cosmid library (Kioussis *et al*,1987) and the 2.9 Kb *Eco* R1 fragment containing this gene was subcloned into pBR322. This fragment includes the entire TNF- $\alpha$  gene as well as 870 bp of upstream and 300 bp of downstream sequences (chapter 7, figure 34). To ensure that the probe was indeed the TNF- $\alpha$  gene and not the TNF- $\beta$  gene, restriction digests were performed and compared with the published maps (Nedwin *et al*,1985). Digestion of the probe with the enzymes *Eco* R1, *Pvu* II, *Sac* I, and *Hind* III generated fragments of the expected size for TNF- $\alpha$  (figure 17).

Having established the identity of the probe, lymphocyte DNA from normal individuals was digested with 26 different restriction endonucleases before hybridizing with the human TNF- $\alpha$  gene. The HLA tissue types of these individuals was not determined. The number of individuals analyzed for each enzyme as well as the sizes of the fragments generated are listed in table 12. None of the enzymes investigated revealed any polymorphisms within the TNF- $\alpha$  locus.

Although the human TNF-α locus did not appear to be polymorphic in the normal population with these enzymes, a limited study was performed to assess whether there was any RFLP associated uniquely with AS. Southern blots that had been previously hybridized with HLA-B27 probes were rehybridized with the TNF-α probe. Genomic DNA from 36 English HLA-B27 positive AS patients, 6 HLA-B27 positive and 21 HLA-B27 negative controls digested with *Pvu* II were analyzed. In addition, DNA from 7 Polish HLA-B27 positive AS patients, 4 HLA-B27 positive and 3 HLA-B27 negative controls were digested with *Eco* R1 and *Hind* III. Southern hybridization analysis did not reveal any RFLPs within the TNF-α gene distinguishing patients from controls in either the English or Polish groups using these enzymes (figure 18).

### 3.3. DISCUSSION

The possibility that the TNF- $\alpha$  gene may be involved in the development of ankylosing spondylitis was investigated using RFLP analysis. While the association of AS with HLA-B27 is very striking, it is not absolute since 4% of patients are negative for this antigen, suggesting that other genes may also be involved. The tumour necrosis factor  $\alpha$  and  $\beta$  genes (TNF- $\alpha$  and  $\beta$ ) have been localized within the MHC between the complement factor genes and the HLA-B locus (Inoko *et al*,1987; Carroll *et al*,1987; Ragoussis *et al*,1988). TNF- $\alpha$  has been shown, amongst other activities, to induce the expression of class 1 genes and stimulate the production of collagenase and the resorption of bone by osteoblasts (Bertolini *et al*,1986). Consequently, this molecule has aroused a lot of interest as a possible candidate for involvement in the development of AS.

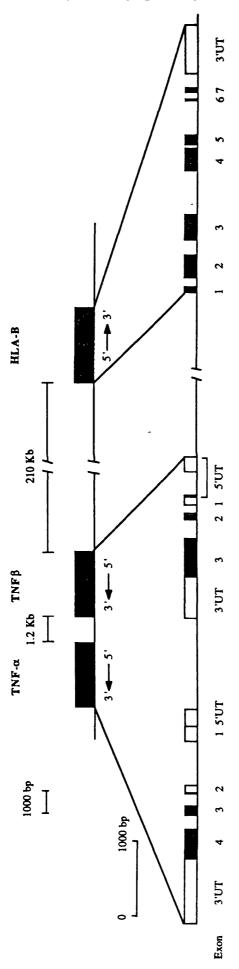
Since this gene has been shown to be polymorphic in the mouse, it was thought that informative polymorphisms may be found for the human gene also. For example, mice of the b or s haplotypes have a Pst 1 fragment of 9.5 kb and a Bam H1 / Xba 1 fragment of 6.5 Kb. Mice of the a or a haplotypes also have a 9.5 Kb a 1 fragment but the a 1 fragment is 4.3 Kb whereas mice with a 1 fragment and a 6.5 Kb a 1 fragment (Gardner a 1,1987). The murine TNF-a gene, on the other hand, has been shown to be non-polymorphic for 12 restriction enzymes (Jacob a 1,1988).

In contrast to the murine gene, the human TNF-α gene was found to be non-polymorphic in the general population for the restriction enzymes studied. This low level of polymorphism has also been found by other workers, with one polymorphism identified with the enzyme *Nco* 1 (Badenhoop *et al*, 1989). This enzyme gives rise to 2 fragments of 10.5 and 5.5Kbp. In addition, both alleles segregate with susceptibility to Type I (insulin dependent) diabetes mellitus depending on the extended haplotype. Those patients with the haplotype HLA-A1,-B8,-DR3 carry the 5.5Kbp allele while those with the HLA-Bw62,-DR4 haplotype carry the 10.5Kbp allele.

This linkage of TNF-a with some extended haplotypes has been suggested as an explanation for some HLA associated autoimmune diseases (Badenhoop et al, 1989). Although the level of polymorphism of the human TNF- $\alpha$  gene is very low, it does not rule out the possibility that a RFLP exists that is associated with AS. However, it does make such an association unlikely. Indeed, this was confirmed in both English and Polish populations in a limited study comparing AS patients and control DNA digested with the restriction enzymes Pvu II, Eco R1 and Hind III: no RFLP was identified with these enzymes that was more common in the patient groups or controls.

In summary, this study has shown that, in contrast to the murine gene, the human TNF- $\alpha$  gene is non-polymorphic for the enzymes analyzed and no RFLP for the enzymes Eco~R1, Pvu~II or Hind~III have been identified that is associated with the disease in the English or Polish groups studied.

## 3.4. FIGURES AND TABLES



white boxes indicating the untranslated regions. The tandem arrangement of the TNF-α and TNF-β genes is shown and the transcriptional exon/intron organization of the TNF-α, TNF-β and HLA-B genes are shown with the solid bars representing the coding regions and the Figure 16: Physical map of the TNF and HLA-B region showing the relative positions of each gene within the human MHC. The orientation of each gene is also indicated with an arrow pointing in the 5' to 3' direction.

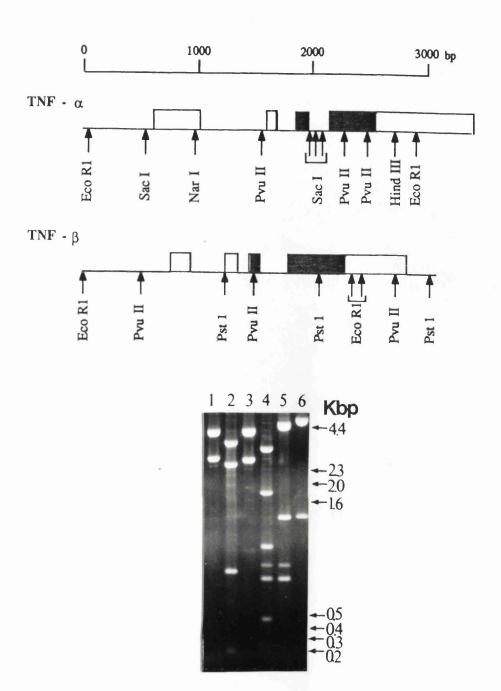


Figure 17: a) Comparison of the structural organization and restriction maps of the human TNF- $\alpha$  and TNF- $\beta$  genes. The solid bars represent the mature coding regions while the white bars show the untranslated regions. The positions of restriction enzyme sites are indicated with arrows and the name of the enzyme.

Source: Nedwin et al (1985)

b) Confirmation of the identity of the human TNF- $\alpha$  probe. The human TNF- $\alpha$  gene was subcloned into pBR322 by Kioussis *et al* (1987) and digestion with *Eco*R1 (lane 1), *Pvu*II (lane 2), *Hind*III (lane 3), *Hind*III and *Nar*I (lane 4), *Nar*I (lane 5) and *Sac*I (lane 6) generated restriction fragments of the expected sizes. The sizes of the  $\lambda$  *Hind*III and pAT153 *Hinf*I markers are indicated on the right.

Table 12: The sizes of restriction fragments detected following digestion of human genomic DNA with various restriction enzymes and hybridization with the human TNF- $\alpha$  probe. The different restriction enzymes used and the sizes of the generated fragments as well as the number of subjects studied are listed below.

Enzyme	Number of Subjects	Fragment sizes (bp)
Alu 1	9	740, 370
Apa 1	8	5020, 3170
Ava 1	10	3800
Ava II	8	1660, 1450, 1070
Ban II	9	1700, 1382, 1120
Bbe 1		28900
Bgl II	. 8 8 8	18600
Bst EII	8	7250
Cfo 1	9	15500, 1250, 4900, 4170
Dde 1	17	1020, 890, 750
Dra 1	8	5380, 2090
Eco R1	15	3320
Eco RV	33	17400
Hae II	9	1120, 980, 850
$H$ in d $\Pi$	9	1180, 1020, 500
Hin d III	9 9 9	16600
Hinf 1	9	1020, 760, 630, 590
Msp 1	9	1070, 1000
Pst 1	24	3310
Pvu II	16	2240, 1170, 780
Sac I	9	6600, 1320
Sac II	. 8	30900
Sal 1	9	>23000
Sau 9A	9	2140, 1480, 1382
Taq 1	44	1820
Xba I	8	10700

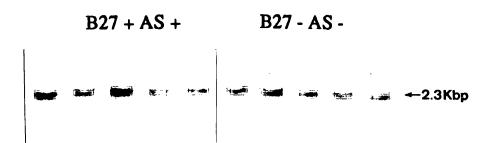


Figure 18: Comparison of *Pvu*II digested genomic DNA from 5 HLA-B27<sup>+</sup>AS<sup>+</sup> and 5 HLA-B27<sup>-</sup>AS<sup>-</sup> English subjects hybridized with the human TNF-α probe. The 2.3Kbp as well as the other *Pvu*II fragments generated were found to be non-polymorphic. DNA was electrophoresed through 0.8% TBE agarose gels before Southern transfer. Filters were washed at 0.1xSSC/0.5% SDS at 65<sup>0</sup>C for 1 hour before exposing for 3 days at -70<sup>0</sup>C.

## **CHAPTER 4**

STRUCTURE
AND
SEQUENCE
OF THE
HLA-B27 GENE
FROM AN
ANKYLOSING
SPONDYLITIS
PATIENT

### 4.1. INTRODUCTION

#### 4.1.1. STRUCTURE OF THE HLA-B27 ANTIGEN

#### a) Identification of HLA-B27 subtypes

Due to its extraordinary association with ankylosing spondylitis, the HLA-B27 antigen has been well characterized using alloantisera (Breur et al,1984), monoclonal antibodies (Grumet et al,1982), cytotoxic T lymphocytes (CTL) (Breuning et al,1984) and isoelectric focussing gel electrophoresis (IEF) (Choo et al,1986a). These techniques have demonstrated the existence of at least eight different genetic variants of the HLA-B27 antigen and sequence analysis of some of these has revealed amino acid substitutions in the hypervariable domains (Vega et al,1986; Rojo et al,1987) (table 13).

Studies using HLA-B27 specific monoclonal antibodies gave the first indication of the heterogeneity of the HLA-B27 molecule. The B27M1 monoclonal antibody defined a constant B27M1 epitope shared by all HLA-B27 molecules but the reactivity with another monoclonal antibody B27M2 was variable (Grumet *et al*,1982).

Subtypes of HLA-B27 have been identified using CTL prepared *in vitro* from HLA-B27 positive blood donors that would specifically lyse other HLA-B27 positive PBMC infected with either EBV or influenza A virus. While these CTL showed HLA-B27 restricted lysis, this appeared to depend on the subtype of the HLA-B27 molecule that was present on the cell surface. These subtypes were designated as HLA-B27.1, HLA-B27.2, HLA-B27.3 and HLA-B27.4 and were also shown to react differently with the B27M2 monoclonal antibody (Toubert *et al*,1984; Vega *et al*,1986) (table 13).

Breuning et al (1982) showed that stimulating PBMC from donor W and from HLA-A, -Band - C identical donor K with irradiated PBMC from the other resulted in only a proportion of HLA-B27 positive target cells being lysed. These HLA-B27 subtypes were designated HLA-B27W, HLA-B27K and nonW/nonK. One dimensional IEF, which enables purified class I molecules to be separated according to their electric charge, has allowed the nonW/nonK group of antigens to be further divided into the B27C and B27D subtypes (Breur-Vriesendorp et al,1986; Neefjes et al,1986) (table 13).

Choo et al. (1986a) identified six variants of HLA-B27 designated HLA-B27a, -B27b, -B27c, -B27d, -B27e and -B27f by one dimensional IEF (table 13) with HLA-B27a being the most acidic and HLA-B27f the most basic. Attempts to correlate these variants with serological studies showed that HLA-B27b and HLA-B27c cannot be separated serologically whereas the other subtypes can. Indeed, HLA-B27a shows two serological reactivities with the HLA-B27 specific monoclonal antibody P56.1 (Choo et al,1986b), so that this subtype is now subdivided further into HLA-B27a.1 and HLA-B27a.2. The variants HLA-B27a and HLA-B27d were recognized by the monoclonal antibody B27M2 whereas HLA-B27b and HLA-B27c showed only weak reactivity and HLA-B27e and f showed no reactivity (table 13). Therefore, a total of seven variants were identified by Choo and coworkers.

# b) Comparison of the amino acid sequences of the HLA-B27 variants detected with different techniques

Amino acid analysis has shown that some of the variants detected by different techniques are the same while the variants HLA-B27a.2, c and f appear to be unique (table 13). This overlap has resulted in new WHO nomenclature for the HLA-B27 variants as summarized in table 13. The HLA-B27 subtypes will hereafter be referred to by this new nomenclature. The primary structure of these antigens also shows that the majority of amino acids are conserved with only one to four substitutions that occur within the polymorphic domains  $\alpha 1$  and  $\alpha 2$ , thus supporting the idea that HLA-B27 consists of a family of closely related HLA-B locus alleles (Choo *et al*,1986b). This is in contrast to the conventional B locus alleles which differ by over 20 amino acids. The high level of homology would therefore be responsible for the serological specificity defined as HLA-B27.

# c) Racial distribution of the different HLA-B27 subtypes and a possible evolutionary pathway for their development

The HLA-B\*2705 variant is present in 90% of HLA-B27 positive Caucasians, 45% of HLA-B27 positive Orientals and in most HLA-B27 positive Blacks and American Indians (Choo et al, 1988). Other HLA-B27 variants occur only within certain races. For example, HLA-B\*2704 and HLA-B\*2706 are only found in Orientals whereas HLA-B\*2703 has only been observed in Blacks and HLA-B\*2702 and HLA-B\*2701 in Caucasians (Choo et al, 1988).

By comparison of the amino acid sequences and population distribution of the HLA-B27 variants, an evolutionary pathway has been proposed for the creation of these variants (Vega et al,1986; Choo et al,1988) (figure 19). HLA-B\*2705 is considered to be the original allele since it is present in all populations, and subsequent diversification of this molecule may have begun after the separation of the races. HLA-B\*2705 is thought to have given rise to HLA-B\*2702 in the Caucasian population and to HLA-B\*2704 in the Oriental population by means of gene conversion. In support of this, the sequence of HLA-B\*2702 from residue 75 to 93 is identical to that in HLA-Bw58. In the Oriental group, HLA-B\*2704 presumably gave rise to HLA-B\*2706 by a similar event since the sequence of HLA-B\*2706 between positions 114 to 130 is the same as that for HLA-B\*. HLA-B\*2701 is also thought to have arisen in Caucasians by gene conversion since it shows sequence identity with HLA-B44 from positions 72 to 81 whereas HLA-B\*2703, on the other hand, probably arose by point mutation within the Black population since it differs from HLA-B\*2705 by only a single nucleotide within the coding region at codon 59 (Choo et al,1988).

#### d) HLA-B27 subtypes and ankylosing spondylitis

Although HLA-B27 is now known to be heterogeneous, studies of AS patients have failed to reveal a consistent association of the disease with a particular subtype of this antigen. Grumet *et al*, (1985) found that the B27M2(-) variant is more common in AS patients, particularly among Orientals and the B27M2(+) variant seems to be associated with susceptibility to Reiter's syndrome in this group. Suarez-Almazor *et al*, (1986) noted that in families having a number of members with AS, all

of those with the disease shared the same HLA-B27 haplotype i.e., the haplotype of the eldest AS sufferer. Other studies failed to find any such correlation (Karr et al,1982; Breur-Vriesendorp et al,1987). For example, tryptic peptide mapping failed to reveal any disease specific features of the HLA-B27 antigen (Karr et al,1982) while population studies failed to associate a particular subtype with the presence of disease (Breur-Vriesendorp et al,1987).

#### e) Effect of amino acid substitutions on the function of HLA-B27

Comparison of HLA-B27 with other HLA-B molecules has revealed two amino acid residues that are specific to the HLA-B27 family of antigens namely, a lysine residue at position 70 and an arginine at position 97 (Weiss *et al*,1988). However, these are conservative substitutions since these sites are also occupied by the basic amino acids in other class I molecules, namely aspartic acid at position 70 and arginine, threonine, tryptophan or serine at position 97 (chapter 2, figure 8). Consequently, it has been suggested that they are unlikely to affect the structure or biological activity of the HLA-B27 molecule (Weiss *et al*,1988).

The residue at position 70 is found within the highly variable region of the a1 domain with the side chain pointing into the antigen binding site, suggesting a direct involvement in the binding of antigen (Bjorkman et al,1987a). The amino acid occupying position 97 forms part of the floor of the antigen binding site and therefore also has a role in antigen binding. A previous study showed that HLA-B27 behaves as a dominant restriction element over other class I antigens in antiviral-CTL responses (Gomard et al,1984). Anti-EBV-CTL and anti-influenza virus-CTL derived from HLA-B27 positive donors were tested against a panel of target cells, of which 43% were positive for HLA-B27. About 94% of the HLA-B27 donors had produced HLA-B27-restricted anti-virus CTL and that 50% had not used the other HLA-A and -B molecules as restricting elements, producing CTL that would be restricted only by HLA-B27. Consequently, it can be postulated that the unique substitutions of the HLA-B27 group of antigens may be involved in the preferential binding of some viral proteins to HLA-B27. Whether this has any relevance to AS remains to be determined although it has been suggested that an abnormally high response to particular infectious agents could alter host cells in some way (Gomard et al,1984).

Schwimmbeck et al, (1987) reported a six amino acid sequence that is shared between HLA-B27 and Klebsiella pneumoniae nitrogenase which has been proposed to initiate an immune cross-reactivity between these two molecules. The fact that no single HLA-B27 subtype is associated with AS suggests that this cross-reactive epitope should be common to all the subtypes yet HLA-B\*2702 and HLA-B\*2704 have only five of these amino acids (Weiss et al,1988) (table 14). In addition, the sequences of the HLA-B\*2704, HLA-B7 and B8 antigens are the same in this region. If AS is the result of molecular mimicry between HLA-B27 and enteric bacteria, it would appear from sequence comparisons that this particular epitope is not responsible.

#### 4.1.2. THE HLA-B27 GENE

While no differences have been detected between the HLA-B27 antigen of AS patients and that of healthy individuals, it is possible that differences exist at the nucleotide level that cannot be identified using antibodies, CTL or IEF. Several groups have attempted to identify such differences by isolating and sequencing the HLA-B27 gene from both normal individuals and patients.

#### a) Organization of the HLA-B27 gene

The HLA-B\*2705 gene was isolated from the lymphocyte DNA of a healthy individual (Weiss et al,1985) and sequencing of this clone established that the exon/intron organization of the HLA-B27 gene is similar to that of other HLA-B class I genes, consisting of seven exons and a 3UT region (chapter 2, figure 5). This gene is typical of an HLA-B sequence as the termination codon is at the end of exon 7 rather than at the end of an 8th exon that is contiguous with the 3'UT region, as found in HLA-A and C alleles (Sood et al,1985; Gussow et al,1987).

#### b) Comparison of the genes encoding the HLA-B27 subtypes

The differences observed between the subtypes of HLA-B27 have been confirmed by sequence analysis of the genes encoding some of these molecules. For example, sequencing of the HLA-B\*2705 and HLA-B\*2702 genes from the BRUG cell line has identified four nucleotide differences over a stretch of 14 bp (Seeman et al,1986). Two of these substitutions are within codon 80 so that a total of three amino acid changes have occurred, namely aspartate to asparagine-77, threonine to isoleucine-80 and leucine to alanine-81. As discussed previously, this region of HLA-B\*2702 is identical with the corresponding sequence of HLA-Bw58 at both the protein and nucleotide levels. This supports gene conversion as the mechanism that gave rise to HLA-B\*2702 (Choo et al,1988). The substitution of asparagine for aspartate at position 77 results in one less acidic charge which correlates with the findings of IEF (Choo et al,1988). The restriction maps of these alleles are identical except for an additional Xba 1 site in the 3' flanking region of HLA-B\*2702.

The HLA-B\*2703 gene isolated from an EBV transformed B-lymphoblastoid cell line established from a healthy individual has also been sequenced (Choo et al,1988). This gene differs from HLA-B\*2705 only at codon 59 in exon 2 where TAT of HLA-B\*2705 is altered in HLA-B\*2703 to CAT resulting in a tyrosine to histidine substitution in the al domain. As this single base change is the only difference between HLA-B\*2703 and the proposed ancestral B\*2705, it is thought that this change arose from a point mutation (Choo et al,1988).

The HLA-B27 subtypes are also known to show different linkage disequilibria with HLA-C locus alleles (Arnett *et al*,1978; Breur-Vriesendorp *et al*,1986). For example, HLA-B\*2705 is in linkage disequilibrium with the HLA-Cw1 and Cw2 alleles while the HLA-B\*2702 subtype is only linked with HLA-Cw2 and HLA-B\*2704 and HLA-B\*2706 with HLA-Cw blank (Arnett *et al*,1978).

# d) Comparison of HLA-B27 sequences from patients and healthy individuals

Sequencing of the coding regions of the HLA-B27 gene from cell lines derived from AS patients has failed thus far to reveal any changes from the sequence of normal individuals (Trapani et al,1985b; Szots et al,1986; Coppin et al,1986). Szots et al, (1986) isolated an HLA-B27 cDNA clone from the B-cell line, LG-2, derived from an affected individual. The differences between the sequence of this clone and that of the coding region of the gene isolated from a normal individual were identified as cloning artefacts and thus not significant (Weiss et al,1985; Szots et al,1986). Coppin et al, (1986) cloned the HLA-B27 genes from the DNA of three EBV transformed B-cell lines derived from individuals of different disease status but no differences were found between the nucleotide sequences of these clones and that reported by Weiss et al, (1985). Trapani et al, (1985a) had earlier described a 3.5 Kb Taq1 RFLP associated with the presence of HLA-B27. Isolation and partial sequencing of this fragment from an AS patient showed that this sequence derived from intron 6- exon 7- intron 7 is identical to the sequence of Weiss et al (1985) except for the deletion of a G residue at position 3223 in intron 7. This residue is found in all the HLA-B27 subtypes sequenced so far as well as in the B7 gene and is therefore probably an error (Weiss et al,1985; Seeman et al,1986; Choo et al,1988).

### 4.1.3. AIM

Analysis of the nucleotide sequence of the HLA-B27 gene has only been performed using cell lines. In particular, only one cell line derived from a patient has been used to determine the sequence of the HLA-B27 coding regions of disease haplotype (Coppin *et al*,1986). In order to confirm the identity of HLA-B27 sequences of the disease and normal haplotypes, the gene has been isolated, and sequenced, from a genomic library made from the lymphocytic DNA of an AS patient who is heterozygous for the HLA-B27 allele.

### 4.2. RESULTS

# 4.2.1. CONSTRUCTION OF A PARTIAL $\lambda$ GT 10 LIBRARY

Hybridization of the pB27-3FR probe to EcoR1 digested DNA detects 4 fragments of 7.9 Kbp, 7.6 Kbp, 6.8 Kbp and 6.5 Kbp (see chapter 2, figure 13) of which the 6.8 and 6.5 Kbp fragments contain complete HLA-B genes (Weiss *et al*,1985; Ways *et al*,1985). The 7.9 and 7.6 Kbp fragments, however, are derived from the HLA-C locus and cross hybridize with this probe. The HLA-B27 gene from an AS patient heterozygous for HLA-B27 was isolated from DNA digested to completion with EcoR1 by cloning DNA fragments of 6.5 Kbp into  $\lambda$ gt10. A total of 7.02x10<sup>4</sup> recombinant phages was obtained corresponding to a cloning efficiency of 7.8x10<sup>4</sup> recombinants per  $\mu$ g of insert DNA.

Screening of this library with the pB27 3FR probe revealed 1 plaque that hybridized very strongly in duplicate as well as 13 others that hybridized more weakly. Upon rescreening of the strong positive (figure 20, panels a,b) plus two of the weaker positives, only the strong positive hybridized with the pB27-3FR probe (figure 20, panels c,d).

Phage DNA was purified from 4 of the plaques that hybridized upon rescreening and digestion with EcoR1 showed that each phage contained an insert of 6.5 Kb. As expected, 3 fragments were generated from digestion of  $\lambda$ gt 10 clones with EcoR1: the insert fragment of 6.5 Kb and two vector fragments of 32.7 and 10.6 Kb each (figure 21).

# 4.2.3. IDENTIFICATION AND SEQUENCING OF THE HLA-B27 CLONE

A restriction map of the sub-clone pBSB27-2 was determined by digesting with the restriction enzymes, *PvuII*, *Pst1*, *BgIII*, *SacI*, *XbaI SspI* and *BamH1*. This restriction map is shown in figure 22 and is identical to that of the HLA-B27 gene isolated by Weiss *et al* (1985).

The identity of this clone was confirmed as HLA-B27 by sequencing the 550bp Bg/II/Pst1 fragment. This fragment includes the 3' end of exon 2, intron 2 and most of exon 3 and contains the region encoding the 6 amino acids shared with Klebsiella pneumoniae nitrogenase. The sequence of this fragment was identical to that reported previously (Weiss et al,1985) indicating that the gene isolated from an AS patient was of the HLA-B\*2705 subtype.

The sequence of the entire HLA-B27 gene of disease haplotype was determined according to the strategy illustrated in figure 22. The sequence of this gene, contained within a 4258 bp fragment, is shown in figure 23. The exon/intron organization of this HLA-B27 gene was found to be similar to that of other HLA-B alleles. This gene consisted of 8 exons with a translation termination codon at the end of the seventh exon. The eighth exon would therefore not be translated. The variant TATA sequence TCTAAA was positioned 52 bp upstream of the ATG translation initiation codon with the CAAT box 26 bp further upstream. The poly A signal AAATAAA was located at nucleotide 3941 with the poly A site 17 bp downstream from this.

The sequence of this HLA-B27 gene of disease haplotype was compared with the sequences of HLA-B27 genes of normal haplotype isolated by others (Weiss *et al*,1985; Coppin *et al*, 1986; Seemann *et al*, 1986) (figure 23). The HLA-B27 gene isolated by Weiss *et al* (1985) was the same subtype as the gene isolated in this study. The coding regions of these two genes were identical. Coppin *et al* (1986) isolated a HLA-B27 gene from a cell line derived from an AS patient. The coding regions of this clone were also shown to be identical to the sequence of Weiss *et al* (1985). Although the HLA-B27 gene of normal haplotype isolated by Seemann *et al* (1985) was also of the same subtype, nucleotide differences were found within exon 3 at position 1638 and within exon 4 at positions 2315 and 2405 (figure 23). These changes did not, however, alter the amino acid sequences.

Nucleotide differences were noted within intervening sequences. While only four differences were found within introns 1 and 2 from the HLA-B27 gene sequence of Weiss and coworkers (1985), many more changes were seen when a comparison was made with the sequence of Seemann *et al* (1986) (figure 23). The intron sequences of the HLA-B27 gene isolated by Coppin *et al* (1986) were not published so that no comparison could be made.

Sequences homologous to the binding sites of regulatory trans-acting factors have also been identified (figure 23). A sequence identical to the NF1 transcription factor binding site was identified at position 600, upstream of the TATA box. Potential binding sites for the transcription factor Sp1 have been identified at position 851 in intron 1 and positions 1236, 1256, 1344 and 1349 in intron 2. In addition, sequences identical to the binding site of the AP2 trans-acting factor have been found at nucleotides 875 in intron 1 and 1283 in intron 2.

Some of these potential binding sites in the HLA-B27 gene of disease haplotype are abolished by sequence differences within the HLA-B27 genes reported by Weiss's and Seemann's groups. Both the HLA-B27 sequence reported here and by Weiss *et al* (1985) have a potential Sp1 binding site at position 851. This site is destroyed in the sequence of Seemann *et al* (1986) by the change of the G residue at position 857 for a C residue. In addition, sequence alterations in the gene reported by Seemann and coworkers abolish the Sp1 binding sequence found at position 1256. The C residues at position 1256 and 1258 of the HLA-B27 gene of disease haplotype and that of Weiss *et al* (1985) are replaced by a G and a T respectively in the sequence of Seemann *et al* (1986). In addition, the insertion of a cytosine at position 1284 in the HLA-B27 gene of disease haplotype creates a potential binding site for the *trans*-acting factor AP2.

Sequences identical to the class I regulatory element and the interferon responsive sequence were located 149 bp and 128 bp respectively upstream of the TATA box (figure 23). These will be discussed in more detail in chapter 5.

### 4.3. DISCUSSION

The HLA-B27 gene has been isolated from a patient suffering from ankylosing spondylitis. This patient was heterozygous for the HLA-B27 allele ensuring that the isolated gene would be of the disease haplotype. While Coppin and McDevitt (1986) compared the sequence of HLA-B27 coding regions from an AS patient with that of a HLA-B27 gene of normal haplotype, their gene of disease haplotype was in fact isolated from a cell line derived from an AS patient. This is the first study to present the sequence of an entire HLA-B27 gene of disease haplotype isolated directly from an AS patient.

All HLA-B genes that have been cloned have been contained in either a 6.5 or a 6.8 Kb EcoR1 fragment (Ways et al,1985; Weiss et al,1985).  $\lambda$ gt10 was therefore chosen as the vector for the library construction since EcoR1 inserts of up to 7.6 Kb can be cloned assuming a packaging efficiency of 105% wild type length (Huynh et al,1985). In order to increase the probability of isolating a HLA-B sequence, a library was therefore constructed in  $\lambda$ gt10 using only DNA of approximately 6.5 Kbp.

If it is considered that the total size of a human genome is  $3x10^9$  bp, then a library of  $4.3x10^5$  recombinants would be required in order to have a complete genome represented with an average size of 7 Kbp. The  $7.8x10^4$  recombinants prepared in this study represents 18% of a complete human genome or two genome equivalents. If this is so, it may have been expected that one clone of each allele would have been detected during the library screening. It is possible that the other HLA-B allele, HLA-B8, carried by the patient from which the library was derived is contained within a 6.8 Kbp fragment which may not have been cloned since the library was restricted to DNA that co-migrated with the 6.5 Kbp 1 HindIII size marker.

The identity of the clone was confirmed by restriction mapping and sequencing to be HLA-B27 of the HLA-B\*2705 subtype. The sequence of this HLA-B27 gene of disease haplotype extended 162 bp upstream and 80 bp downstream from that published previously for normal haplotype by Weiss *et al*, (1985). The positions of the 5' and 3' splice sites, and thus the lengths of the exons, were identical between these genes. No differences were detected in the coding regions of the clone isolated in this study compared with the HLA-B27 gene of normal haplotype isolated by Weiss *et al*(1985) but nucleotide changes were noted from a similar sequence reported by Seemann *et al* (1986). However, these alterations did not alter the predicted amino acid sequence of the gene product. This indicates that the HLA-B27 antigen of an ankylosing spondylitis patient is identical to that of a healthy individual confirming the implications of Coppin and McDevitt (1986) that a pathogenic variant of the HLA-B27 antigen is not responsible for the development of AS.

The absence of differences between the coding sequences of the HLA-B27 genes from patients and healthy subjects does not eliminate a direct role for HLA-B27 in the pathogenesis of the disease. It may be that the regulation of HLA-B27 gene expression in AS patients is abnormal when compared with healthy individuals. The promoter sequences of the HLA-B27 genes of disease and normal haplotype are identical. This suggests that if the regulation of HLA-B27 gene expression is altered in AS patients, then the disease causing abnormality (ies) may lie within unknown regulatory sequences or within the cellular factors that control the HLA-B27 gene expression. Differences were noted in the nucleotide sequences of introns when comparisons were made between the HLA-B27 gene isolated here

and those published by both Weiss et al (1985) and Seeman et al (1986). Some of these changes altered the potential binding sites for trans-acting transcription factors.

While potential Sp1 binding sites found within the HLA-B27 sequences of disease haplotype are destroyed in the sequence of normal haplotype as reported by Seemann et al (1986), these are unlikely to be relevant to the disease as these sites are also found in the gene of normal haplotype reported by Weiss et al (1985). On the other hand, the AP2 binding site at position 1283 may be of importance to the disease as this site is not present in either of the sequences published for the normal haplotype. While it is unknown if this trans-acting factor has any role at all in the regulation of HLA-B27 expression, it is possible that such an alteration in the sequence of the HLA-B27 gene may be responsible for incorrect regulation of its expression. In order to determine the relevance of this site to the regulation of HLA-B27 gene expression, it would be necessary to first establish if this site is capable of binding DNA binding proteins using an oligonucleotide specific for this site in a gel mobility shift assay. Using cell extracts from patient and control cells it may be possible to see a difference in binding activity. Mutation analyses could be used to determine if these sites are important in the regulation of HLA-B27 expression. Class I genes whose sequences have been altered within the DNA binding sites of interest could be transfected into HLA loss mutant cell lines and the expression of the mutated genes assayed for at the mRNA level by Northern hybridization or S1/RNase mapping and at the protein level by binding of specific antibodies.

Although the predicted sequence of the HLA-B27 antigen in an AS patient is no different from that predicted for a healthy individual, this does not rule out the possibility of a direct role for HLA-B27 in the development of AS. For example, the molecular mimicry model as discussed in chapter 1, sections 1.2.2.b and 1.3.3.a may still apply. Alternatively, HLA-B27 gene expression may not be regulated normally. The regulation of HLA-B27 gene expression by IFNs will be discussed in more detail in chapter 5.

# 4.4. FIGURES AND TABLES

Table 13: Comparison of HLA-B27 variants

	152		Val	•	•	Glu	•	Glu	
domain	116		Asp	•			•	Тут	
Alpha 2 domain	114 116		His		,	•	,	Asp	
	81		Ala	,	Leu	Leu	Leu	Leu	
	80		Thr	Ile	•		•	•	
lomain	11		Asn	•	Asp	Ser	Asp	Ser	
Alpha 1 domain	74 77		Тут	Asp	Asp	Asp	Asp	Asp	
,	59	1	Tyr	1	His	•	•	•	
vith MAb	P56.1		+	+	+	•	+	•	
Reactivity with MAb	B27M2		•	•	+	3	+	>	
tion	$CL\Gamma q$		•	B27.2	•	B27.3	B27.1	í	
Method of detection	IEFb W/Kc CTLd		ı	B27K	B27J	B27C	B27W	B27D	
Methox	q		B27f	B27e	B27d	B27b	B27a	•	
WHO	HLA-B27	alleles <sup>a</sup>	B*2701	B*2702	B*2703	B*2704	B*2705	B*2706	

a HLA-B27 alleles were designated B\*2701 through to B\*2706 in the order of basic to acidic IEF bands in the Tenth International

Histocompatibility Workshop

b Choo et al,1986

<sup>c</sup> Breur-Vriesendorp et al, 1986; IEF variant 'B271' equivalent to HLA-B27d has recently been identified from another American

Black individual (Ivanyi, cited in Choo et al, 1988)

d Toubert et al,1984

Source: Choo et al, 1988

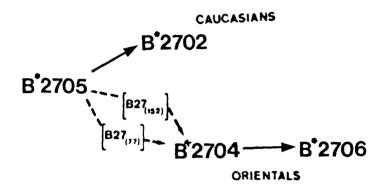


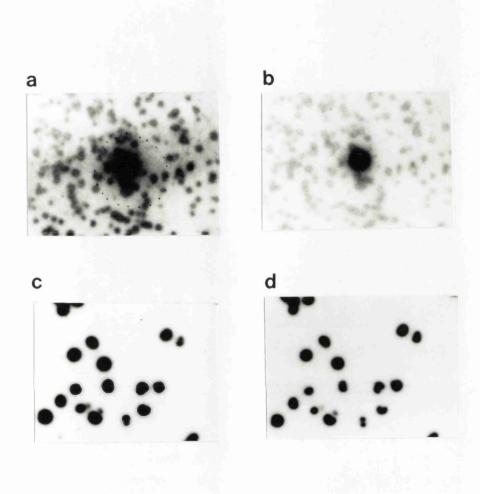
Figure 19: Proposed evolutionary pattern for HLA-B27. The HLA-B\*2705 subtype is tentatively placed at the origin because it is the major subtype in the human population and is widely distributed in different ethnic groups. B27(152) and B27(77) are hypothetical intermediary forms of HLA-B27 differing from HLA-B\*2705 by a single amino acid change of Asp77 to Ser77 and of Val152 to Glu152 respectively.

Source: Vega et al (1986)

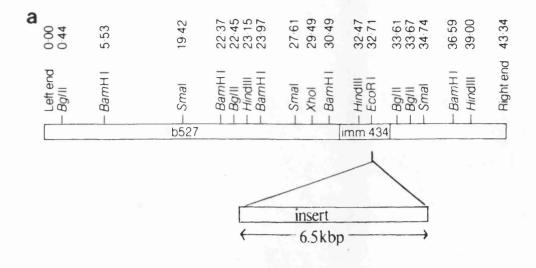
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E <b>2705</b>		D	F:	Ε	Ţ	9	i	C	ĸ	Α	K	A	Q	Ţ	D	R	Ε	D	L	R	T	L	L	ĸ	Υ	Y	Ν	G	S	Ε	Α
2 <b>702</b>																															
<b>₽2704</b>																				•											
67		•		Ν		•	٠	Υ	•	•	G	•	ŀ	•	•	•	٠	S	١.	•	N	٠	R	G	٠	•	•	•	٠	٠	•
58																															
544																				•											
851				N			•	F		Ţ	N	7	١.	•	Y	•	•	Ν	١.	•	•	R	•	•	•	•	•	٠	•	•	•
858			G			R	N	М		٠	S	•	١.		٧	•	•	Ν	١.	•	I	Α	•	•	•	٠	٠	•	•	•	•
Bu62	•		•		•	•	•	S		Ţ	N	T	Ŀ	٠	٧	•		<u> </u>	J٠	٠	N	•	R	G	٠	•	•	•	•	•	•

**Table 14:** Comparison of the HLA-B27 amino acid sequences with other HLA-B antigens between residues 60 to 90. The 6 amino acid region shared between HLA-B\*2705 and *Klebsiella pneumoniae* is boxed and compared with other HLA-B27 subtypes and HLA-B antigens.

Source: Weiss et al (1988).



**Figure 20:** Autoradiograph of (a,b) primary and (c,d) secondary screening of the λgt10 genomic library. The library was prepared from 6.5 Kbp *Eco*R1 digested genomic DNA isolated from an AS patient heterozygous for the HLA-B27 allele. Panels(a,b) show the strong positive signal in duplicate following initial screening of the library with pB27-3FR while panels (b,c) show some of the positive signals revealed in duplicate following secondary screening of the strong positive from panels (a,b) with the same probe.



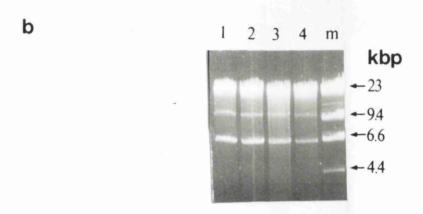


Figure 21: a) Restriction map of recombinant  $\lambda gt10$  bacteriophage showing the positions of EcoR1 sites.

b) Fragments generated following digestion of 4 recombinant  $\lambda gt10$  bacteriophages with EcoR1 (lanes 1-4). Sizes of the  $\lambda Hin$ d III markers (lane m) are indicated on the right hand side.

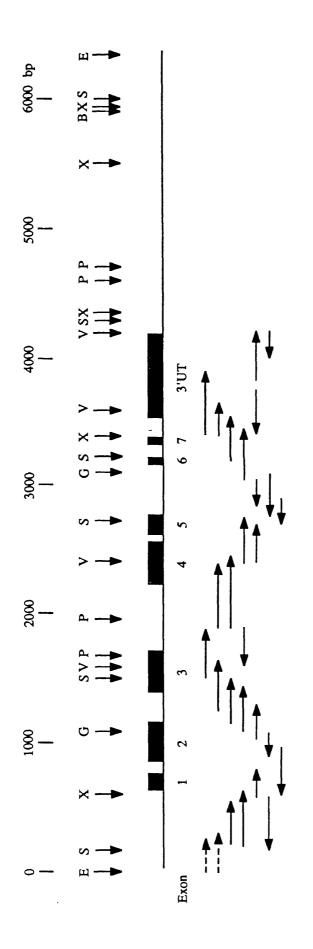


Figure 22: Restriction map and sequencing strategy of the 6.5Kbp *EcoR1* fragment containing the HLA-B27 gene of disease haplotype. The general structure of the HLA-B27 gene is indicated with black boxes representing the exons and 3UT region. The restriction endonucleases sites are indicated with arrows above with the following abbreviations: E, *EcoR1*; G, BgII; X, Xba 1; V, PvuII; P, PstI; S, SacI. The sequencing strategy is indicated under the HLA-B27 gene. The directions of the arrows indicate the direction of sequencing and the dotted arrows indicate that region sequenced by double stranded sequencing.

Figure 23: Nucleotide sequence of the HLA-B\*2705 gene of disease haplotype. Nucleotides are numbered from the *EcoR*1 cloning site. The promoter (TCTAAA), CAAT box, the mRNA start site and polyadenylation signal (AAATAAA) are in bold face. The exons are also in bold face and separated according to codons. The known regulatory regions are boxed and named as are the potential binding sites for *trans*-acting transcription factors. This sequence is compared with the sequences of HLA-B27 genes isolated by others. The following abbreviations refer to these sequences: B\*2705-W and B\*2705-S are the HLA-B\*2705 sequences of normal haplotype isolated by Weiss *et al* (1985) and Seemann *et al* (1986) respectively; B\*2705-C is the coding sequence of the HLA-B\*2705 gene of disease haplotype isolated by Coppin *et al* (1986); B\*2702 is the HLA-B\*2702 sequence reported by Seemann *et al* (1986) while B\*2703 is the HLA-B\*2703 sequence isolated by Choo *et al* (1988). The nucleotide differences are indicated with a / symbol representing a missing nucleotide.

	AAT	ATG	TAT	GGC	TGC	GAC	GTG	GGG	CCG	GAC	GGG	CGC	CTC	CTC	CGC	GGG	TAC	CAC	CAG	GAC	GCC	TAC	
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g*2703																					_	193	
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## **CHAPTER 5**

CLASS I
GENE REGULATION
IN
LYMPHOBLASTOID
CELLS
DERIVED FROM

DERIVED FROM
ANKYLOSING SPONDYLITIS
PATIENTS

### 5.1. INTRODUCTION

The expression of class I genes is regulated during development and between tissues in the adult (Daar et al,1984; Ozato et al,1985). Expression is also altered in response to viruses and immune modulators such as interferons (IFNs) and tumour necrosis factor  $-\alpha$  (TNF- $\alpha$ ) (Fellous et al,1982; Collins et al,1986).

# 5.1.1. TISSUE DISTRIBUTION OF CLASS I ANTIGENS

The expression of class I genes is developmentally regulated with class I antigens only becoming detectable upon differentiation of embryonal cells. Class I mRNA and surface antigen cannot be detected *in vivo* until the midsomite stage of mouse embryogenesis (Ozato *et al*,1985) whilst *in vitro* studies have shown that the undifferentiated murine embryonal carcinoma cell line, F9 does not express class I until differentiation is artificially induced by retinoic acid (Rosenthal *et al*,1984).

Detailed studies of the distribution of class I antigens in normal adult tissues have shown that there is a great variety in the expression levels between cell types (Daar et al,1984; Guillemot et al,1988). Cell types such as fibroblasts, neural cells and muscle cells express low levels of class I antigens while cells of the immune system express high levels. Table 15 shows the distribution of class I antigens in normal tissues as reported by Daar et al, (1984).

# 5.1.2. REGULATION OF CLASS I GENE EXPRESSION BY CYTOKINES

### a) Regulation of class I gene expression by interferon

#### The major classes of interferons

Three classes of interferon (IFNs) each synthesized by different cell types are now recognized (Langer *et al*,1988).  $\alpha$ -interferon (IFN- $\alpha$ ) and  $\beta$ -interferon (IFN- $\beta$ ) are produced by leukocytes and fibroblasts respectively while  $\gamma$ -interferon (IFN- $\gamma$ ) is synthesized by T cells or activated macrophages. At least 14 IFN- $\alpha$  polypeptides encoded by a multi-gene family have been identified in man and are about 25% homologous with IFN- $\beta$ . IFN- $\gamma$  shows little homology with either IFN- $\alpha$  or IFN- $\beta$  (Langer *et al*,1988).

#### Elevation of class I expression by the interferons

Treatment of cells with IFNs leads to an elevated expression of many proteins, some of which are induced by all IFNs while others are specific for the particular IFN used. Increased levels of major histocompatibility complex proteins have been detected in a wide variety of cell lines after treatment with all types of IFNs (Fellous *et al*,1979; Heron *et al*,1978; Basham *et al*,1982) (table 16). For

example, human lymphoblastoid and fibroblast cell lines showed enhanced binding of monoclonal antibodies specific for class I molecules following exposure to IFN- $\alpha$  and IFN- $\beta$  (Fellous *et al*,1982). Furthermore, electrophoretic analysis of iodinated surface proteins showed directly that the amount of surface protein was increased rather than more molecules becoming exposed due to a change in the conformation of the cell membrane. This increase is preceded by an enhanced rate of synthesis and thus elevated steady state levels of class I specific mRNA, rather than by a decrease in turnover (Fellous *et al*,1982; Burrone *et al*,1982).

The enhancement of class I expression is thought to be specific since other cell surface markers do not change. For example, melanoma cell lines treated with IFN do not show elevated binding of the monoclonal anti-melanoma antibody, NU-4-B (Basham *et al*,1982) and treatment of the Raji B cell line increases expression of HLA-A, B, C antigens but not HLA-DR antigens (Burrone *et al*,1982).

#### b) Regulation of class I gene expression by TNF-α

 $TNF-\alpha$  increases the expression of class I antigens in normal and tumour cells in vitro

Recombinant human TNF- $\alpha$  increases the surface expression of class I antigens on human vascular endothelial cells, human dermal fibroblasts (Collins *et al*,1986) and rat glial cells (Massa *et al*,1987). Elevated surface expression has also been reported in the tumour cell lines Colo 205, SW 480 and SW 620 (Pfizenmaier *et al*,1987).

In most cells, increases in surface expression induced by TNF- $\alpha$  are preceded by a specific increase in the steady-state levels of class I mRNA; no expression of class II MHC genes is detectable at either the mRNA or protein levels (Collins *et al*,1986). TNF- $\alpha$  did not increase class I expression in the HLA-negative cell line K 562 but did enhance IFN- $\gamma$  induced HLA gene transcription (Pfizenmaier *et al*,1987). However, while treatment of Colo 205 cells with TNF- $\alpha$  did elevate class I antigen expression the steady state levels of mRNA did not increase. These results together suggest that TNF- $\alpha$  can enhance both constitutive and IFN- $\gamma$  induced HLA class I expression at either the transcriptional or post-transcriptional level of gene expression in some tumour cells.

#### TNF-\alpha does not increase the expression of class I antigens in vivo

Since TNF- $\alpha$  appears capable of altering gene expression in untransformed cell types, it has been suggested that TNF- $\alpha$  may have a similar effect *in vivo*. Weber *et al*, (1988) used tumour cells with varying basal levels of class I expression to study the effect of IFNs and TNF- $\alpha$  on the *in vivo* enhancement of MHC expression. Both IFN- $\alpha$  and IFN- $\gamma$  increased the level of class I but not class II antigens on the cell lines MCA-101(low basal class I expression), MCA-102,-106 (intermediate), and MCA-105 (high) *in vitro* whereas TNF- $\alpha$  had no significant effect. Tumours were generated by injection of these cell lines into mice and the effect of cytokines on the expression of class I within these tumours was assessed. Tumours generated from MCA-102 cells showed an increased class I but not class II expression when the mice were treated with IFN- $\alpha$  or IFN- $\gamma$  but not with TNF- $\alpha$ . None

of these cytokines could upregulate class I or class II expression on MCA-101 tumours in vivo (Weber et al, 1988).

# 5.1.3. CIS -ACTING REGULATORY ELEMENTS OF CLASS I GENE TRANSCRIPTION

### a) The promoter

Sequences that show homology with consensus eukaryotic promoter sequences are located in the 5' region of of MHC class I genes. Whilst murine class I genes have CAAT and TATAAA sequences located 50 and 25 bp upstream of the cap and transcription start site respectively (e.g., Kimura et al,1986), most human class I genes have the variant promoter sequence TCTAAA (e.g., Weiss et al,1985). Instead of the CAAT and TATAAA sequences, the HLA-Cw3 gene has the variant sequences CGGT and TCTGAA respectively (Sodoyer et al,1985). These variants have been suggested as responsible for the reduced expression of HLA-C compared with the levels seen for HLA-A and HLA-B (Guillemot et al,1988).

### b) Regulatory sequences in the 5' promoter region

Studies using deletion mutants and sequence comparisons have identified a number of regulatory sequences in the 5' promoter region. These are summarized in figure 24.

### Positive and negative control by the class I regulatory element

The region -195 to -165 bp upstream of the H-2L<sup>d</sup> cap site is responsible for a 4 to 5 fold decrease in transcription in undifferentiated F9 cells and is also capable of acting as a repressor of heterologous promoters irrespective of its orientation. After differentiation of F9 cells by retinoic acid, the repressor activity reverses so that it acts as an enhancer (Miyazaki et al,1986; Sugita et al,1987; Israel et al,1987). This region, which has also been shown to enhance transcription in NIH 3T3 and mouse 3T6 cells, has been designated the class I regulatory element (CRE). Thus, the CRE is a cisacting element that, depending on the developmental state, may act as a repressor or enhancer of class I transcription.

While this region is highly conserved between the classical human and murine class I genes, genes from the murine Qa region show less homology. Indeed, the Qa gene Q10 has three substitutions which reduce the enhancing activity (Kimura et al,1986) (figure 24). This region also shows homology with the GC rich region found upstream of the TATA box in a number of genes such as the SV40 enhancer, the HSV tk promoter, as well as the 21 bp repeats within the SV40 promoter.

#### The IFN responsive sequence

A 30 bp consensus sequence was identified by Friedman et al (1985) by comparing the 5' regions of human genes that had been previously shown to be inducible by IFN. The region '165 and '136 is essential for the induction of class I expression by IFNs (Kimura et al,1986; Reid et al,1989) and overlaps with the CRE (figure 24). This region is discussed in more detail below.

#### Enhancer B

Kimura *et al.* (1986) identified a region 3' of the CRE between nucleotides  $^{-}$  99 and  $^{-}$  61 that also has enhancer activity. This sequence, designated enhancer B, is also conserved between class I genes and shares some homology with the SV40 and HSV tk promoters. No homology is observed, however, with the  $\beta_2$ microglobulin 5' flanking region, even though this latter region has enhancer activity similar to that observed for the H-2K<sup>b</sup> promoter (Kimura *et al.*,1986).

### c) Sequences required for induction by interferon

### IFN- $\alpha/\beta$

Analysis of deletion mutants fused to a CAT gene has revealed that in the embryonic fibroblast cell line BLK SV and L fibroblasts, the induction of the murine class I promoter by IFN- $\alpha/\beta$  requires the presence of both the IRS and the CRE (Israel et al,1986; Korber et al,1988). In contrast, for the fibroblast cell lines NIH3T3 and BL5 the presence of the IRS alone was sufficient for induction by IFN- $\alpha/\beta$  (Sugita et al,1987; Korber et al,1988). When combined with the CRE, the IRS can function as an inducible enhancer of heterologous promoters. For example, promoters such as that for conalbumin (Israel et al,1986) or SV40 (Sugita et al,1987), can be made responsive to interferon  $\alpha/\beta$  if fused to the IRS and this occurs regardless of its orientation relative to the cap site (Israel et al,1987). However, not all enhancers can function in combination with IRS. Fusion of either enhancer B or the SV40 enhancer with the IRS did not permit induction of class I promoter activity by IFN $\alpha/\beta$  (Israel et al,1986).

#### IFN-Y

Although the IRS is involved in transcriptional regulation of class I genes by all IFNs (e.g., Israel et al,1986; Blanar et al,1989) recent studies indicate that different sequences within this region are independently involved in regulation by IFN- $\alpha$  and IFN- $\gamma$  (Korber et al,1987). In contrast to IFN- $\alpha/\beta$ , deletion of the CRE does not result in the loss of IFN- $\gamma$  inducibility of murine class I promoter activity in L cells (Korber et al,1987). This suggests that, unlike IFN- $\alpha/\beta$ , induction by IFN- $\gamma$  is independent of interactions between the trans-acting factors.

Other sequences outside the IRS appear to contribute to the response to IFN- $\gamma$  in L cells since this region alone accounts for less than 40% of the overall induction of expression (Korber *et al*,1987). In addition, the expression of H-2L<sup>d</sup> gene under the control of a non-IFN-regulated promoter can still be induced by IFN- $\gamma$ , indicating that a region 3' of the transcription initiation site, together with the IRS, controls the induction of class I promoter activity by IFN- $\gamma$ . The 3' regulatory sequence has been

suggested to function in post-translational processing by either increasing RNA stability or altering the rate of class I mRNA translation (Korber et al,1987).

## 5.1.4. TRANS-REGULATION OF CLASS I GENE EXPRESSION

The *cis*-acting regulatory sequences found in the 5' promoter region of class I genes are binding sites for *trans*-acting transcription factors. Such factors have been identified in assays where oligonucleotide probes specific for discrete regions of the 5' sequence are used to selectively bind nuclear proteins and the migration of these complexes through gels is retarded relative to the unbound probe. Competition experiments using mutant sequences and methylation interference identify bases within the binding site that are critical for binding the nuclear protein.

### a) Identification of CRE binding proteins in vitro

Nuclear extracts from L fibroblasts and the mouse T lymphocyte cell line LH8 contain binding activities for three distinct sequences within the CRE, namely regions I, II and III (Shirayoshi et al,1987) (figure 24). While binding to regions I and II has also been demonstrated in F9 cells, no correlation has been made between the presence of region III specific factors and the state of cellular differentiation (Shirayoshi et al,1987). The CRE is comprised of two overlapping 18 bp repeats as well as two 11 bp inverted repeats (Kimura et al,1986) and figure 24 shows that these repeats correspond to the three regions: region I extends from nucleotide ~161 to ~173; region III includes nucleotides ~161 to ~189 and thus overlaps region I; and region II is situated between ~185 to ~203.

Recent studies indicate that the sequence TGGGGATTCCCCA contained within region I is capable of binding a number of *trans*-acting factors. This sequence exhibits dyad symmetry centred at nucleotide ~166 and specific binding occurs with the four consecutive guanine residues from positions ~172 to ~169 in the coding sequence and from ~165 to ~162 in the non-coding sequence (Baldwin *et al*,1987). The nuclear factor H2TF1 from HeLa cells, mouse erythroleukemia cells and Balb/c fibroblasts has been shown to bind this sequence (Baldwin *et al*,1987) as well as the two factors KBF1 and KBF2 from mouse 3T6 fibroblasts and a murine thymoma cell line (Israel *et al*,1989). KBF2, but not KBF1 activity, was found in undifferentiated EC cells and upon differentiation KBF1 binding occurred (Israel *et al*,1989). The transcription factor NF-kB has also been shown to bind region I, recognizing six of the eight guanine residues contained within the binding site (Baldwin *et al*,1988). *In vivo* competition experiments and analysis of mutant sequences indicate that binding of a *trans*-acting factor to the enhancer sequence is necessary for the basal expression of the class I promoter (Israel *et al*,1987; Baldwin *et al*,1987).

The central portion of region II has been proposed as the binding site since methylation of the guanine residues from -200 to -191 of both strands interfered with binding (Shirayoshi *et al*,1987) (figure 24). Although factors that bind region II have not been as extensively studied as region I binding factors, conflicting reports about purified AP-1 protein binding to region II have been published (Korber *et al*,1988; Burke *et al*,1989).

The two areas between ~189 to ~179 and ~173 to ~162 both appear to be essential for binding to region III and are contained within each half of the large inverted repeat (Shirayoshi *et al*,1987) (figure 24).

### c) Identification of CRE binding proteins in vivo

In vivo studies of Balb/c mice showed that murine tissues also contain proteins that bind discrete subregions of the CRE (Burke et al,1989). Using probes for each subregion, it was found that prior to birth only region II binding activity was observed and that at the time of birth, the appearance of a region I binding protein coincided with increased class I mRNA levels. This finding supports the notion that binding of a trans-acting factor to region II is primarily responsible for low expression levels such as that seen in fetal tissues while region I binding activity is involved in controlling developmental expression of class I gene.

Tissue specific class I gene expression in the adult appears to be more complex than developmental expression. Comparisons of adult tissues showed that binding of a trans-acting factor to region II was independent of class I gene expression whereas region I binding activity was detected only in tissues that express class I genes at relatively high levels (Burke et al,1989). However, both of these regions are capable of enhancing class I gene expression with neither region alone exerting a full effect. In adult brain tissue where class I expression is not detectable, little or no region I specific but high levels of region II specific binding protein was observed. It has been suggested that tissue specific expression in vivo is due to either chromatin structure or the DNA methylation pattern. Thus binding to region I in the brain may be inhibited in vivo. Alternatively, a negative trans-acting factor which represses transcription of class I genes may be present in these tissues (Burke et al,1989). As discussed in section 5.1.3., negative regulation of class I gene expression has been observed in undifferentiated F9 cells (Miyazaki et al,1986). It is possible that the factor KBF2 may act as a negative trans-acting factor since binding by this protein is only observed in the undifferentiated state and is displaced by KBF1 upon cell differentiation (Israel et al,1989).

In contrast to observations *in vitro*, no factor has been observed that bound region III *in vivo* (Burke *et al*,1989). Since the expression of this activity *in vitro* shows cell type specificity it is not clear why no expression is observed *in vivo*.

### d) Trans-acting factors bind to the IRS upon treatment with interferon

### IFN-α

Nuclear proteins that bind the IRS upon treatment with IFN- $\alpha$  have been identified (Shirayoshi *et al*,1988). Binding of a constitutive *trans*-acting factor (B1) to the IRS of the H-2L<sup>d</sup> gene was observed in the mouse T cell line LH8 and Ltk- fibroblasts regardless of IFN treatment. Within 1 hour of IFN $\alpha$ / $\beta$  treatment, however, two new binding activities (B2 and B3) were observed. Both the constitutive and induced factors appear to bind the same region between residues ~152 and ~143 (figure 24).

The synthesis of the factor B2 occurs in the presence of cycloheximide suggesting that a preexisting protein, possibly the constitutive factor B1, undergoes post-translational modification to become B2 which then induces class I transcription in response to treatment with IFN- $\alpha/\beta$  (Shirayoshi et al,1988). Binding by B3 was inhibited by cycloheximide, indicating that it is synthesized de novo.

Specific binding of the induced factor to the IRS appears to be directly responsible for the induction of expression by IFN- $\alpha/\beta$  since mutations within the binding site but not in other regions abolished activation of class I genes by IFN. Furthermore, the binding motifs of other IFN-inducible genes, for example 2'-5' oligoadenylate synthetase, competed for the binding site of the class I promoter (Shirayoshi *et al*,1988). The consensus sequence AGTTTCNNT(C)TC(T)CT has been proposed as the binding motif for IFN inducible genes (Shirayoshi *et al*,1988).

#### IFN-γ

Binding of a nuclear protein (IBP-1) to the IRS of the H-2K<sup>b</sup> promoter has been observed in HeLa cells treated with IFN-γ (Blanar et al,1989). Methylation of the guanosine residues at positions - 144 and -146 of the non-coding strand only interferes with this binding. While residue -146 is common to the IRS binding sites involved in induction by both IFN-α and IFN-γ, the other residues are not, indicating that the binding sites of the trans-acting factors induced by these IFNs are different. Korber et al, (1988) used DNase I protection assays to show binding of one or more nuclear factors in the mouse myeloma cell line MPC 11 to H-2D<sup>d</sup> promoter sequences after treatment with IFN-γ. Similar to the findings of Blanar et al, (1989), binding was observed to the IRS region between -157 to -140 but, in addition, binding occurred between -205 to -188 and -108 to -90 which are homologous to region II of the CRE and enhancer B of the H-2K<sup>b</sup> gene respectively (Kimura et al,1986). Further, binding was observed prior to treatment of MPC 11 cells with IFN-γ, suggesting that these cells constitutively express this binding activity (Korber et al,1988).

In contrast to class I induction by IFN- $\alpha$ , the IBP-1 binding sequence alone is not sufficient to confer IFN- $\gamma$  responsiveness to heterologous promoters (Blanar *et al*,1989). This is in agreement with the finding that a sequence 3' of the initiation site is also required for induction by IFN- $\gamma$  (see section 5.1.3.). Further, IBP-1 appears to be synthesized *de novo* in response to IFN- $\gamma$  since treatment with cycloheximide blocked induction of its DNA binding activity.

Sequence comparisons of genes responsive to IFN- $\alpha/\beta$  and/or IFN- $\gamma$  have revealed similarities between upstream sequences of the HSP-70, Igk and IFN- $\beta$ 1 genes and the H-2K<sup>b</sup> promoter IBP-1 binding site, yet competition experiments using probes derived from the homologous regions of these genes showed that only the IFN- $\beta$ 1 sequence bound to the IBP-1 binding site (Blanar *et al*,1989). Therefore, IFN- $\alpha$  and IFN- $\gamma$  appear to stimulate production of different nuclear factors which bind to slightly different regions of the class I IRS,

### f) TNF-α induces production of a NF-κB-like factor

Primary T lymphocytes treated with TNF- $\alpha$  show elevated levels of NF- $\kappa$ B like activity (Lowenthal *et al*,1989). The expression of the interleukin-2 receptor- $\alpha$  promoter was shown to be enhanced by TNF- $\alpha$  in normal human T lymphocytes at physiological concentrations i.e., 1-100 U/ml.

Deletion of bases within a 12 bp NF- $\kappa$ B-like element abolished this induction. As discussed previously, the NF- $\kappa$ B binding site shares a high degree of sequence homology with class I major histocompatibility complex antigens and  $\beta_2$ microglobulin. Although the presence of cycloheximide did not inhibit the production of TNF- $\alpha$  in this system, cycloheximide does block its induction of class I mRNA expression. While this suggests that TNF- $\alpha$  does not induce binding of NF- $\kappa$ B per se, it is possible that TNF- $\alpha$  may alter the expression of class I genes by the activation of NF- $\kappa$ B-like transcription factors.

### 5.1.5. AIM

As discussed in chapter 1, aberrant expression of the MHC has been suggested to be involved in the development of autoimmune disease (Bottazzo et al,1983; Hanafusa et al,1983). Studies of the regulation of MHC expression by immune modulators have shown that IFN- $\gamma$ can induce class II expression in normally non-expressing thyrocytes, suggesting that abnormal MHC expression due to inappropriate regulation by cytokines may be involved in disease development (Todd et al,1985). It seems possible that aberrant regulation of class I expression by IFNs and other cytokines such as TNF- $\alpha$  may alter recognition by class I restricted, autoreactive cytotoxic T lymphocytes.

In order to study the regulation of HLA-B27 expression, EBV immortalized B cell lines were established from two HLA-B27+AS+ patients, one HLA-B27-AS+ and one HLA-B27+AS- control. The effect of IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  on class I expression by these cell lines was analyzed by antibody staining with the anti-class I monoclonal antibody W6/32 and Northern hybridization with the pB27-3'UT probe as well as an oligonucleotide probe derived from a region conserved between HLA-B27 subtypes but not between HLA-B alleles.

### 5.2. RESULTS

# 5.2.1. MORPHOLOGY OF EBV IMMORTALIZED CELL LINES

EBV immortalized B lymphoblastoid cell lines were established from peripheral blood lymphocytes isolated from one HLA-B27 negative (J:B27-AS+) and two HLA-B27 positive AS patients (i.e., B:B27+AS+ and E:B27+AS+) as well as one HLA-B27 positive unaffected individual (M:B27+AS-). The B:B27+AS+, E:B27+AS+ and J:B27-AS+ cell lines grew as expected for B lymphoblastoid cells: the cells were quite small and grew as clumps in suspension with an average doubling time of 24-48 hours. In contrast, the HLA-B27 positive cells taken from a healthy individual (M:B27+AS-) were larger and grew much faster as a single cell suspension with a doubling time of 12 hours. The morphologies of these cell lines are compared in figure 25.

# 5.2.2. REGULATION OF ENDOGENOUS HLA CLASS I EXPRESSION BY CYTOKINES

Since ankylosing spondylitis may result from aberrant regulation of HLA-B27 gene expression, attempts were made to study the regulation of this gene by cytokines within cell lines derived from patients and controls.

### a) Cytokine levels used

Many studies have been published describing the effects of IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  on class I expression. Some of these were compared and from these suitable concentrations of these cytokines were determined in order to study their effect on class I expression in B lymphoblastoid cells (table 16). The following concentrations were chosen as they appeared sufficient for a response at both the mRNA and protein levels: IFN- $\alpha$  400 U/ml; IFN- $\gamma$  200 U/ml; TNF- $\alpha$  20 U/ml. mRNA levels were assessed after 6 hours and protein levels determined after 48 hours of treatment. While TNF- $\alpha$  is cytotoxic for many cell types (Sugarman *et al*,1987), no such activity was observed against the cell lines used here at 10 U/ml or 50 U/ml after 48 hours (data not shown).

### b) Surface expression of class I antigens

The effect of each cytokine on the surface expression of class I protein was assessed by the level of binding of fluoroceinated anti-class I antibody W6/32. The amount of antibody bound to the cell surface was measured by flow cytometry. Figure 26 and table 17 shows the response of the patient cell lines to treatment with each cytokine.

The M:B27<sup>+</sup>AS<sup>-</sup> cell line did not appear to express class I protein at its surface (table 17). Further, treatment with any of the cytokines had no effect on the expression of class I antigens at the cell surface.

The two HLA-B27 positive cell lines B:B27+AS+ and E:B27+AS+ both expressed surface class I protein prior to cytokine treatment (table 17; figure 26a,b). The E:B27+AS+ cell line expressed higher levels of class I surface antigens than B:B27+AS+ and did not respond to cytokine treatment to

the same degree as B:B27+AS+ (figure 26). The surface expression of class I antigens on both cell lines was marginally increased by IFN- $\alpha$ . Treatment with IFN- $\gamma$  also increased the surface protein levels although B:B27+AS+ responded to a much greater degree than did E:B27+AS+. Only slight increases in surface class I antigen levels were observed following exposure of both cell lines to TNF- $\alpha$ . Treatment with IFN- $\gamma$  and TNF- $\alpha$  together increased the levels of surface proteins to a higher level on E:B27+AS+ than observed for either cytokine alone. This suggests that these cytokines have a synergistic effect on the enhancement of class I expression in this cell line. This was not observed for the B:B27+AS+ cell line following such treatment: the levels did not reach that observed for IFN- $\gamma$ .

The J:B27-AS+ cell line also expressed surface class I antigen (table 17). In contrast to the two HLA-B27+ patient cell lines, expression of class I proteins decreased following treatment of J:B27-AS+ with IFN- $\alpha$ . The levels increased markedly in response to IFN- $\gamma$  while only a slight increase was observed with TNF- $\alpha$  (table 17, figure 26c).

### c) Class I mRNA expression

### Hybridization with the HLA-B27 3'UT region probe

The effect of the cytokines on the expression of HLA-B27 was examined by hybridizing total RNA prepared from the cytokine treated cell lines with the HLA-B 'specific' 3'UT region probe. This probe which detects a class I mRNA species of the expected 1.6Kb is described in more detail in section 7.2.3.a. The results are shown in figures 26 and 27 and table 17. To quantitate the hybridization signal of class I specific mRNA, the lanes were scanned with a densitometer and the results expressed as the amount of class I mRNA relative to the amount of β-actin mRNA (table 17). This probe detected an mRNA of 1.9Kb. The change in class I mRNA expression was then determined relative to the untreated control (table 17). These values were then plotted as relative levels for each cytokine treatment (figure 26). The Northern hybridization signals observed for each cell line are shown in figure 27. In agreement with that observed for the surface expression of class I, each cell line responded differently to cytokine treatment at the RNA level.

It can be seen that the expression of class I mRNA in B:B27<sup>+</sup>AS<sup>+</sup> cells (figures 26a, 27a; table 17) was elevated in response to treatment with both IFN-α and IFN-γ whereas slight decreases were observed with TNF-α and IFN-γ/TNF-α together. The increases observed with the IFNs did not reflect the changes observed at the protein level. The levels of class I mRNA were enhanced from 1.00 to 1.70 following IFN-α treatment which was greater than that observed for the surface protein. IFN-γ, on the other hand increased the amount of class I protein to a greater degree than the corresponding mRNA: twice as much protein was detected following IFN-γ treatment whereas the mRNA increased from 1.00 to 1.65. The levels of surface protein following treatment with TNF-α and IFN-γ/TNF-a together were greater than that of the class I mRNA. The amount of mRNA following these treatment were less than observed for the untreated cells while the protein levels were marginally elevated compared with the untreated cells..

Expression in E:B27+AS+ cells at the RNA level, on the other hand, was only slightly enhanced after treatment with IFN- $\alpha$  from 1.00 to 1.11 (figures 26b, 27b; table 17). While IFN- $\gamma$  and

TNF- $\alpha$  did not appear to effect mRNA expression levels in E:B27<sup>+</sup>AS<sup>+</sup> cells, these two cytokines together increased class I mRNA expression significantly. This increase was slightly greater than that observed at the level of surface antigen: an increase of 1.00 to 1.44 compared with 1.00 to 1.27 respectively.

In contrast to the HLA-B27 positive patient cell lines, class I mRNA levels, were decreased in J:B27-AS+ by IFN- $\alpha$  but increased by IFN- $\gamma$  and TNF- $\alpha$  (figures 26c, 27c; table 17). Although the class I mRNA levels were decreased by IFN- $\alpha$  from 1.00 to 0.47, the amount of surface protein was increased by this cytokine from 1.00 to 1.60. IFN- $\gamma$  increased the class I mRNA level markedly from 1.00 to 2.09 whereas the protein only increased slightly. The increased mRNA level observed with TNF- $\alpha$  was marginal (1.00 to 1.29) compared with the protein level (1.00 to 1.65). Although the effect of IFN- $\gamma$ /TNF- $\alpha$  on class I mRNA levels was not determined for this cell line, an slight increase was observed at the protein level (1.00 to 1.32).

The RNA isolated from M:B27+AS- did not hybridize to the pB27-3'UT probe although there was quite clearly RNA present on the blot since hybridization to  $\beta$ -actin was observed (figure 27d). This is in agreement with the lack of class I expression observed at the cell surface of these cells.

### Hybridization with an HLA-B27 specific oligonucleotide probe

In order to examine the effect of IFNs and TNF- $\alpha$  on the expression of the HLA-B27 mRNA specifically, class I gene sequences were compared and the region within exon 2 from nucleotide 913 to 941 was shown to be conserved between HLA-B27 subtypes but not between other HLA-B alleles or class I loci. This sequence was chosen for an oligonucleotide probe (chapter 7, figure 36). A search against the Microgenie human data bank revealed no homologous sequences with up to 4 mismatches other than HLA-B27. This pB27-oligo probe was therefore considered specific for HLA-B27. After checking that the majority of oligonucleotide sequences synthesized were of the correct size and not contaminated with incomplete sequences (figure 28), the probe was used to hybridize the same Northern blots that had been previously probed with pB27-3UT.

Using the formula Tm=4x(G+C) + 2x(A+T) for DNA-DNA hybrids in 6xSSC (Mason *et al* (1985), the Tm of this 18mer oligonucleotide was calculated as  $56^{\circ}$ C. Hybridization was carried out at  $10^{\circ}$ C below this value (46°C) and the filters washed at increasing temperatures to remove imperfect hybridization matches. By comparing the hybridization patterns of this probe with that of the pB27-3'UT and  $\beta$ -actin probes, it was clear that the oligonucleotide hybridized to RNA of the same size as that detected by the pB27-3'UT probe i.e., 1.6 Kb (figure 27). It is likely therefore that the oligonucleotide hybridized specifically to class I mRNA. However, increasing the stringency to above the hybridization temperature at  $52^{\circ}$ C did not remove the probe from the HLA-B27 negative RNA.

The regulation of class I mRNA as detected by the pB27-oligo probe appeared to be different from that observed with the pB27-3'UT probe (figures 26,27; table 17). Class I mRNA expression in the B:B27<sup>+</sup>AS<sup>+</sup> cell line seemed to be unaffected by IFN- $\alpha$  but was decreased by IFN- $\gamma$ , TNF- $\alpha$  and a combination of these two. Treatment of E:B27<sup>+</sup>AS<sup>+</sup> and J:B27<sup>-</sup>AS<sup>+</sup> with all the cytokines decreased mRNA expression.

# 5.2.3. EXPRESSION OF THE HLA-B27 PROMOTER IN CELL LINES

The Northern blot analysis discussed above did not seem to permit analysis of how the HLA-B27 gene is regulated by cytokines relative to other HLA-B genes. A more direct approach was therefore attempted by fusing the promoter region from the HLA-B27 gene of an affected individual (see chapter 4) to the reporter gene chloramphenical acetyl transferase (CAT). By introducing such a construct into the EBV immortalized cell lines and selecting positive transfectants with G418 resistance, it should be possible to analyze the regulation of HLA-B27 from an AS patient directly.

### a) pB27CAT construct

The HLA-B27 promoter region from the gene of disease haplotype isolated as described in chapter 4 was ligated to a vector carrying the CAT gene. This vector also carried the aminoglycosyl 3' phosphotransferase (AGPT) gene of Tn5 for resistance to G418 and the ampicillin resistance gene. By having the CAT gene under the control of the HLA-B27 promoter, the activity of this region could be assessed by assaying for CAT activity. In addition, G418 resistance would allow stable cell lines to be established by positive selection of transfected cells. The cloning strategy of pB27CAT is outlined in figure 29.

pB27CAT was constructed in two stages. Firstly, the vector pTCFCAT was prepared as shown in figure 29a. The 2200bp\*HindIII/BamH1 fragment of the cosmid pTCF (Grosveld et al, 1982) was replaced with the 1650bp HindIII/BamH1 fragment from pSV0CAT that contains the CAT gene (Gorman et al,1982). Secondly, the HLA-B27 promoter was isolated as an HindIII/Nar1 fragment from the cloned HLA-B27 gene as shown in figure 29b. The Nar1 site was located 12bp downstream from the HLA-B27 ATG translation initiation codon. This was the only unique restriction endonuclease site within this region. However, it was thought that the presence of a protein synthesis initiation signal from the HLA-B27 gene in addition to the initiation codon from the CAT gene would obscure the expression of enzymatically active CAT. Thus attempts were made to eliminate the HLA-B27 initiation signal by incubating the Nar1 digested fragment with the Large fragment of DNA polymerase in the presence of dTTP followed by a second incubation with only dATP present by making use of the 3'→5' exonuclease activity of the Klenow fragment. The single stranded 5' overhang was removed by digestion with mung bean nuclease. Following addition of HindIII linkers, the promoter was ligated into the pTCFCAT vector.

To ensure that the clone used for transfections had the promoter in the correct orientation, Xba1/Bam H1 double digests were performed on minipreparations of pB27CAT DNA. The expected fragments for each orientation are shown in figure 29c. With the promoter in the correct orientation (pB27CAT-1), fragments of approximately 6300 bp and 1600 bp were expected (lane 1) compared with fragments of approximately 5500 bp and 2400 bp for the opposite orientation (pB27CAT-2) (lane 2).

The smaller Xba 1/Bam H1 fragment of pB27CAT-1 was sequenced from the Xba 1 site to 442bp into the CAT gene in order to determine if the cloning strategy was successful. It was discovered, however, that the ATG initiation codon from the HLA-B27 gene had not been removed. Although, the reading frame of the CAT gene had not been altered, it was predicted that the extra HLA-

B27 amino acid sequence would inactivate the CAT protein (figure 30a). However, it was thought that this protein might display a low level of CAT activity when compared with the vector pTCFCAT. In order to test this, both pB27CAT-1 and pTCFCAT were transfected into HeLa cells and, indeed, some CAT expression from the HLA-B27 promoter was observed (figure 30b). When compared with 57% observed for the positive control pSV2CAT, the conversion of chloramphenicol to the reaction product acetylchloramphenicol was low at 1.3%. However, the CAT activity level of pB27CAT-1 was four times that observed for the promoterless pTCFCAT. The conversion level for pTCFCAT was the same as that seen for the similarly promoterless negative control pSV0CAT.

### b) Transfection of suspension cells by electroporation

In order to analyze the regulation of the HLA-B27 promoter from an AS patient, the pB27CAT-1 construct would have to be transfected into EBV immortalized cell lines. Transfection of suspension cells by calcium phosphate precipitation is known to be inefficient (Andreason *et al*, 1988), therefore attempts were made to set up conditions for transfection by electroporation. The cell line B:B27+AS+ was electroporated at varying voltages and capacitances in order to determine under what conditions pSV2CAT activity and therefore transfection was at an optimum

As a general rule, effective electroporation occurs at a point where the cell viability is about 20% (Andreason *et al*,1988). Therefore, as a relatively inaccurate measure of estimating the appropriate electroporation voltage and capacitance, the point at which the viability of the cells was 20% was determined. However, when assaying for CAT activity using a diffusion assay, transfection did not appear to have taken place under these particular conditions (data not shown). For example, at a capacitance setting of 25µFd, 20% of the cells remained viable at about 750V (figure 31a) yet no CAT activity was observed. Indeed, transfection did not occur at other capacitance and voltage settings when the viability was 20%. For example, when the cells were electroporated at a density of 4x10<sup>6</sup> cells/ml HBS, 20% viability was observed at 250µFd, 390V and 500µFd, 350V (figure 31b,c). Burkitt's lymphoma cells, on the other hand, are successfully transfected by electroporation at 960µFd, 250V at a density of 8x10<sup>6</sup> cells in 250µl of RPMI-1640 (M.Patel, personal communication). While about 20% viability was observed under these conditions (figure 31d), no transfection of the EBV immortalized B lymphoblastoid cells was achieved under these conditions.

### c) G418 sensitivity of EBV immortalized B lymphocytes

If conditions for transfection of the B cell lines by electroporation had been successfully determined, the cells would have been stably transfected with pB27CAT-1 and the regulation of CAT activity by cytokines assessed. The positive transfectants would have been selected for by their resistance to G418. Whilst the electroporation conditions were being examined, the sensitivity of the EBV immortalized cell lines to G418 was determined. The B:B27+AS+ cells were exposed to increasing concentrations of G418 and their viability assayed over a period of two weeks (figure 32). Since the appropriate concentration of G418 for selection of stable cell lines is the minimum which kills 100% of control cells after 7-10 days (Andreason *et al*,1988) it can be seen that the cells used in this study would be killed by 1000U/ml (figure 32).

### 5.3. DISCUSSION

Attempts were made to determine if AS is the result of aberrant regulation of the HLA-B27 gene by immune modulators. Sequencing of an HLA-B27 gene of the disease haplotype did not reveal any differences within the characterized regulatory regions when compared with a gene isolated from a healthy individual (see chapter 4). This suggests either that the expression of the HLA-B27 gene in AS patients may be due to abnormalities outside the regulatory region sequenced or that differences exist in the cellular factors responsible for the control of HLA-B27 expression. Studies were performed in order to test the second possibility. In order to test if HLA-B27 expression is regulated normally by immune modulators, attempts were made to compare the response of class I expression in patient and control cells to the cytokines IFN-α, IFN-γ and TNF-α. EBV immortalized B lymphoblastoid cell lines were derived from HLA-B27 positive and negative patients and an HLA-B27 positive healthy individual and the level of class I expression within these cells was assessed at both the protein and mRNA levels following cytokine treatment.

While many studies have shown IFN-α and IFN-γ to be potent enhancers of class I expression in lymphoid cells, the cell lines used here did not respond equally to such treatment. For example, while the E:B27+AS+ and B:B27+AS+ cell lines are both derived from HLA-B27+ patients, their response to treatment differs. E:B27+AS+ expressed much higher basal levels of class I than B:B27+AS+ and differences in expression levels before and after treatment of E:B27+AS+ were smaller than that observed for B:B27+AS+. EBV immortalized B lymphocyte cell lines already express very high levels of HLA class I surface antigens (L.MacKenzie, personal communication) so that attempts to look at induction of their expression by cytokines is difficult since any enhancement will only be slight over a very large background. Therefore, minor increases in expression levels could be easily missed. It is likely that the different responses of E:B27+AS+ and B:B27+AS+ are due to the high expression levels observed for E:B27+AS+.

The J:B27-AS+ cell line responded differently to cytokine treatment when compared with the other cell lines. In particular, IFN-α reduced class I mRNA while elevating surface class I expression. On the whole, however, the induction of class I mRNA as detected with pB27-3'UT seemed to correlate well with induction of surface antigen. Although the steady state levels of class I mRNA was measured in this study, this correlation is in agreement with the induction of class I protein expression by interferons being the result of elevated transcription rates (Guillemot *et al*,1988).

The M:B27<sup>+</sup>AS<sup>-</sup> cell line did not express class I at either the mRNA or protein level. The morphology and growth kinetics of these cells were clearly different from the others, suggesting that it was not actually a B cell line. Although this was not confirmed, it is known that expression levels differ between cell types therefore it is possible that the lack of class I expression may be due to the cell type. Since M:B27<sup>+</sup>AS<sup>-</sup> grew as a suspension culture, it is likely that this cell line was of the haemopoetic cell lineage. As these cells are known to express class I, it was therefore unexpected that no class I expression was observed. If these cells were of early B cell origin, it is possible that the methylation pattern of the class I genes was inappropriate for expression. Furthermore, the EBV integration site may have inactivated the expression of the class I genes.

Each cell line responded differently to cytokine treatment: EBV immortalization clearly results in lymphoblastoid cell lines with different phenotypes. Consequently, it is possible that this immortalization results in differing responses of each cell line to cytokine treatment. The levels of cytokines and/or the incubation times chosen for this study may have been inappropriate for the cell lines used. Clearly, a much larger panel of cell lines derived from both patients and healthy controls would have to be studied for any conclusions about the regulation of HLA-B27 gene expression in AS patients to be reached with any certainty. Since the expression of class I genes in B cells may not be of direct relevance to the development of AS, it would be necessary to study such regulation in many different cell types, in particular synovial fibroblasts from the sacro-iliac joints.

While the effect of cytokines on the expression of HLA-B27 in these cell lines was of most interest, it proved difficult to study the endogenous levels of this particular allele specifically. For example, the monoclonal antibody W6/32 recognizes an epitope common to all HLA-A, -B, -C antigens. No antibodies that specifically recognize HLA-B27 were available for this study so that only the effect of cytokines on surface class I expression generally could be assessed. In addition, the pB27-3 UT probe is, as described previously, not truly HLA-B locus specific. In order to overcome this, an oligonucleotide probe was designed that was believed to show such specificity for HLA-B27. This was found not to be the case since all HLA-B allele mRNAs remained bound to the pB27-oligo probe. Hybridization conditions at which only HLA-B27 mRNA remained stably bound to the oligonucleotide probe but not to other HLA-B allele mRNAs could not be determined.

The effect of cytokines on the expression of class I mRNA as detected by the pB27-oligo probe differed from that detected with the pB27-3'UT probe. Since the pB27-3'UT is not specific for the HLA-B locus, this probe is most likely detecting the effect of treatment on all class I mRNAs. The pB27-oligo probe, while not specific for the HLA-B27 allele, may be specific for the HLA-B locus. Thus the effects of IFNs and TNF-α observed on the mRNAs detected with this probe may reflect changes in the expression of HLA-B alleles specifically. Hakem *et al* (1989) showed that the expression of HLA-B7 and HLA-A3 were differentially regulated by IFN-α. Treatment of Jurkat T cells with IFN-α increased the the expression of HLA-B7 while expression of HLA-A3 remained unchanged. While these alleles were found to respond to the same degree to IFN-α in EBV immortalized cell lines (Hakem *et al*, 1989) it is possible that the regulation of HLA-B alleles in B lymphoblastoid cells from AS patients differs from that in other B lymphoblastoid cell lines. It is possible that the response of different class I alleles to cytokine treatment may be of relevance in the synovial fibroblasts of AS patients.

Attempts were made to look specifically at the control of HLA-B27 expression by fusing the HLA-B27 promoter to the reporter CAT gene. A vector was designed that would be suitable for studying the expression of a CAT gene driven by a heterologous promoter within mammalian cells. This construct also carried the amino glycosyl 3' phosphotransferase (AGPT) gene under thymidine kinase promoter control for selection of stable transfectants with G418.

The 5' region of the HLA-B27 gene of disease haplotype was fused to this CAT vector. This 5' region had been previously shown to be all that is required for induction of class I gene expression by IFNs (Chamberlain *et al*, 1988). Sequencing of the pB27CAT-1 construct revealed that the ATG

translation initiation codon of the HLA-B27 gene had not been removed. Attempts were made to exploit the 3' $\rightarrow$ 5' exonuclease activity of the Klenow fragment by limiting the concentration of free nucleotides present in the reaction mix. By having only dTTP present, the Klenow fragment should have removed nucleotides in a 3' $\rightarrow$ 5' direction until it reached the first T where it would continually replace this residue with those T's in the reaction mixture. Replacing dTTP with dATP, the enzyme should then have removed nucleotides until the first A residue found in the ATG initiation codon. Although this cloning strategy was not completely successful, the reading frame of the CAT gene was not altered so that no premature stop codons would have been generated.

While the effect of the additional HLA-B27 sequence was not determined, a low level of expression of the pB27CAT-1 plasmid was observed. However, the enzyme activity of this fusion protein was reduced when compared with that of pSV2CAT. Either a small number of translation events were initiating from the ATG codon of the CAT gene resulting in the synthesis of a small amount of functional CAT protein or the B27CAT fusion protein had some low residual activity. It is possible that this extra amino acid sequence affected the enzyme activity by altering protein folding or the stability of the fusion protein may have been different from that of the CAT enzyme. Expression of this construct would probably be improved if the HLA-B27 ATG codon was removed. T4 DNA polymerase may have been the enzyme of choice for the removal of nucleotides since the 3'→5' exonuclease activity of this protein is more active than that of the Klenow fragment.

Suspension cells in culture are inefficiently transfected using calcium phosphate precipitation (Andreason et al, 1988). However, attempts to improve the efficiency by using electroporation did not prove to be successful. Transfection by electroporation is dependent on many variables such as the condition and concentration of the cells, the electroporation medium, the DNA used as well as the voltage and capacitance. While many different voltages and capacitances were explored here, little attempt was made to alter other parameters. It is possible that either the type and/or the volume of the electroporation medium used was inappropriate. For example, Lowenthal et al (1989) successfully transfected human PBMC at 960µFd, 250V with only 250µI of cells resuspended at 4x10<sup>7</sup> cells/ml of growth medium. Hepes buffered saline is the highest resistance buffer recommended for use with the BioRad Gene Pulser and, consequently, may not be the medium of choice as it may affect the fragility of the cells by generating too much heat (BioRad Gene Pulser manual). In addition, the density of cells may have been too low for successful transfection. Increasing the number of cells exposed to the electric pulse would increase the chances of transfection occurring. Further, while DNA was added to the cells at a concentration within the range suggested i.e., 10µg/ml, no carrier DNA was included. Transfection efficiency may have been improved had carrier DNA been present. Further, supercoiled DNA was used for the transfections. While this form of DNA is appropriate for transient transfections, linear DNA gives a higher efficiency of stable transfections. It is likely that assaying for successful transfection using a transient system may not be appropriate when the goal is to establish stable cell lines. Clearly, more parameters would need to be studied for transfection by electroporation to be considered a useful technique for establishing stable B lymphoblastoid cell lines.

A diffusion assay was used for determining CAT activity in the electroporated EBV immortalized cell lines. This assay is based on the differential phase solubilities of the cofactor

AcetylCoA and the reaction product acetylchloramphenicol. It is a very rapid means of determining CAT activity, however, since it depends on separation of phases, any jostling of the reaction vessel can disrupt this separation. Consequently, the background of these reactions tended to be very high, reducing sensitivity of the assay. Assaying for CAT activity is much more sensitive using the standard thin layer chromatography procedure based on the differential mobilities of the substrate chloramphenicol and the reaction products. This assay technique may have been the method of choice since the levels of CAT activity in the electroporated EBV cell lines was very low, if present at all.

No attempt was made to assess the possibility that AS is the result of abnormalities within unknown regulatory sequences. In order to test this, the HLA-B27 promoter of both normal and disease haplotypes fused to a CAT gene could be transfected into HeLa cells. The effect of cytokine treatment on the regulation of each of these promoters could then be determined.

While attempts to study the regulation of HLA-B27 expression in AS cells compared with normal cells have not been successful, foundations have been laid for further studies in this area. Many more variables would need to be explored in order to establish B lymphoblastoid cell lines stably transfected with the CAT construct carrying the HLA-B27 promoter of disease haplotype. The optimum levels of cytokines for the induction of class I expression at both the RNA and protein levels would be determined before studying the regulation of this HLA-B27 promoter in many different cell lines derived from patients and healthy subjects.

### 5.4. FIGURES AND TABLES

Table 15: Detailed tissue distribution of HLA-A, B, C antigens in normal human tissues

Tissue	Staining with anti- HLA-A,B,C
Cells of the endocrine system	
Thyroid (follicular, parafollicular)	$_{+}b$
Parathyroid	+
Pituitary (acidophils, basophils, chromophobes)	+
Pancreatic islets of Langerhans Adrenal	+
3 cortical zones	$^{++}a$
Medulla	++
Gastrointestinal tract: Epithelium of	
Tongue	++
Esophagus	++c
Stomach	тт
Fundus	+
Antrum	· +
Duodenum	++
Brunners glands	+/-d
Ileum	++
Appendix	++
Colon	++
Rectum	++
Gall bladder	+
Liver:	
Sinusoidal lining cells	++
Hepatocytes	+/-
Biliary epithelium	++
Respiratory and cardiovascular systems: Epiglottis	
Lingual surface epithelium	++
Mixed glandular tissue	++
Trachea	
Surface epithelium	++
Mixed glandular tissue	++
Tonsillary epithelium	++
Lung (bronchial and alveolar epithelium) Heart	++
Myocardium	+
Intercalated discs	++
Endothelium	
Capillaries	++
Larger vessels	++
Nervous system:	
Peripheral Control	++
Central	
Neurones Occasional unidentified cell	<del>-</del>
	++
Dura Uraganital system:	++
Urogenital system: Kidney glomeruli (endothelium, mesangium)	++
Kidney tubules	++
Epithelium	тт
Ureter	++
J1004	, ,

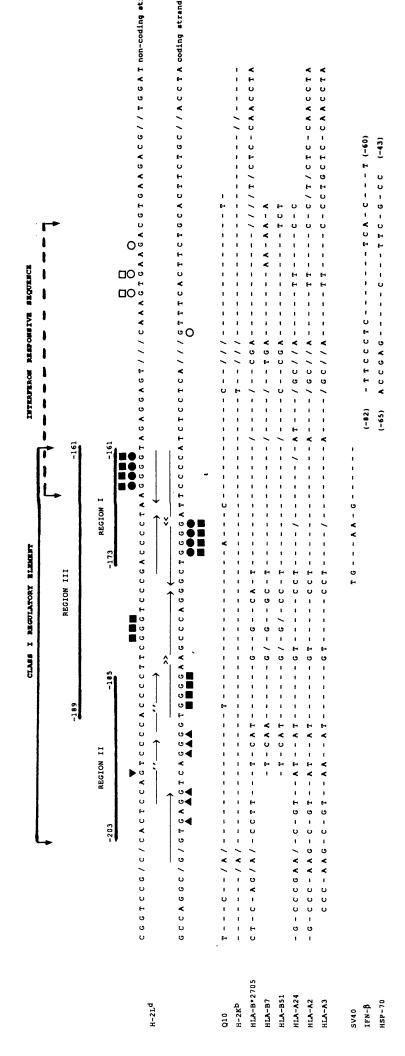
Bladder	++
Prostate	++
Urethra	++
Testis	
Gern cell, spermatozoa	+
Sertoli and Leydig cells	+
Epidydymis	
Epithelium	+
Spermatozoa	-
Miscellaneous:	
Breast epithelium	
Ductal Glandular	++
	++
Pancreas  Execution mostion	
Exocrine portion	<del>-</del>
Ductal epithelium Parotid	++
Acinar epithelium	
Ductal epithelium	-
Muscle	++
	0
Skeletal	± <sup>e</sup>
Smooth	+
Cornea	
Outer squamous epithelium	+
Endothelium, Descemet's membrane and	-
substantia propria	
Possible dendritic cells at periphery	++
Langerhans cells, interstitial dendritic cells	++
Lymphatics	++
Fibroblasts	++
Placenta-villous trophoblast -	-
Epidermis	+
<ul> <li>a: (++) = strong binding of anti-class I antibodies</li> <li>b: (+) = weak binding</li> <li>c: basal or deeper layer(s) positive</li> </ul>	
d: (-) = no binding	
e: (±) = very weak staining	
•	
Source: Daar et al, (1984)	
, ,	

**Table 16:** Summary of previous studies of the effect of IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  on HLA class I gene and antigen expression

Reference	Cell type	Units		Time	Effect
		IFN-α IFN-γ	$TNF-\alpha$		
Heron et al, 1978	fresh human PBMC	5-10000	1	16 h	maximum reached at 50 U/ml for 2 hours
Fellous et al,	Ramos	1000	1 .	12 h	Aclass I mRNA
1702	Chevalier Namalva	400 1000	7 4 4	24 h 24 h 24 h	Tclass I anugen fclass I mRNA and antigen no change in class I mRNA
	Daudi	2000	2	24 h	or antigen class I mRNA
	K562-pre erythroid	1000	2	24 h	antigen not detectable class I mRNA and antigen
	FS11-fibroblast SV-80-fibroblast	1000	1 1	19 h 16 h	not detectable class I mRNA and antigen class I mRNA and antigen
Burrone et al, 1982	Molt 4	2000	9	p 9	♦in class I mRNA and antigen reached maximum after 6 days although increase was observed after 10 hours
Rosa et al, 1983	murine cells transfected with HLA-A3	1000	Ī	1,2,4,10 h	+25% antigen 1300% mRNA after 4 hours
Витопе <i>et al</i> , 1985	Molt 4	2000	2	24 h	2 fold antigen detection of new HLA-B specificities

Reference	Cell type	Uni	Units / ml		Time	Effect
		IFN-a	IFN-7	TNF-a		
Friedman et al, 1985	T98G human neuroblastoma cells	10-1000			2 h	maximum increase observed at 10 U/ml
Shirayoshi et al,	LH-8 T cell line	800			various	B2 and B3 DNA binding proteins appear after 3 hours
	Ltk-	800		-	various	as for LH-8 cells
Israel <i>et al,</i> 1986	HeLa cells transfected with H-2K <sup>b</sup> promoter	200	200			♦CAT activity ♦CAT activity
	mouse 3T6 cells transfected with H-2K <sup>b</sup> promoter	00			24 h	2-3 fold increase in endogenous evels class I mRNA levels CAT activity
Korber et al, 1987	transfected L cells	800	0.2-1000		12h-4d 3d	maximal effect at 2 U/ml for 72 h results comparable with IFN-γ
Nissen et al 1987	fresh human PBMC	200			18 h	β2-microglobulin on all cells class I on B cells, monocytes, and T cells
Pfizenmaier et al 1987	Colo 205 SW 620 SW480 K 562			500 500 500 500	24 h 24 h 24 h 24 h	class I antigen class I antigen class I antigen no class I antigen
Gerrard et al, 1988	Jurkat T cells Molt 4 T cells		400 ng/ml		2 d	class I antigen

Reference	Cell type	IFN-α	Units / ml IFN-y	TNF-a	Time	Effect
Lapierre <i>et al</i> , 1988	human endothelial cells	100 1000 1000 1000	200 200 200 200 200 200	00 00 00 00 00 00 00 00 00 00 00 00 00	24 h	class I Ag class I Ag class I Ag no efect class I Ag
Weber et al, 1988	murine tumour cell lines	1000	0001	10 ug /ml	48 h 48 h 48 h	class I Ag class I Ag no effect
Blanar et al, 1989	Нега		1000		30,45,90, 120,150,180 minutes	maximum level of IBP-1 DNA binding protein seen at 45 mins
Hakem <i>et al</i> 1989	Jurkat T cells EBV-B-LCL HKK	1000	001 001		48 h 48 h 48 h 48 h	HLA-B7, no effect on HLA-A3 HLA-B7 no effect on HLA-A3 HLA-B7, HLA-A3 HLA-B7, HLA-A3
	HLA-B7/A3 transfected L cells	1000	001		48 h 48 h	HLA-B7, HLA-A3 HLA-B7, HLA-A3
Lowenthal et al, 1989	fresh human PBMC			100		NF-kB induction
Osborn et al, 1989	Jurkat T cells			100	20 h	NF-kB induction



element of the murine H-2L<sup>d</sup> gene have been shown to bind protein. Methylation of the guanine residues indicated on the coding and nonbinding to this region following treatment with IFN- $\alpha$  (O) and IFN- $\gamma$  ( $\Box$ ). The arrows between the strands of the H-2L<sup>d</sup> sequence indicate responsive sequence overlaps with the class I regulatory element and methylation of the guanine residues indicated interfered with DNA coding strands of the H-2L<sup>d</sup> 5' region interfered with this binding to region I (♠), to region II (♠) and to region III (■). The interferon Figure 24: Sequence comparisons of the 5'upstream regulatory regions of class I genes. Three regions within the class I regulatory the repeat regions. Comparisons of this region is made with the other murine genes H-2Kb and Q10 as well as with human class I sequences. In addition, sequence similarities have been observed between this region and SV40, IFN-β and HSP-70.

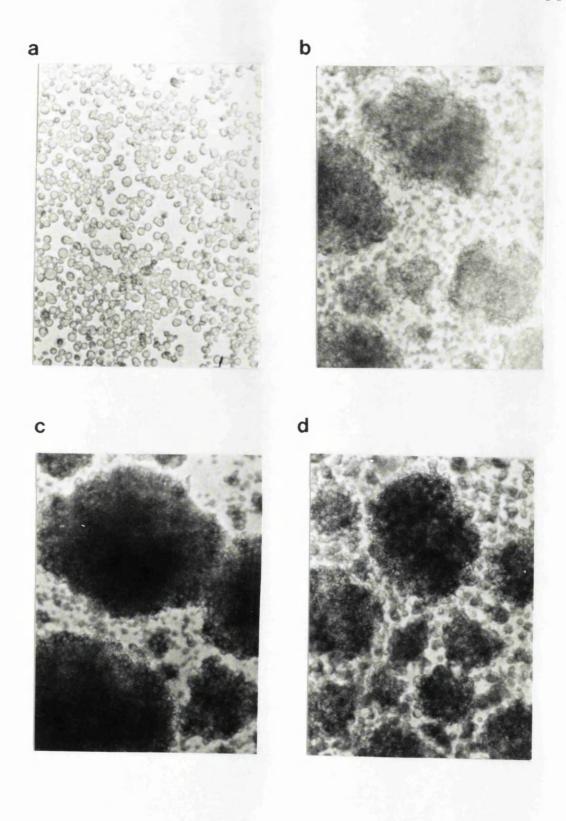
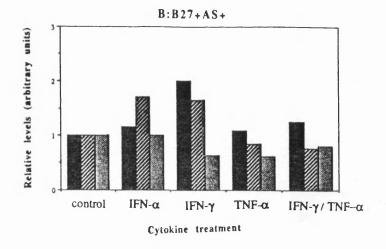
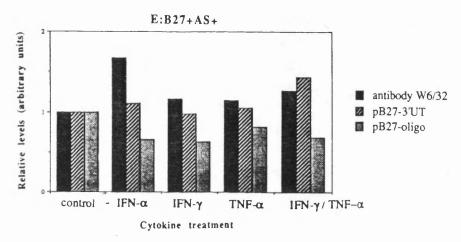


Figure 25: Comparison of the morphologies of the EBV transformed immortalized B lymphoblastoid cell lines prepared from 3 AS patients and 1 healthy individual.

(a) M:B27+AS-, (b) J:B27-AS+, (c) B:B27+AS+, (d) E:B27+AS+





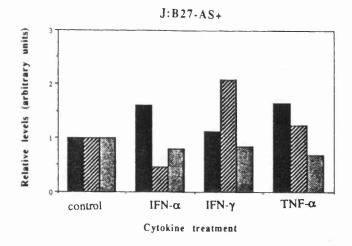


Figure 26: The effects of cytokine treatment on class I expression by the EBV immortalized B lymphoblastoid cells. Solid bars represent the changes in surface class I expression as detected with the anti-class I antibody W6/32. The hatched bars show the effect of the cytokines on the class I mRNA detected by Northern hybridization of total RNA isolated from each cell line with the pB27-3'UT probe. The dotted bars represent the binding of the pB27-oligo probe to the same RNA. The level of W6/32 binding has been corrected for background fluorescence while the Northern hybridization values have been normalized for the amount of RNA present in each track as detected with  $\beta$ -actin. The cell line is indicated above the relevant graph. The values plotted in these graphs are listed in table 17.

Table 17: Comparison of the effects of each cytokine on the EBV immortalized B lymphoblastoid 1 1 5 cell lines at both the protein and mRNA levels. Surface class I protein levels are expressed as mean fluorescence intensity (MFI) and were determined by binding of the fluoresceinated anti-class I antibody W6/32 (Ab). The background MFI obtained without Ab was subtracted from the MFI with Ab and expressed as MFI - background (bgd). These values were then made relative to the untreated control (MFI wrt control). The intensity of hybridization patterns revealed with each of the probes pB27-3'UT, pB27-oligo and β-actin were determined by densitometry. Each of the values obtained with pB27-3'UT and pB27-oligo were normalized for the amount of RNA in each track as determined by hybridization with β-actin (pB27-3'UT/pB27-oligo wrt β-actin). These values were then made relative to the untreated control (pB27-3'UT/pB27-oligo wrt control). The values made relative to controls were then plotted and are shown in figure 26). ND = not determined; NH = no hybridization observed.

Cells	MFI - Ab	MFI + Ab	MFI - bgd	MFI wrt control	pB27-3'U'	pB27-3'UT pB27-ollgo β-actin	o β-actin	3·UT wrt β-actin	3.UT wrt control	oligo wrt β-actin	oligo wrt control
B:B27+AS+	91	629	538	1.00	3.120	1.512	3.638	98.0	1.00	0.42	1.00
g-IFN	81	701	620	1.15	5.035	1.436	3.438	1.46	1.70	0.42	8.
7 IFN	92	1152	1076	2.00	3.039	0.576	2.149	1.42	1.65	0.27	0. <b>2</b>
TNF-0	61	9/9	585	1.09	5.023	1.778	6.918	0.72	0.84	0.26	0.62
γIFN/TNF-α	8	492	029	1.25	3.680	1.883	5.576	99.0	0.77	0.34	0.81
E:B27+AS+	738	5249	4511	1.00	2.632	1.518	2.432	1.08	1.00	0.62	1.00
g-IFN	265	5533	5268	1.67	3.619	1.228	3.024	1.2	1.11	0.41	99.0
4 IFN	327	5542	5215	1.16	3.616	1.320	3.422	1.06	86.0	0.39	0.63
TNF-α	298	5482	5184	1.15	3.163	1.437	2.805	1.13	1.05	0.51	0.82
γIFN/TNF-α	279	6029	5750	1.27	5.168	1.416	3.303	1.56	1.44	0.43	69.0
J:B27-AS+	Š	5	700	-	755.0	6370	0.780	00 0	00.1	0.84	1.00
control	\$ 8	1422	1324	1.00	1.086	1 558	2 3 1 4	0.47	0.47	0.67	0.80
G-IFIA	7 2	1568	1489	1.12	3.759	1.287	1.820	2.07	5.09	0.71	0.85
TNF-0	123	2308	2185	1.65	1.641	0.774	1.330	1.23	1.24	0.58	69.0
γIFN/TNF-α	%	1843	1747	1.32	2	Ð	Q	Ð	Ð	2	2
M:827+AS-							į	!	į	ğ	Ş
control	89	89	0	2	HN	HN	1.304	Ž	Q I	2 !	2 9
α-IFN	71	29	4	Ð	HZ	HZ	3.797	£	2	<u>a</u> !	2 4
Z IEN	61	110	49	£	HN	HZ	2.418	2	R	2	₹!
TNF-a	2	20	9	S	HZ	HZ	2.362	Ð	2	2	2 5
γIFN/TNF-α	72	70	7-	2	2	Ð	2	2	£	2	Ž

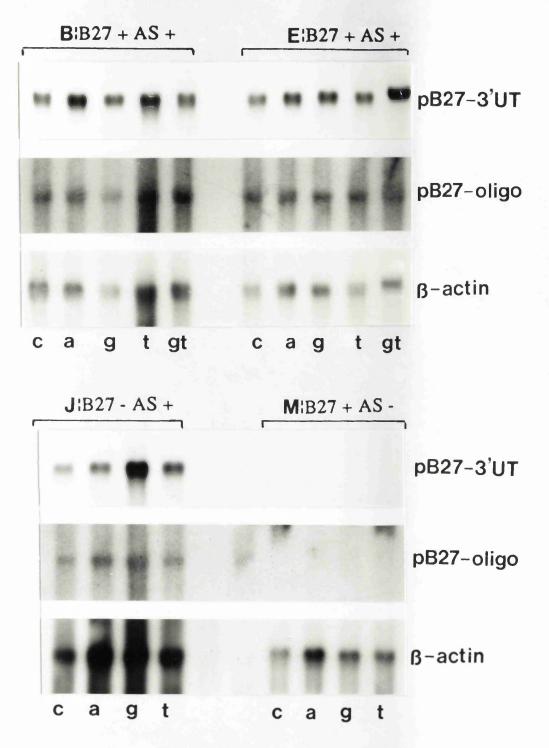
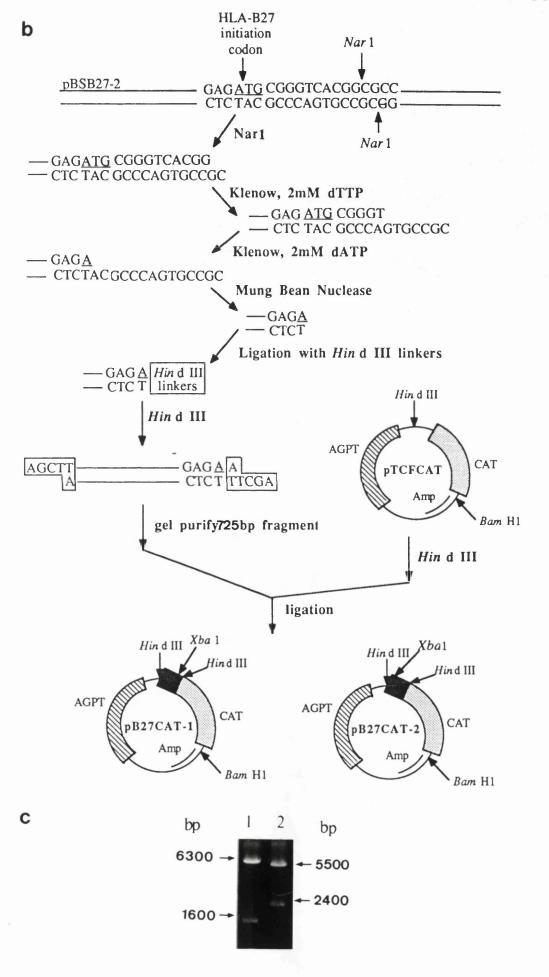


Figure 27: The effects of cytokine treatment at the RNA level in the EBV immortalized cell lines. RNA was prepared from the B lymphoblastoid lines following (c) no treatment, (a) IFN- $\alpha$ , (g) IFN- $\gamma$ , (t) TNF- $\alpha$  and (gt) IFN- $\gamma$  and TNF- $\alpha$  treatment. 20µg of total RNA was electrophoresed through 1.5% formaldehyde/agarose gels at 45V for 5 hours before transferring to Hybond-N. Class I mRNA was detected by hybridizing with the pB27-3'UT and pB27-oligo probes. Following hybridization, the filters were washed at 0.1xSSC/0.5% SDS 65 $^{\circ}$ C for 1 hour and 2xSSC/0.5% SDS 52 $^{\circ}$ C for 15 minutes before exposing 2 hours at room temperature and 2 days at -70 $^{\circ}$ C respectively. The amount of RNA in each track was determined by hybridization with human  $\beta$ -actin and washing to 0.1xSSPE/0.5% SDS at 42 $^{\circ}$ C and exposing for 3 hours at room temperature. The particular cell line is indicated above each set of Northern blots.



Figure 28: Purity of the pB27-oligo probe as determined by polyacrylamide gel electrophoresis. The oligonucleotide (lane 1) appeared to be 18 nucleotides long when compared with a 17mer (lane 2) and a 20mer (lane 3). This probe also appeared to be pure as smaller, incomplete molecules were not detected.



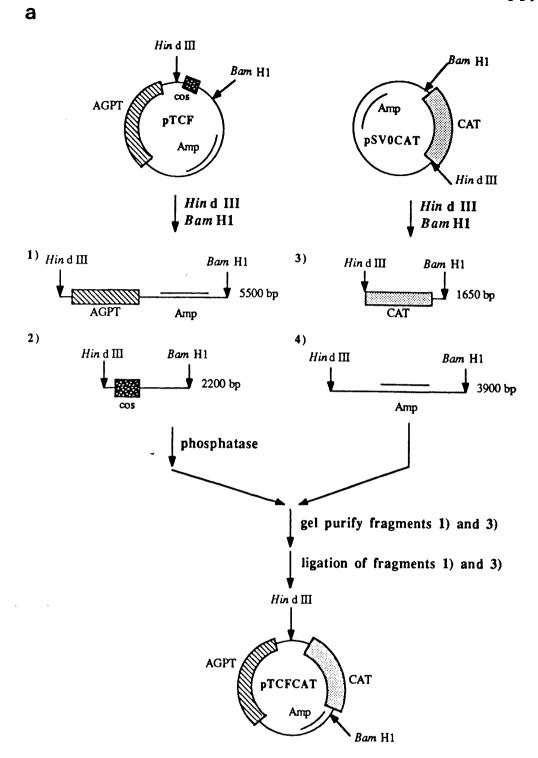


Figure 29: a) Strategy for the preparation of the CAT vector pTCFCAT.

b) Strategy for the preparation of the HLA-B27 promoter region of disease haplotype and construction of the pB27CAT plasmids pB27CAT-1 and pB27CAT-2.

c) Orientation of the HLA-B27 promoter region relative to the CAT gene as determined by digestion with Xba1/BamH1: Lane 1, pB27CAT-1; Lane 2, pB27CAT-2.

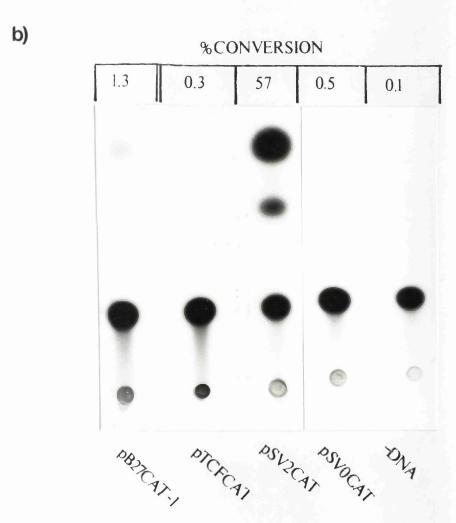
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CAGGTCGACTCTAGAGAAGCCAATCAGTGTCGCCGGGGTCCC

AGT TCTAAA GTCCCCACGCACCCACCCGGACTCAGAATCTCCT

CAGACGCCGAG ATG CGG GTC ACG GCA AGC TTG GCG

AGA TTT TCA GGA GCT AAG GAA GCT AAA ATG GAG



AAA AAA ATC ACT GCA TAT

Figure 30: a) Sequence of pB27CAT-1 from the Xba1 site of the HLA-B27 promoter to 24 nucleotides into the CAT gene. The CAAT and TATA boxes of the HLA-B27 promoter are boxed while the HindIII linker is underlined and indicated with an asterisk. The ATG initiation codon of the HLA-B27 gene is numbered 1 and the ATG codon of the CAT gene is numbered 2...

b) The level of CAT activity of pB27CAT-1 as determined by thin layer chromatography. The activity of this plasmid following transfection into HeLa cells is compared with the promoterless controls pTCFCAT and pSV0CAT as well with the CAT gene under the control of the SV40 promoter as found in pSV2CAT. The percentage conversion of chloramphenicol to acetyl chloramphenicol for each construct is indicated above.

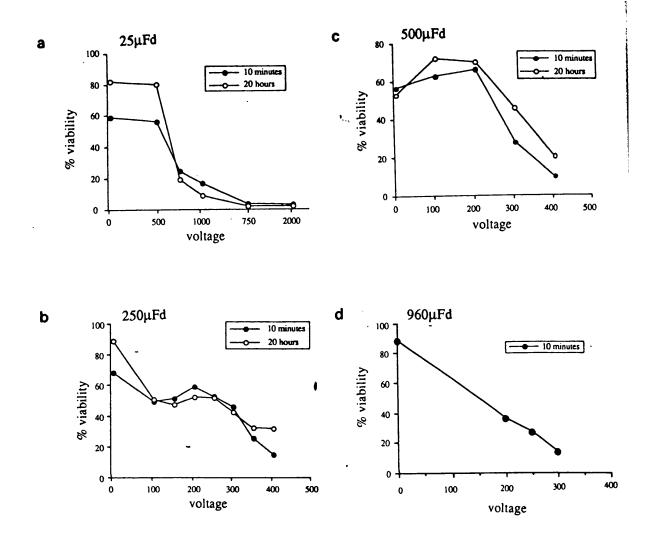


Figure 31: Killing of B:B27+AS+ by electroporation.  $3x10^6$  cells in 0.8 ml HBS were exposed to varying voltages at capacitances of (a)  $25\mu$ Fd, (b)  $250\mu$ Fd and (c)  $500\mu$ Fd. In addition,  $8x10^6$ cells in  $250\mu$ l of RPMI-1640 were electroporated at (d)  $960\mu$ Fd. The cell viabilities were assayed by dye exclusion after 10 minutes on ice ( $\bullet$ ) and 20 hours ( $\circ$ ) at  $37^0$ C following electroporation.

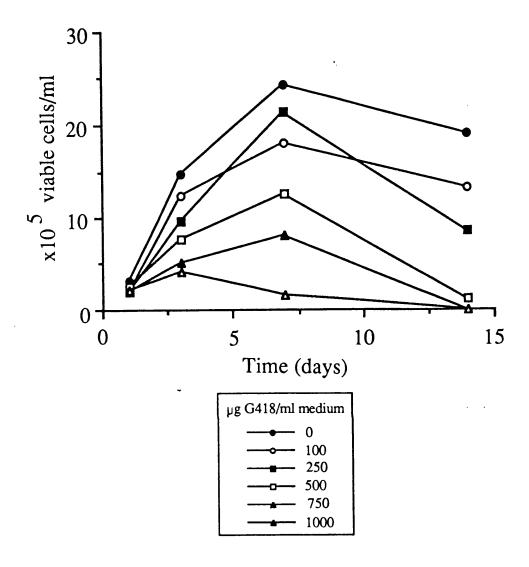


Figure 32: Sensitivity of the B:B27+AS+ cell line to increasing concentrations of G418.  $3.75 \times 10^5$  cells/ml were exposed to between 0-1000 $\mu$ g G418/ml medium and their viability assessed by dye exclusion after 1, 3, 7 and 14 days.

## CHAPTER 6

**GENERAL** 

**DISCUSSION** 

### 6.1. GENERAL DISCUSSION

The remarkably high association between HLA-B27 and AS has suggested either that HLA-B27 plays a direct role in the development of the disease or that it is in linkage disequilibrium with the true 'AS gene' and is merely its marker. The involvement of HLA-B27 in AS has been studied using RFLP analysis with probes for the HLA-B27 locus, by cloning and sequencing the HLA-B27 gene directly from an AS patient and analyzing the regulation of HLA-B27 expression in B lymphocyte cell lines derived from 3 patients and 1 control. In addition, the likelihood of TNF- $\alpha$  being involved in disease development was studied by RFLP analysis.

Probes for the HLA-B27 locus were used to search for a disease related RFLP in genomic DNA from AS patients and healthy controls digested with the restriction enzymes *PvuII*, *EcoR1* and *HindIII*. Subjects from English and Polish populations were analyzed. Using full length HLA-B27 and pB27-5' cDNA probes a 14Kbp *PvuII* fragment was detected by Southern hybridization that was more frequent in English HLA-B27+AS+ subjects than in healthy controls (p<0.01). This suggests that the sequence contained within this fragment may have a role in disease development in English HLA-B27+ individuals.

Using the same probes, a *PvuII* fragment of 8.9Kbp was shown to be less frequent in HLA-B27+AS+ individuals than in HLA-B27+ healthy controls in both populations studied (p<0.01). McDaniel *et al* (1987) reported that a 9.2Kbp *PvuII* fragment that was significantly more frequent in HLA-B27+AS+ patients than in controls. While a fragment of this size was not detected here, it is likely that the 8.9Kbp and 9.2Kbp *PvuII* fragments are the same. In contrast to McDaniel and coworkers, the 8.9Kbp fragment was not detected using probes derived from the 3' region of the HLA-B27 locus. Differences in populations may explain the differences in frequencies observed in these studies.

This *PvuII* RFLP was found to be linked to the HLA-A3/-A9 alleles by Ahearn *et al* (1989) and analysis of the available tissue types of the subjects studied here confirmed this. While the study of Ahearn and coworkers did not show any disease association with this fragment, a negative correlation was observed in this study. It is therefore a possibility that AS is associated with an extended haplotype. More extensive tissue typing would need to be done to confirm this.

In order to eliminate a mutation within the HLA-B27 gene as the cause of AS, the gene was cloned and sequenced directly from an AS patient. This showed that the predicted amino acid sequence the HLA-B27 antigen of disease haplotype was no different from that of HLA-B27 proteins of the normal haplotype. This suggests that an alteration in the HLA-B27 antigen itself is not responsible for the development of AS. Only 4 differences were found from the sequence reported by Weiss *et al* (1985) of a normal HLA-B27 gene of the same subtype. These were all found within introns 1 and 2. However, many more differences were found when the HLA-B27 gene sequenced here was compared with the sequence reported by Seeman *et al* (1986) for a HLA-B27 gene of the same subtype. This indicates that class I alleles are even more polymorphic than can be detected serologically.

Potential binding sites for Sp1 and AP2 were identified within introns 1 and 2. In particular, creation of an AP2 binding site within intron 2 of the HLA-B27 gene of disease haplotype may indicate

that the regulation of HLA-B27 expression in AS patients is abnormal. However, the existence of a potential transcription factor binding site within the HLA-B27 gene sequence does not necessarily mean that the expression of this gene is under that factor's control. Many studies could be performed in order to ascertain the relevance of such DNA binding sites to the expression of HLA-B27. Gel mobility shift assays using cell extracts from patient and control cells may be show differences in the proteins that bind the AP2 sequence. Mutation analyses could be used to determine if these sites are important in the regulation of HLA-B27 expression.

Of particular interest to the study of AS is the regulation of HLA-B27 expression in patients as compared with healthy controls. Attempts were made to study the regulation by cytokines of the endogenous HLA-B27 gene in B cell lines derived from 3 AS patients and 1 healthy control. EBV immortalized B lymphoblastoid cell lines were established from the peripheral blood lymphocytes of 2 HLA-B27+AS+, 1 HLA-B27-AS+ and 1 HLA-B27+AS- individual. B lymphoblastoid cell lines are relatively easy to establish in culture as their extraction from subjects is not particularly invasive. However, EBV immortalized B cells already express very high levels of HLA class I surface antigens. This high basal level of expression may explain why each cell line studied here responded differently to cytokine treatment.

Since this approach gave little information as to the regulation of the endogenous HLA-B27 gene in AS patients when compared with healthy individuals, attempts were made to study the regulation of the HLA-B27 promoter within these cells more directly. A construct was made with the CAT gene under the control of the HLA-B27 promoter of disease haplotype. However, only low levels of expression of this construct were observed in HeLa cells. Conditions for electroporation and G418 sensitivity were explored for future studies of the cytokine regulated expression of this promoter in the patient and control B lymphoblastoid cell lines.

The relevance of B lymphocyte cell lines to a disease such as AS is questionable. In agreement with the disparate levels of class I surface expression observed on these cell lines, van der Gaag *et al* (1985) showed that the levels of HLA-B27 expression on mononuclear leukocytes varied a great deal between healthy and diseased subjects. Further, they could not correlate the expression of HLA-B27 on the surface of these cells with susceptibility to AS. This suggests that if the control of HLA-B27 expression is directly involved in disease development, it would be more relevant to study this in cells present at the site of inflammation i.e., synovial cells of the sacro-iliac joint. Unfortunately, this would involve a particularly invasive process of removal.

The studies presented here have attempted primarily to ascertain the involvement of HLA-B27 in the development of AS. If HLA-B27 is not the 'AS gene' but is in linkage disequilibrium with this gene, a search for an RFLP that increases the risk for developing AS using probes from the HLA-B27 locus will be limited since only those sequences that are homologous to HLA-B27 will be detected. Such a study will not detect the 'AS gene' if it is not homologous. On the whole, attempting to find a disease related RFLP is like looking for a 'needle-in-a-haystack'. The number of restriction enzymes, the number of subjects from different populations and the variety of probes required makes such a study difficult.

It would appear that AS is not the result of a mutation in the sequence of the HLA-B27 antigen. It is still possible that HLA-B27 carries an epitope that cross reacts with one present on an infecting organism i.e., the molecular mimicry model may apply. Alternatively, inappropriate expression of HLA-B27 may be responsible for the disease. The promoter sequence of the disease HLA-B27 gene is identical to that of normal genes, therefore, if abnormal expression is the cause of the disease, this abnormality may lie within another gene(s) encoding a HLA-B27 specific *trans*-acting factor(s). However, it is unlikely that HLA-B27 expression would be controlled by factors specific for this allele. More likely, the factors involved in the control of HLA-B27 expression are the same as for other HLA-B alleles generally if not for other class I loci. Since expression of HLA-B alleles generally does not appear to be at fault in AS patients, the disease appears to be the result of either molecular mimicry or a mutation within another gene(s).

The gene encoding TNF- $\alpha$  was considered an alternative candidate 'AS gene'. In addition to its proximity to the HLA-B locus, TNF- $\alpha$  has been shown to enhance expression of class I genes in certain cells and to induce collagenase synthesis by synovial cells. RFLP analysis of genomic DNA isolated from normal individuals was performed in order to determine the degree of polymorphism of this locus. Using a large panel of restriction enzymes, the TNF- $\alpha$  gene was found to be non-polymorphic in these individuals. A small comparison of PvuII, EcoR1 and HindIII digested DNA from English patients and controls also did not reveal a disease associated RFLP of the TNF- $\alpha$  gene or any gross structural abnormality in the gene. This study has not ruled out the possibility that a minor mutation exists that alters the gene significantly.

The TNF- $\alpha$  protein may play a role in AS as an immune modulator. For example, TNF- $\alpha$  has been shown to enhance the motility of polymorphonuclear cells, particularly those that are HLA-B27 positive, suggesting that TNF- $\alpha$  may cause inflammation mediated injury.

Several newly identified genes i.e., the G genes and BAT genes have been localized to between the class III and class I regions of the human MHC. If the development of AS is due to another gene in linkage disequilibrium with the HLA-B27 gene, it is possible that any of these genes may be the 'AS gene'. These coding regions were identified by searching for CpG islands. The function of these genes is as yet unknown so that it is impossible at the present time to elucidate their possible role in disease development.

Localization of two HSP-70 genes to the MHC suggests these as candidate 'AS genes'. Heat shock proteins are synthesized in response to stress such as heat and oxygen free radicals. While oxygen free radicals are produced against pathogenic organisms, they can also be responsible for tissue injury during inflammation. Heat shock proteins are synthesized during fever and inflammation (Polla *et al* 1988) and are known to protect against oxidative injury within inflamed joints. It is conceivable that non-functional HSP-70 proteins, as might be found in AS, can reduce protection against oxidative injury giving rise to tissue damage.

Alternatively, HSP-70 may serve as the disease initiating antigen. In support of this, antibodies to the mycobacterial HSP-65 protein have been reported in AS (Bernstein, 1989). In addition, 40% of AS sera recognized the 93D *Drosophila* heat shock puff in an immunofluorescence

assay. While these antigens may not be involved in disease development, it is possible that a similar, as yet unidentified, antigen is.

Other genes not yet discovered which might be responsible for the onset of AS may exist within the MHC. Genes that do not have CpG islands may be linked to the HLA-B locus. It might be possible to identify these genes using oligonucleotides probes specific for exon/intron splice junctions.

For a molecular mimicry model to apply, HLA-B27 negative patients must carry an antigen that has a similar cross reactive epitope. No particular class I allele appears to be more common in these patients, suggesting that either the disease experienced by these patients is different or that antigenic cross reactivity is not the cause of AS. While molecular mimicry cannot be ruled out, it appears more likely that the HLA-B27 gene is in linkage disequilibrium with the true 'AS gene'.

# CHAPTER 7

# METHODS AND MATERIALS

# 7.1. MATERIALS

#### 7.1.1. SOURCES OF MATERIALS

anti-class I monoclonal antibody W6/32: gift from Dr.P.Lydyard, Immunology Department,

University College and Middlesex Hospital School of Medicine (UCMSM).

ampicillin; from Beecham Research labs (Brentford, UK)

BioRad Gene Pulser: BioRad (Hemel Hempstead, UK)

calf intestinal phosphatase: Boerhinger Corporation Ltd.

dATP, dCTP, dGTP, TTP: from Pharmacia Fine Chemicals Ltd. (Uppsala, Sweden).

DNA polymerase Klenow fragment: Gibco/BRL (Paisley, Scotland).

G418: Gibco/BRL (Paisley, Scotland).

L-glutamine: 200 mM supplied by GIBCO/BRL (Paisley, Scotland).

human β-actin probe: kindly provided by Dr Primus Mullis, Medical Molecular Biology Unit,

UCMSM.

human IFN-a: gift from Dr. Peter Lydyard, Immunology Department, UCMSM.

human IFN-y. gift from Dr. J. Taverne, Immunology Department, UCMSM.

Hybond-N: supplied by Amersham International plc (Amersham, UK).

Kodak X-OMAT S and XAR 5 film: supplied by Gelman Sciences (Northampton, UK).

λgt10 packaging extracts: Amersham International plc (Amersham, UK).

lymphopaque: Gibco/BRL (Paisley Scotland)

mouse TNF-α: gift from Dr. J. Taverne, Immunology Department, UCMSM.

pB1 probe: HLA-B27 cDNA probe kindly provided by Elisabeth Weiss, Department of Immunology,

University of Munich.

pEcoR1fragment in pUC13: HLA-B27 6.5Kbp fragment subcloned into pUC13 and kindly

provided by Elisabeth Weiss, Department of Immunology, University of Munich.

Polaroid type 667 film: from Polaroid (St. Albans, Herts., UK).

proteinase K: Boerhinger Corporation Ltd.

random primers: from Pharmacia Fine Chemicals Ltd. (Uppsala. Sweden).

restriction endonucleases: Gibco/BRL (Paisley Scotland) or Boehringer Corporation Ltd (BCL)

(Lewes, UK).

Ribonuclease A (RNAse A) and Ribonuclease T<sub>1</sub> (RNAse T<sub>1</sub>): Sigma (Poole, UK).

Sequenase kit: United States Biochemicals Corporation,

streptomycin: supplied by Middlesex Hospital dispensary.

T4 ligase: Boerhinger Corporation Ltd.

T4 DNA polynucleotide kinase: Gibco/BRL (Paisley, Scotland).

tetracycline: from Sigma (Poole, Dorset, UK)

Ultra-Turrax homegenizer: supplied by Sartorius (Belmont, Surrey, UK).

Tissue culture media, foetal calf serum and plasticware: Gibco/BRL (Paisley, Scotland).

# 7.1.2. BUFFERS AND SOLUTIONS

acrylamide: 38% (w/v) acrylamide, 2% (w/v) N,N-methylene bisacrylamide in water to give a 40% solution.

ampicillin: prepared as a 100 mg/ml stock solution in water and stored at -20 $^{\circ}$ C. This was used at a working concentration of 100  $\mu$ g/ml.

DNA loading buffer: 40% sucrose containing 0.4mg/ml bromophenol blue and autoclaved.

**chloroform**: chloroform and isoamyl alcohol mixed at a ratio of 24:1 and equilibrated with TE pH 7.5 **10x core buffer**: 500mM NaCl, 500mM Tris-HCl pH7.5, 100mM MgCl<sub>2</sub> made up in water and stored at -20<sup>0</sup>C until required.

100 x Denhardt's (Denhardt, 1966): 2% (w/v) bovine serum albumin, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) Ficoll 400 made up in water.

DNA loading buffer: 40% sucrose made in water with 0.01% bromophenol blue.

GTE: 50 mM glucose, 10 mM EDTA in 25 mM Tris/HCl pH 8.0.

herring testes DNA: 10mg/ml solution made in water and sonicated and autoclaved to shear the DNA.

**IPTG:** 100mM solution of isopropyl-β-D-thiogalactopyranoside made by dissolving 23.8 mg in 1ml of water.

10xMOPS running buffer: 0.4M Mops (pH7.0), 100 mM sodium acetate, 2mM EDTA pH8.0 made up in water, autoclaved and stored at room temperature.

oligolabelling buffer: solutions A, B and C in a ratio of 100:250:150. Solution A was made up of 1ml of 1.25M Tris-Cl pH8.0 / 0.125M MgCl<sub>2</sub>,  $18\mu$ l  $\beta$ -mercaptoethanol, 5  $\mu$ l each of 0.1M dATP, dTP, dGTP and stored at -20<sup>0</sup>C. Solution B is 2M Hepes made to pH6.6 with NaOH and stored at  $4^{0}$ C. Solution C is a mixture of random primers made to 90U/ml in TE and store at -20<sup>0</sup>C.

PBS: 100mM NaCl, 2mM KCl, 5mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM KHPO<sub>4</sub> pH7.2

penicillin: supplied by Middlesex Hospital dispensary.

phenol: analR grade equilibrated with TE pH7.5

RNA loading buffer: 50% glycerol, 0.01M NaH<sub>2</sub>PO<sub>4</sub> (pH7.0), 0.4% bromophenol blue made up in water, autoclaved and stored at room temperature.

spermidine: 100mM solution made in water.

20x SSC: 3M sodium chloride, 0.3M tri-sodium citrate

20xSSPE: 3M sodium chloride, 200mM sodium phosphate pH7.7, 20mM EDTA

SM: 100mM NaCl, 10mM MgSO<sub>4</sub> in 50mM Tris-HCl pH 7.5

T4 polynucleotide kinase buffer: 800mM Tris-HCl pH7.6, 120mM MgCl<sub>2</sub>, 60mM dithiothreitol (DTT) made up in water and stored at -20<sup>0</sup>C.

TAE: 40mM Tris, 1mM EDTA in water, pH adjusted to 8.0 with glacial acetic acid.

TBE(sequencing): 100mM Tris, 100mM Boric acid, 2.0mM EDTA, pH8.35.

TBE: 90mM Tris, 90mM Boric acid, 1.25mM EDTA in water, pH8.35.

TE: 10mM Tris-HCl, 1mM EDTA in water, pH7.5-9.0 as stated.

TEN: 40mM Tris-HCl pH7.5, 10mM EDTA, 150mM NaCl in water.

tetracycline: prepared fresh as a 15 mg/ml stock solution in water and diluted to a working concentration of 15µg/ml.

**XGAL:** 2% solution was made up by dissolving 20mg of 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside in 1ml of dimethyl formamide.

#### **7.1.3. MEDIA**

#### a) Bacterial

λgt10 broth: 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5% (w/v) sodium chloride, 0.2% (w/v) glucose, 25% (w/v) magnesium sulphate, 0.2% (w/v) maltose made up in water and adjusted to pH 7.5 with sodium hydroxide.

λgt10 agar: 1.5% (w/v) Difco technical agar in λgt10 broth.

Luria Bertani medium (LB): 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 1% (w/v) sodium chloride made up in water and adjusted to pH 7.5 with sodium hydroxide.

L-agar: 1.5% (w/v) Difco technical agar in LB.

H agar: 1.2% (w/v) Difco technical agar in 1% bacto tryptone, 0.8% (w/v) sodium chloride.

H top agar: 0.5% Difco technical agar in 1% (w/v) bacto tryptone, 0.8% sodium chloride.

SOB: 2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride made up in water and adjusted to pH 7.2 with sodium hydroxide.

2xTY: 1.6% (w/v) bacto tryptone, 1% (w/v) bacto yeast extract, 0.5% (w/v) sodium chloride made up in water.

All procedures involving the manipulation of bacterial strains were performed under conditions of good microbiological practice according to the guidelines laid down by the Genetic Manipulations Advisory Group.

#### b) Tissue culture

Dulbecco's modified Eagle's medium (DMEM): HeLa cells grown in DMEM with 10% foetal calf serum.

1 x RPMI-1640 medium with L-glutamine: EBV immortalized lymphoblastoid lymphocytes grown in RPMI-1640 medium with 10% heat inactivated foetal claf serum.

foetal calf serum: Heat inactivated at 520C for 20 minutes.

G418: made to 100mg/ml in serum free RPMI-1640 and stored at -20<sup>0</sup>C

10xHBS: 8.18% (w/v) NaCl, 5.94% (w/v) Hepes, 0.2% Na<sub>2</sub>HPO<sub>4</sub>, stored at  $40^{\circ}$ C and adjusted to pH7.12 with 1N NaOH just prior to use.

human IFN-α: supplied as 3x10<sup>4</sup>U/ml in RPMI-1640.

human IFN-y: supplied as 1x10<sup>5</sup> U/ml in RPMI-1640 and diluted to 20U/ml.

mouse TNF-α: supplied as 1x10<sup>6</sup>U/ml in RPMI-1640 and diluted to 1U/ml.

penicillin: 600mg per vial was dissolved in 5mls of RPMI 1640 which was then used to dissolve

streptomycin. Mixture stored at -200C.

streptomycin: 600 mg vial dissolved in same 5 mls as used to dissolve penicillin.

# 7.1.4. RADIO-ISOTOPES

Deoxycytidine 5' [ $\alpha^{32}$ P] triphosphate (3000 Ci/mmole), adenosine [ $\gamma^{32}$ P] triphosphate (3000 Ci/mmole), deoxyadenosine 5'[ $\alpha^{35}$ S] triphosphate, [acetyl-1-<sup>14</sup>C] butyryl CoA (4mCi/mmole), [acetyl-1-<sup>14</sup>C] acetyl CoA (40-60mCi/mmole) and [Dichloroacetyl-1,2-1<sup>4</sup>C] chloramphenicol (40-60mCi/mmole) were all purchased from New England Nuclear Corporation (Boston, MA, U.S.A.).

#### 7.1.5. GLASS AND PLASTICWARE

For DNA work, all non-disposable plasticware and glassware was washed with nitric acid and rinsed thoroughly with distilled water before autoclaving or baking overnight at 180<sup>0</sup>C.

For RNA work all non-disposable plasticware and glassware was soaked overnight in 3% Decon and rinsed thoroughly in distilled water before washing with absolute ethanol and sterilising by either autoclaving or baking overnight at  $180^{\circ}$ C.

For tissue culture, all plasticware was purchased from Nunc.

# **7.1.6. SOFTWARE**

For analysis of DNA sequences the Beckman Microgenie Sequence Analysis Program was used. For densitometry of Northern blots, the BioRad 1-D Data Analysis Software for the model 620 video densitometer was used.

# **7.1.7. SUBJECTS**

The 34 unrelated English AS patients were selected from those seen at the Ankylosing Spondylitis Research Clinic, Bloomsbury Rheumatology Unit, University College and Middlesex Hospital School of Medicine, London. While all patients fulfilled the New York criteria, only 2 did not have peripheral joint disease and 14 suffered from uveitis. Only one B27 negative patient was included in this study. This subject suffered from peripheral joint disease but not from uveitis. The 32 healthy English controls were chosen at random from members of the Medical Molecular Biology Unit, University College and Middlesex School of Medicine, London and from donors to the Anthony Nolan Laboratories, London. Of these, 7 (21%) individuals were positive for the B27 antigen.

Twenty Polish patients were selected from those attending the AS clinic at the Institute of Rheumatology, Warsaw.Of these, ten (50%) had peripheral joint involvement but the incidence of uveitis is unknown. There were 13 Polish healthy controls and four (31%) of these carried the B27 antigen.

None of the healthy controls had a family history of AS or other seronegative arthropathies.

# 7.2. METHODS

# 7.2.1. GENERAL DNA TECHNIQUES

#### a) Restriction endonuclease digestion of DNA

DNA was digested with restriction endonucleases in the presence of 1xcore buffer and 0.4mM spermidine unless otherwise recommended by the manufacturer.

#### b) Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were separated by electrophoresis following restriction digests using 0.8% horizontal slab gels made with 1xTBE or 1xTAE and  $0.5\mu g/ml$  of ethidium bromide included. DNA fragments were visualized with a short wavelength (254 $\eta$ m) Ultraviolet transilluminator and photographed using Polaroid type 667 film with an orange filter.

#### c) Fragment purification by electrolelution

DNA fragments were prepared by electroelution. DNA was digested with the appropriate restriction enzyme and, after strand separation through 0.8% TAE agarose, the required DNA fragments were cut from the gel. The fragment was then purified from the agarose using an electroelution chamber in 1.5g/l Tris, 0.56g/l borate, 0.5g/l EDTA at 120V for up to 2 hours. Using this apparatus, the DNA was eluted in a volume of 200µl which was extracted once with phenol/chloroform and once with chloroform before ethanol precipitation overnight at -20°C.

#### 7.2.2. PREPARATION OF PLASMID DNA

# a) Calf intestinal phosphatase treatment of restriction endonuclease digested vectors

After restriction endonuclease digestion of vectors, an aliquot was run on a 0.8% TBE agarose gel to confirm that restriction of the DNA had gone to completion. EDTA pH8.0 was added to the remianing restriction digest to 1mM excess over the Mg<sup>2+</sup> concentration present in the restriction endonuclease buffer, together with 15-20U of claf intestinal phosphatase. This solution was incubated at 37<sup>0</sup>C for 60 minutes and the reaction terminated by heat inactivating the enzyme at 65<sup>0</sup>C for 15 minutes.. The DNA was purified by extracting once with phenol/chloroform, twice with chloroform and ethanol precipitating at -20<sup>0</sup>C. The precipitated DNA was washed twice with absolute ethanol, freeze dried and resuspended in TE pH8.0.

# b) Ligation of DNA fragments into plasmid vectors

Ligations were carried out in 20mM TRis-HCl pH7.6 containing 10mM MgCl<sub>2</sub>, 10mM DTT, 0.6mM ATP and 1U of T4 DNA ligase at 12<sup>0</sup>C overnight.

#### c) Preparation of competent *E.coli*:

A modification of the calcium chloride / rubidium chloride method of Maniatis *et al* (1982) for preparing competent cells was used (T.Lund, personal communication). An overnight culture of *E.coli* grown in LB containing 10mM MgCl<sub>2</sub> was used to inoculate 100ml LB, 10mM MgCl<sub>2</sub> and grown to an A<sub>600</sub> of 0.3-0.4. The bacteria were harvested by centrifuging at 2000g for 10 minutes and resuspended in 50ml of ice cold 10mM MOPS ph 7.0, 10 mM rubidium chloride. Immediately following centrifugation again at 2000g for 10 minutes, the bacterial pellet was resuspended in 50 ml of cold 10mM MOPS pH 7.5, 10mM rubidium chloride, 50mM calcium chloride and left on ice for 30 minutes. The bacteria were pelleted again and resuspended in 3.3ml of cold 10mM MOPS pH 7.5, 10mM rubidium chloride, 50mM calcium chloride no ice until ready for use.

#### d) Transformation of plasmid DNA into competent E.coli

DNA (10ηg) in a minimum volume was added to 100 µl of competent *E.coli* and left on ice for at least 1 hour. The bacteria were then heat shocked at 44°C for 90 seconds and returned to ice for 15 minutes before adding 800µl of SOB, 8µl 1M MgCl<sub>2</sub>, 8µl 1M MgSO<sub>4</sub>, 8µl 2M glucose and incubating at 37°C for 1 hour. After pelleting, the bacteria were resuspended in 200µl of SOB. Dilutions of 1/10 and 1/100 were prepared with SOB and 100µl of each spread onto an LB agar plate with the appropriate antibiotic and incubated overnight at 37°C.

#### e) Large scale plasmid preparations

Large scale cultures of plasmids were prepared by a modification of the method of Ish-Horowitz et al (1981). 5ml overnight cultures prepared from single bacterial colonies were used to inoculate 750mls of sterile LB containing the appropriate antibiotic and grown overnight at 37°C with vigorous shaking. The bacteria were pelleted at 4200 rpm for 30 minutes at 4°C in a Beckman J6-B centrifuge and then resuspended in 50mls of GTE. The cells were lysed by adding 100mls of 0.2M NaOH/1% SDS and the chromosomal DNA was precipitated with the addition of 50mls of 3M potassium acetate pH4.8. After mixing, the precipitate was pelleted by centrifugation at 4200 rpm for 15 minutes at 4°C. The supernatant was filtered through nylon gauze and the plasmid DNA and bacterial RNA precipitated by adding 120 mls of propan-2-ol. After centrifuging at 6000 rpm in a Sorvall GS3 rotor, the pellet was washed with 70% ethanol and resuspended in 5mls of TE pH8.0 before being transferred to a preweighed sterile universal and made to 9g with TE pH8.0. To this was added 10g cesium chloride and 1ml of 5mg/ml ethidium bromide. The plasmid DNA was separated from the RNA according to density by centrifuging for about 24 hours in a Beckman 70Ti rotor at 55000rpm at 18°C. The lower plasmid band was extracted from the gradient and made to 10mls with TE pH8.0. Plasmid DNA was precipitated by adding 20mls of ethanol at room temperature and spinning at 3000 rpm for 20 minutes in a Beckman J6-B centrifuge. After washing with 70% ethanol and freeze drying, the DNA was resuspended in 400µl of sterile H2O and treated with 40U/ml RNase A for 15 minutes at 37°C. The protein was then removed by extracting once with phenol / chloroform:isoamylalcohol and once with chloroform:isoamyl alcohol and the DNA ethanol precipitated.

#### f) Small scale preparations of plasmid DNA

Rapid isolation of plasmid DNA yielding 5-10µg was performed using a scaled down version of the large scale plasmid preparation described above. A single bacterial colony was used to inoculate 10mls of broth and grown overnight in a shaking incubator at 37°C. The bacteria were pelleted by spinning at 3000 rpm for 10 minutes and resuspended in 200µl of GTE buffer and then lysed by addition of 400µl of 0.2 M sodium hydroxide, 1% (w/v) SDS lysed the bacteria and the chromosomal DNA and protein debris were precipitated by mixing with 200µl 3M potassium acetate pH4.3 and pelleted in a microfuge. The supernatant was mixed with 0.6 volumes of propan-2-ol and centrifuged for 5minutes. The pellet was washed with 70% ethanol, and resuspended in 200µl of TE pH8.0 before adding RNAse A/T1 to a concentration of 40U/ml and 200U/ml respectively and incubating at 37°C for 30 minutes. Plasmid DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation. The DNA was then resuspended in 50µl TE pH8.0 and 5µl used for a test digestion with restriction endonucleases.

# 7.2.3. PREPARATION OF PROBES

#### a) Probes

#### Full length HLA-B27 cDNA probe

The plasmid pB1 contains a near full length HLA-B27 cDNA cloned into the *Pst*I site of pBR322 (Szots *et al*, 1986) so that a full length HLA-B27 cDNA probe was prepared from this clone by digesting with this restriction endonuclease. The sequence of the cDNA begins 10bp downstream of the mRNA initiation codon and includes the polyadenylation signal and poly (A) tail. The insert had an internal *Pst*I site so that digestion with this restriction enzyme resulted in two fragments of about 1000bp and 500bp each (figure 33). These fragments were electroeluted together so that the entire HLA-B27 cDNA insert was represented within the one solution.

#### HLA-B27 5' and 3' region probes

In order to prepare probes for the 5' and 3' regions of the HLA-B27 cDNA, pB1 was digested with *Pvu* II and *Pst* I. Two fragments of 550bp and 415bp were (figure 33) were subcloned into pBLUESCRIPT and their identities confirmed by double stranded sequencing. The 415bp fragment (pB27-5') comprises the 5' end of the HLA-B27 cDNA and includes 194bp of exon 3. The 550bp fragment (pB27-3'UT) encompasses the 3' untranslated region of the HLA-B27 cDNA and includes the polyadenylation signal and poly (A) tail but no coding sequences.

#### HLA-B27 3' flanking region probe

A probe for the HLA-B27 3' flanking region (pB27-3'FR) was prepared from the HLA-B27 gene isolated by Weiss and coworkers (1985) (figure 34). The HLA-B27 gene was contained within a 6.5Kbp Eco R1 fragment that had been previously cloned into pUC13. This construct was digested

with Pst I and religated in a large volume to allow recircularization of the plasmid. The 3' flanking region was contained within a 1650bp *Pst* I and *Bam* H1 fragment which was excised prior to hybridization.

#### Tumour necrosis factor $\alpha$ (TNF- $\alpha$ )

An entire human TNF- $\alpha$  gene contained within a 2.9Kbp EcoR1 fragment isolated from a HPB-ALL cos202 genomic library (Kioussis *et al*, 1987). This clone including 870 bp of upstream and 300 bp of downstream sequences (figure 35).

#### Oligonucleotide probe

DNA sequences of HLA-B and other locus alleles were compared. The region from the *Bgl*II site within exon 2 downstream for 18 nucleotides was chosen as a HLA-B27 specific probe as this region was unchanged between HLA-B27 subtypes but not between HLA-B alleles. The sequence comparisons and the sequence chosen are shown in figure 36. The oligonucleotide was synthesized using an Applied Biosystems 381A DNA synthesizer.

#### Human B-actin probe

Human  $\beta$ -actin probe was prepared by digesting the plasmid pHA4-1 with EcoR1 and HindIII and purifying the 3.5Kbp insert fragment by electroelution as described in section 7.2.1. This fragment detects a 1.9Kb human mRNA species.

#### b) Oligolabelling of probes

The method of Feinberg *et al* (1983) was used to label purified fragment DNA; 100ηg was denatured by boiling for 7 minutes and quenched on ice before 10µl of oligolabelling buffer, 30µCi of [α<sup>32</sup>P] dCTP and 2 units of Klenow fragment were added to a final volume of 50µl. The reaction was allowed to proceed overnight at room temperature after which the labelled probe DNA was separated from the unincorporated label using a Sephadex G50 medium column. The labelling reaction was made to 100µl with TE pH7.5 and loaded onto the column, followed by 400µl of TE. Aliquots of 100µl of TE pH7.5 were added and the effluent collected after each aliquot. Each fraction was counted by Cherenkoff counting and the fractions of incorporated label pooled. The labelled DNA was boiled and quenched on ice prior to use.

# c) End-labelliing oligonucleotide probe

An 18mer oligonucleotide (50 $\eta$ g) was end labelled by T4 polynucleotide kinase in the presence of 120 $\mu$ Ci of [ $\gamma^{32}$ P] ATP and 1x kinase buffer in a total volume of 20 $\mu$ l. After 1 hour at 37 $^{0}$ C, the labelled probe was separated from the unincorporated isotope as described for oligolabelled probes but using Sephadex G50 fine.

# 7.2.4. SOUTHERN BLOTTING AND HYBRIDIZATION ANALYSIS OF GENOMIC DNA;

# a) Isolation of genomic DNA from peripheral lymphocytes

Human lymphocyte DNA was isolated as previously reported (Collis,1988). The peripheral lymphocytes from 10 mls of whole EDTA blood were lysed in 90 mls of lysis buffer containing 0.32M sucrose, 10mM Tris pH7.5, 5mM MgCl<sub>2</sub> and 1% (v/v) Triton-X-100. The nuclei were pelleted by centrifugation at 2000g for 20 minutes and then resuspended in 5mls of 75mM NaCl, 24mM EDTA pH8.0. The nuclear membranes were lysed with 0.5% SDS and 0.2mg/ml proteinase K at 37-50°C for 2-12 hours and the DNA cleaned by extracting twice with phenol and twice with chloroform. Addition of 0.5ml of 3M sodium acetate pH 5.2 and 11mls of ethanol precipitated the DNA which was then washed gently in 70% ethanol and resuspended in 0.5ml TE pH 7.5. The DNA was allowed to dissolve at 4°C.

#### b) Digestion of genomic DNA with restriction enzymes

Human lymphocyte DNA (10μg) was digested at 37°C overnight with 4 units of restriction enzyme per μg DNA in 1x core buffer and 4mM spermidine in a total volume of 60μl. To check for complete digestion, 1/20th of the reaction was electrophoresed through 0.5% TBE agarose and compared with 0.5μg of undigested genomic DNA.

#### c) Southern blotting of genomic DNA and hybridization

Southern blotting (Southern, 1975) was carried out using a modification of the method described by Amersham for use with Hybond-N filter membranes. Genomic DNA was digested with the appropriate restriction enzyme and the resulting fragments separated by electrophoresis through 0.8% TAE agarose gels at 60 V for 17-20 hours with the buffer recirculated. The DNA was denatured with 1.5M NaCl, 0.5M NaOH twice for 45 minutes and then neutralized with 1.5M NaCl, 0.5M Tris/HCl pH7.0 twice for 45 minutes at room temperature. Transfer to Hybond-N membrane was carried out overnight in 20xSSC after which the DNA was covalently linked to the membrane by UV irradiation for 3 minutes and then washed briefly in 2xSSC. Membranes were prehybridized at 65°C for a minimum of 4 hours in 6xSSC, 5xDenhardt's, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS, 100µg/ml denatured herring testes DNA. Hybridization was carried out overnight at 65°C in 20mls of fresh hybridization mix containing 0.01M EDTA pH8.0 as well as 100ng denatured oligolabelled probe at  $2x10^6$ cpm/ml plus  $10^5$  cpm of oligolabelled  $\lambda$  to visualize the molecular size markers. The membranes were then washed twice in 2xSSC/0.5% SDS at 65°C for 30 minutes followed by two 30 minute washes in 0.1xSSC/0.5% SDS at 65°C. The filters were exposed against Kodak XAR-5 film at -70°C using intensifying screens. In order to rehybridize, probe was removed from the membrane after use by washing at 90°C in distilled water for 5 minutes.

#### d) RFLP analysis

Patients and controls were analyzed for the presence and absence of fragments visualized with each hybridization probe. The statistical significance of each was determined using chi squared analysis.

# 7.2.5. CLONING OF THE HLA-B27 GENE FROM A λGT10 GENOMIC LIBRARY

# a) Purification and digestion of genomic DNA

Lymphocyte DNA was prepared as described in section 7.2.3. from 25mls of citrate blood taken from a patient with well defined ankylosing spondylitis as described for Southern hybridization. This individual was heterozygous for the HLA-B27 allele with the tissue type HLA-A1, -A2,-B8, -B27. DNA (100 $\mu$ g) was digested to completion with *Eco* R1 overnight at 37°C followed by electrophoresis through a 0.8% TBE/agarose gel at 10V for 48 hours. Genomic DNA that comigrated with the  $\lambda$  *Hin*dIII 6.5 Kb size marker was cut from the gel and electroeluted as described for plasmid DNA fragments in section 7.2.2. Following ethanol precipitation overnight, the DNA was resuspended in 20 $\mu$ l TE pH7.5, giving a final yield of about 3.2 $\mu$ g.

#### b) Ligation, packaging and plating out of genomic library

Of the purified genomic DNA, 150 $\eta$ g was ligated into 1 $\mu$ g of Eco R1 digested  $\lambda$ gt10 vector arms in a total volume of 10 $\mu$ l according to the directions in the Amersham cDNA  $\lambda$ gt10 cloning manual. Ligation reactions were also set up for the appropriate controls. Amersham packaging extracts were used to package 4 $\mu$ l of the insert/vector ligation and 2 $\mu$ l of the control ligations following the manufacturer's instructions.

The size of the library was determined by plating out the packaged DNA on the non-selective strain VCS257 and the selective strain C600 hfl as instructed. This indicated a library of approximately 14000 real recombinants. Since these are all derived from a total of about 150ng of insert DNA, this represents a cloning efficiency of about 95000 recombinants/µg of 6.5 Kb genomic DNA.

In view of this result, 5 more ligation reactions of 250 $\eta$ g of 6.5 Kb insert DNA and 1 $\mu$ g  $\lambda$ gt10 vectors arms were set up and packaged as described. Each of these reactions gave 18700 recombinants so that a library of 104000 recombinants in total was prepared.

#### c) Library screening

In order to isolate the HLA-B27 gene from this library, each entire packaging reaction was plated onto C600 hfl as described by Maniatis *et al*, (1982). Phage DNA was transferred in duplicate to Hybond-N as described by the manufacturer and fixed by UV irradiation for 3 minutes. The filters were then hybridized with the pB27-3' FR probe using the same method as for Southern blots.

After hybridization, one very strong positive signal was observed in duplicate as well as 13 weaker positive signals. The corresponding plaques were picked and transferred to 500µl SM and left at room temperature for 30 minutes before adding a drop of chloroform and storing at 4°C.

The very strong positive plaque as well as the area around this plaque and a putative positive were further plated out at 2000 and 300 plaques per plate. For each of these 15µl was incubated with C600 hfl at 37°C before adding top agarose and plating onto 15cm petri dishes and incubating overnight at 37°C. Duplicate lifts were taken of each dilution and hybridized with pB27-3FR. Following this rescreening the initial strong positive gave 106 hybridization signals after a two hour exposure. The other lifts showed no positives after 2 days exposure. Eight of the 106 positives were picked and stored in SM/ chloroform at 4°C.

#### d) Small scale phage preparation

An overnight culture of C600 hfl grown in LB with 0.4% maltose was diluted to give an A600 of 0.6. The bacteria were then incubated with enough phage from 4 of the 8 positives to give 150,000 pfu and plated onto a 15cm petri dish and grown overnight at 37°C. The phage was eluted by overlaying the plate with 18mls SM and gently shaking for 3 hours at room temperature. The SM was decanted and extracted once with chloroform before treatment with 30µl 20mg/ml RNase and DNase at 37°C for 1 hour. The phage were lysed by addition of lysis buffer to a final concentration of 0.4% SDS, 83mM Tris pH 9.0, 41mM EDTA and incubated at 70°C for 15 minutes. A quarter volume of 8M potassium acetate was added and the mixture left on ice for 15 minutes before spinning at 3000 rpm for 10 minutes. The supernatant was solvent extracted and precipitated with 0.6 volume propan-2-ol. After a second precipitation with ethanol, the DNA was finally resuspended in 50µl of TE. Of this 1µl was run on a gel to confirm the presence of DNA of the right size. Half of the DNA was digested overnight with *Eco* R1 and a third of this was electrophoresed to show an insert of 6.5 Kb present in each. Vector fragments of the expected sizes were also visible. One of these digests was phenol:chloroform extracted, ethanol precipitated and resuspended in 20µl of TE ready for subcloning into pBLUESCRIPT SK-.

#### d) Subcloning into pBLUESCRIPT

pBLUESCRIPT vector was digested with *Eco* R1 at 37°C. After checking the digest was complete, 2U of calf alkaline phosphatase was added and the mixture incubated at 37°C for an hour. The vector DNA was cleaned by extracting with phenol/chlorofrm and then ethanol precipitated.

The 6.5 Kb Eco R1 fragment (500 $\eta$ g) was ligated into 100 $\eta$ g of pBLUESCRIPT SK- in a total volume of 40 $\mu$ l. Competent E.coli DH5 $\alpha$  were transformed with 5 $\mu$ l of this ligation reaction, along with the appropriate controls. The bacteria were grown at 37°C overnight on LB/ampicillin plates that had been previously overlayed with 20 $\mu$ l of 2% X-GAL and 50 $\mu$ l of 0.1 M IPTG.

From the resulting colonies, 12 small scale plasmid preparations were made and digested with *Eco* R1 to check the inserts were 6.5 Kb fragment. From a *Bam* H1 digest the orientation of the inserts within the pBLUESCRIPT vector was determined and designated pBSB27-1 and pBSB27-2.

A restriction map of this HLA-B27 clone was determined by digesting with various combinations of the restriction enzymes *Bgl* II, *Pst* 1, *Bam* H1, *Xba* I, *Sac* I, *Pvu* II, *Eco* R1 and compared with the published maps.

# 7.2.6. DNA SEQUENCING

#### a) 'In gel' ligation of fragments into M13

The fragments to be sequenced were prepared by digesting the pBSB27 plasmid DNA with the appropriate restriction enzymes and separating on a 0.5-1% low melting temperature agarose/TAE gels. The required fragments were cut from the gel and transferred to a preweighed tube so that the weight of agarose and thus the concentration of the DNA could be determined. The insert DNA was heated to 65<sup>0</sup>C for 10 minutes and kept at 37<sup>0</sup>C prioir to ligation.

M13mp18 or M13mp19 vectors were digested prior to ligation with the appropriate enzyme(s) and treated with 1U of phophatase for 1 hour at 37<sup>0</sup>C before solvent extracting and ethanol precipitating. Vector was finally resuspended at 10ng/µl in TE pH 7.5.

Ligations were set up between 100 $\eta$ g of insert DNA and 20 $\eta$ g of vector in a total volume such that the final agarose concentration did not exceed 0.2%. The reactions were allowed to proceed at room temperature overnight. Control ligations were also set up with 10 $\eta$ g of digested vector. The ligations were heated to 65 $^{\circ}$ C for 10 minutes and kept at 37 $^{\circ}$ C prior to infection of *E.coli*.

#### b) Infection of E.coli

A single colony of *E.coli* TG2 or JM101 that had been previously grown on a glucose/minimal medium plate was used to inoculate 10 ml of 2xTY medium and grown overnight at 37°C. This overnight culture was diluted 1ml in 100ml of 2xTY and grown until an A550nm of 0.3-0.4 after which the bacteria were spun down, resuspended in 5mls 50mM CaCl<sub>2</sub> and left on ice until required.

To transform competent TG2 cells, half the ligation reactions were added to 300µl of bacteria and left on ice for a minimum of 1 hour before heat shocking at 44°C for 90 seconds. A transformation control was also set up with 5ηg of uncut M13. For each plate, 40µl of 2% X-GAL, 40µl of 0.1M IPTG, 200µl of 2xTY and 200µl of an overnight culture of TG2 or JM101 were mixed and 270µl added to the transformed bacteria. To each sample, 3mls of H top agar was added and the mixture poured onto H base agar and allowed to set. The number of blue and white plaques were counted after an overnight incubation at 37°C. The uncut M13 gave rise to the expected 200 blue plaques/ηg of DNA and the number of plaques arising from transformation with the ligation reaction of phosphatased vector alone was less than 1%.

# c) Preparation of M13 single stranded templates

A modification of the protocol described by Amersham in their booklet 'M13 cloning and sequencing' was used to prepare single stranded DNA. An overnight culture of *E.coli* TG2 or JM101 was diluted 1/100 with 2xTY medium and 1.5ml aliquots inoculated with a colourless M13 recombinant plaque. After incubating with shaking for 6 hours at 37°C, the phage released into the medium was separated from the bacteria by centrifugation at 3000 rpm for 5 minutes in a Beckman J6-B centrifuge. Of the supernatant, 1ml was removed and transferred to a new eppendorf tube. Phage particles were precipitated by addition of 200µl of 2.5M NaC/20% (w/v) polyethylene glycol 6000

(PEG) at  $^{40}$ C for 15 minutes and pelleted by spinning in a microfuge for 5 minutes. All traces of PEG were removed from the phage pellet before resuspending in 100  $\mu$ l of TE pH 8.0. The viral particles were lysed by extracting with phenol:chloroform:isoamyl alcohol and then chloroform:isoamyl alcohol and the template DNA precipitated with ethanol overnight at -20°C before being finally washed, dried and resuspended in 30  $\mu$ l of TE pH8.0. A check gel was run with about 1-2  $\mu$ l of phage DNA to ensure the presence of DNA for sequencing.

#### d) Preparation of pBLUESCRIPT double stranded templates

Plasmid DNA to be sequenced directly was prepared by the large scale plasmid preparation technique describe in section 7.2.1. 10µg of plasmid DNA was added to an equal volume of freshly prepared 0.4M NaOH and left at room temperature for 10 minutes. Denature DNA was precipitated by addition of 0.1 volume 3M sodium acetate and 4 volumes ice-cold absolute ethanol and left at -700C for 20 minutes. The DNA was recovered by centirifugation in a microfuge for 10 minutes, washed in 70% ethanol and air-dried. The pellet was resuspended in 35µl of water.

#### e) DNA sequencing

DNA sequencing was performed by the chain termination method originally devised by Sanger et al (1977) and detailed by United States Biochemical Corporation for use with their Sequenase kit. Sequencing reactions were labelled with [35-S]-dATP and run on 6% polyacrylamide gels. Gels was then fixed in 10% acetic acid and 10% methanol before drying at 80<sup>0</sup>C for 20 minutes and exosing Kodak X-OMAT AR film.

# 7.2.7. PREPARATION OF CULTURED LYMPHOBLASTOID CELLS AND RNA ANALYSIS

#### a) Preparation of Epstein Barr virus

A fully grown culture of the marmoset cell line B95-8 was kept undisturbed for 14 days before harvesting the culture supernatant by centrifugation. The supernatant was filtered through a sterile  $0.2\mu$  Millipore and aliquoted before storing in liquid  $N_2$ .

# b) Immortalization of peripheral blood lymphocytes with Epstein Barr virus

Venous blood was taken from two HLA-B27 positive AS patients, one HLA-B27 negative patient attending the AS clinic at the Middlesex Hospital, London and one HLA-B27 positive healthy donor to the Anthony Nolan Laboratories, London. The blood was collected in 50ml heparin tubes and diluted with an equal volume of RPMI 1640 medium before layering over an equal volume of Ficoll/Hypaque. After centrifuging at 2000 rpm for 30 minutes in a Beckman J6-B centrifuge, the lymphocytes were collected from the interface and washed twice by mixing with RPMI 1640/3% foetal calf serum (FCS) and spinning at 1500 rpm for 5 minutes. The cells were finally resuspended at  $2x10^5$  cells/ml RPMI 1640/10% FCS. Two drops of Epstein Barr virus preparation were added per ml of cell

suspension and the cells divided between 96 flat bottomed wells in a microtitre plate so that each well contained 2 drops each. Two further drps of medium were added to each weel and the cells were maintained at 37°C in 5% CO<sub>2</sub>. When cell clumps began to form, medium was added to about 2mls until the cells began to grow rapidly. Once established, the cell lines were maintained at 37°C in 5% CO<sub>2</sub> in RPMI 1640 with 0.12mg each of penicillin and streptomycin per ml medium, 10% heat inactivated FCS and 1 mM glutamine.

#### c) Storage of cells

Cells growing in log phase at a minimum of  $5 \times 10^6$  cells/ml were frozen in liquid N<sub>2</sub> in 1.5ml of RPMI 1640 containg 15% DMSO and 15% FCS. Cells were either stored in liquid N<sub>2</sub> or at -70<sup>0</sup>C.

#### d) Recovery of frozen cells

Cells that had previously been stored frozen were thawed rapidly at 37<sup>0</sup>C and washed with RPMI medium containg 25% FCS by spinning down at 1500 rpm for 5 minutes at room temperature. The cells were resuspended in 3ml of RPMI/25% FCS and left overnight at 37<sup>0</sup>C in 5% CO<sub>2</sub> before replacing the medium. The cells were then left to grow for a week after which more medium was added slowly over a period of days until the cells were growing rapidly. The lymphocytes were then maintained in RPMI 1640/10% FCS.

The viability of cells was determined by dye exclusion assay using 0.4% erythrocin B.

#### b) Induction of HLA-B27 expression by cytokines

The induction of class I gene xpression was studied using the human IFN- $\alpha$ , human IFN- $\gamma$  and mouse TNF- $\alpha$  with specific activities of  $3x10^4$  U/ml;  $8x10^5$  U/ml and  $1x10^6$  U/ml respectively. EBV immortalized lymphoblastoid cells were resuspended at  $10^6$  viable cells per ml and incubated for 6 hours with each cytokine so that the final concentration of IFN- $\alpha$  was 400U/ml; of IFN- $\gamma$  was 200U/ml and of TNF- $\alpha$  was 20U/ml. Following this treatment,  $2x10^6$  cells were removed and incubated for a further 2 days for staining with the anti-class I antibody W6/32. The remaining cells were pelleted at 1500 rpm for 5 minutes and washed twice before being resuspended in 0.5ml of PBS. The cells were snap frozen in liquid nitrogen and stored at -70°C until required for RNA isolation.

#### c) RNA isolation

The RNA extraction technique was adapted from Chirgwin *et al*, (1979). Cells that had been previously resuspended in 0.5ml of PBS were lysed with 10 mls of homogenization buffer containing 50% (w/v) guanidinium isothiocyanate, 25mM tri-sodium citrate pH 7.0, 0.1% (w/v) sodium sarkosyl and 70μl of β-mercaptoethanol and genomic DNA sheared by homogenizing with an ultra-turrax for 10 seconds. The homogenate was layered over a 3ml cushion of 5.7M CsCl / 0.1 M EDTA pH 7.0 in a beckman SW41 tube. The RNA was pelleted by spinning at 25000 rpm in a SW41 rotor for 22 hours in a Beckman centrifuge L8-centrifuge at 18°C. Following centrifugation, the supernatant was aspirated off and the CsCl/EDTA cushion discarded. The tubes were inverted for 5 minutes after which time the pellets were dissolved in 0.5ml of TE pH 7.5. The RNA was precipitated at -70°C until required by

addition of 0.1 volume of 3M sodium acetate pH 5.2 and 3 volumes cold absolute ethanol . The RNA was pelleted by spinning in a microfuge at  $^{40}$ C for 30 minutes, washed in cold 70% ethanol and resuspended in 100 $\mu$ l of TE pH 7.5. Assuming that a  $^{40}\mu$ g/ml solution of RNA has an absorbance at 260nm of 1, the concentration of extracted RNA was determined by measuring the absorbance at this wavelength (Maniatis *et al.*, 1982).

# c) Northern blotting of total RNA and hybridization analysis

RNA samples were prepared by dissolving 15µg of RNA in TE pH 7.5 to a total volume of 12µl and were denatured by heating to 65<sup>0</sup>C for 15 minutes after addition of 25µl of deionized 50% formamide, 5µl of 10xMOPS running buffer and 8µl of 37% formaldehyde. Following denaturation, 5µl of sterile RNA loading buffer was added and the samples electrophoresed through denaturing gels of 1.5% agaose, 2.2M formaldehyde and 1xMOPS running buffer at 100V for 6 hours. Northern transfer to Hybond-N was carried out overnight in 20xSSC following the protocol for Southern blots omitting the denaturing and neutralizing steps.

Filters hybridized with the pB27-3FR and human  $\beta$ -actin probes were prehybridized for at least 4 hours in 6xSSC, 1x Denhardt's, 0.5% SDS, 250µg/ml denatured herring sperm DNA at 30°C for 4 hours and hybridization with the oligonucleotide probe at 2x10<sup>6</sup>cpm/ml was carried out at 30°C overnight in fresh mixture. Following hybridization with the pB27-3FR probe, filters were washed 2xSSC/0.5% SDS and then 0.1xSSC/0.5% SDS for 1 hour each at 65°C. Northern blots hybridized with  $\beta$ -actin were washed in 2xSSPE/0.1% SDS for 15 minutes at 42°C, 1xSSPE/0.1% SDS for 30 minutes at 42°C and 0.1xSSC/0.1% SDS for 15 minutes at room temperature. Filters were exposed to Kodak XAR5 film at -70°C between intensifying screens.

Filters hybridized with the class I specific oligonucleotide probe were prehybridized for 4 hours in 5xSSC, 20mM sodium phiosphate pH7.0, 10xDenhardt's, 7% SDS and 100μg/ml denature herring testes DNA at 46<sup>0</sup>C. The filters were hybridized at the same temparature overnight in the same solution following addition of 50% dextran sulphate to a final concentration of 10% and 5 ηg/ml labelled oligonucleotide probe. Following hybridization, the blots were washed twice in 4xSSC/0.1% SDS for 15 minutes at room temperature, once with 2xSSC/0.1% SDS for 15 minutes at room temperature and once with 2xSSC/0.1% SDS for 15 minutes at 37<sup>0</sup>C before exposing against Kodak XAR5 film at -70<sup>0</sup>C between intensifying screens. The filters were then washed further at 46<sup>0</sup>C and 52<sup>0</sup>C in 2xSSC/0.1% SDS for 15 minutes each and exposed again.

# 7.2.8. CELL TRANSFECTION AND ANALYSIS

# a) Transfection by calcium phosphate precipitation, harvesting and extract preparation

Cells were transfected using the calcium phosphate technique outlined in Gorman (1986) with slight modifications. HeLa cells were plated out at  $7x10^5$  cells in DMEM/10% (v/v) FCS per 10cm petri dish. The following day half the medium was removed from the cells which were then returned to the incubator for 2 hours. Recombinant CAT plasmid DNA (10µg) was added to 3µl 2M CaCl<sub>2</sub> in a

removed, the cells glycerol shocked with 15% (v/v) glycerol in 1xHBS, washed with serum-free medium and fed with complete medium.

#### a) Cell transfection by electroporation

Lymphoblastoid cells were pelleted at 1500 rpm for 5 minutes and washed once with HBS before finally resuspending at  $5 \times 10^6$  viable cells/ml HBS. Using a BioRad Gene Pulser, 0.8ml of cells was electroporated at varying capacitances and voltages and placed immediately on ice for 10 minutes. Prior to adding 14.2mls of RPMI/10% (v/v) FCS, an aliquot was removed for assaying viability. The remaining cells were transferred to an incubator and left for 20 hours before assaying their viability again. The cells were then left for 48 hours before harvesting.

# b) Harvesting and cell extract preparation

After 48 hours the cells were harvested by washing once with  $Ca^{2+}/Mg^{2+}$  free PBS before leaving in 1ml of TEN for 5 minutes and transferring to an eppendorf tube. The cells were pelleted and the supernatant removed and replaced with 100 $\mu$ l 0.25 M Tris-HCl pH7.8. The cells were pelleted again and the pellet resuspended and subjected to 3 freeze/ thaw cycles in liquid nitrogen and a 37 $^{0}$ C water bath to lyse cells. Endogenous acetylases were inactivated by heating at  $60^{0}$ C for 10 minutes. The cell debris was spun down and the supernatant transferred to a new tube and stored at -20 $^{0}$ C until ready for use. The protein content of the HeLa extract was determined with a Pierce BCA protein Reagent Kit as recommended.

#### c) CAT assays using ascending chromatography

Equalled amounts of cell extract were made to 90μl with 0.25M Tris-HCl pH7.5 and mixed with 35μl water, 1μl [<sup>14</sup>C] chloramphenicol and 20μl freshly prepared 4mM acetyl CoA. The reaction was stopped after 30 to 120 minutes at 37<sup>0</sup>C by addition of 1 ml ethyl acetate. The chloramphenicol was extracted by vortexing and spinning in a microfuge at high speed for 5 minutes. The top organic phase, containing all forms of chloramphenicol, was transferred to another tube and dried under vacuum before resuspending in 20μl ethyl acetate and spotting onto a TLC plate. Ascending chromatography was carried by placing the plates in a 95:5 mix of chloroform:methanol. The chromatography plates were air dried and exposed to Fuji RX X-ray film at -70<sup>0</sup>C. The percentage of chloramphenicol converted to its acetate forms was established by cutting out the spots from the TLC plates and counting in a liquid scintillation counter.

#### d) Diffusion CAT assays

The two phase CAT assay developed by NEN research products was used to determine CAT activity following transfection of lymphoblastoid cells. The reactions were set up in a 7ml scintillation vial by mixing 50 $\mu$ l of cell extract, 200 $\mu$ l of 1.25mM chloramphenicol/100mM Tris-HCl pH 8.0 and 0.1 $\mu$ Ci of [acetyl-1-<sup>14</sup>C] butyryl CoA or 5 $\mu$ Ci [acetyl-1-<sup>14</sup>C] acetyl CoA to give a final acetyl CoA concentration of 100mM. This was overlayed with 5ml of Econofluor and left at 37 $^{0}$ C for 1 hour before counting for 0.1 minute in a liquid scintillation counter.

# e) Assessment of TNF-α sensitivity

Cells in log phase of growth were counted diluted to  $5x10^5$  cells/ml. The cells were exposed in duplicate to 10U/ml and 50U/ml of TNF- $\alpha$  in a total volume of 1ml for 48 hours. The viability of the cells was then assessed by dye exclusion.

# f) Assessment of G418 sensitivity

Confluent lymphoblastoid cells were spun down and washed with medium before resuspending at  $3.75 \times 10^5$  cells per ml. 0.8ml of cells were mixed with increaing concentrations of G418 in a total volume of 1 ml in flat bottomed multiwell plates. The cells were incubated in the presence of G418 and counted at intervals of 1, 3, 7 and 14 days. The viability of the cells was also assessed at these times by dye exclusion assay.

# 7.3. FIGURES AND TABLES

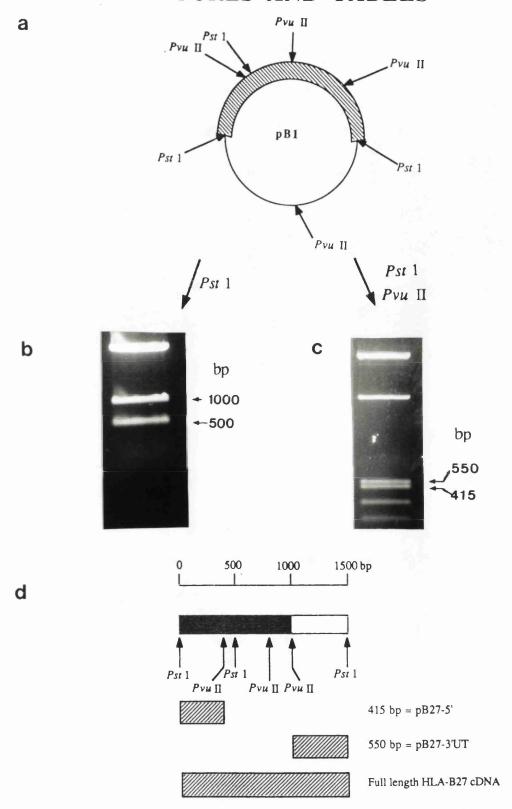


Figure 33: Preparation of the full length HLA-B27 cDNA, 5' and 3'untranslated region probes. Each of these probes was prepared from the plasmid pB1 (Szots et al, 1986). The positions of the Pst1 and PvuII sites within pB1are shown in part (a). (b) The full length HLA-B27 cDNA probe was prepared by digesting pB1 with Pst1 and electroeluting the 500 and 1000bp insert fragments together. These fragments are indicated with arrows. (c) The pB27-5' and pB27-3'UT probes were prepared by digesting pB1 with Pst1 and PvuII and purifying the 415bp and 550bp insert fragments respectively. These fragments are also indicated with arrows. (d) The coding region of the HLA-B27 cDNA is represented by the solid bar while the untranslated region is shown as a white bar. The positions of the pB27-5', pB27-3'UT probes and full length cDNA probes relative to the HLA-B27 cDNA reported by Szots et al (1986) are indicated with hatched bars.

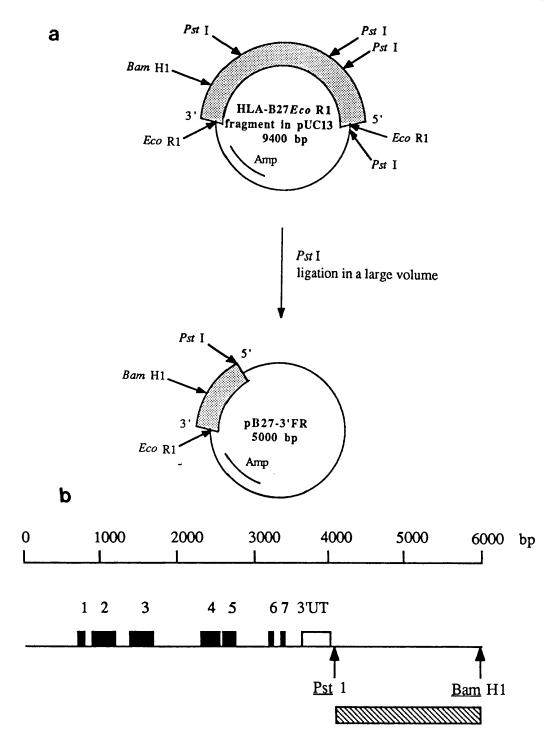


Figure 34: (a) Preparation of the pB27-3'FR probe from the 6.5Kbp HLA-B27 EcoR1 fragment that had been previously subcloned into pUC13 (Weiss et al, 1985). This plasmid was digested with Pst1 to remove the majority of the HLA-B27 5' sequence. A plasmid containing the 3' HLA-B27 sequence was then formed by ligating the resulting DNA restriction fragments in a large volume. This allowed the vector/insert fragment to recircularize. The pB27-3'FR probe was prepared by digesting the resulting plasmid with Pst1 and BamH1 and the positions of these sites are indicated.

(b) The position of the pB27-3'FR probe relative to the HLA-B27 gene. The coding regions of this gene are shown as solid bars while the 3'UT region is shown as a white bar. The 1.6Kbp pB27-3'FR probe is indicated beneath the HLA-B27 gene as a hatched bar.

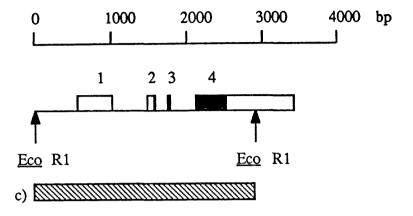


Figure 35: The position of the human TNF- $\alpha$  probe relative to the coding sequence isolated by Kioussis *et al* (1987). The probe for the human TNF- $\alpha$  gene encompasses 870bp of upstream as well as 300bp of downstream sequence. The mature coding regions are represented as solid bars while the untranslated regions are shown as white bars. The TNF- $\alpha$  probe is indicated as a hatched bar.

B*2705 (Weiss)	+895 TGG GAC CGG GAG ACA CAGA	TC TGC AAG GCC AAG GCA	+988 3AG ACA CAGATC TGC AAG GCC AAG GCA CAG ACT GAC CGA GAG GAC CTG CGG ACC CTG CTC CGC TAC TAC
B*2705 (Seeman)	TGG GAC CGG GAG ACA CAG	TC TGC AAG GCC AAG GCA	TOG GAC COG GAG ACA CAGATC TOC AAG OCC AAG OCA CAG ACT GAC COA GAG GAC CTO COG ACC CTO CTC COC TAC TAC
B*2705 (Coppin)	TGG GAC CGG GAG ACA CAG	TC TGC AAG GCC AAG GCA	3AG ACA CAG ATC TGC AAG GCC AAG GCA CAG ACT GAC CGA GAG GAC CTG CGG ACC CTG CTC CGC TAC TAC
B*2703 (Choo)	TGG GAC CGG GAG ACA CAG	TC TGC AAG GCC AAG GCA	TOG GAC COG GAG ACA CAGATC TOC AAG GCC AAG GCACAG ACT GAC COA GAG GAC CTO COC ACC CTO CTC COC TAC TAC
B*2702 (Seeman)	TGG GAC CGG GAG ACA CAGA	TC TGC AAG GCC AAG GCA	TGG GAC CGG GAG ACA CAG ATC TGC AAG GCC AAG GCA CAG ACT GAC CGA GAG AAC CTG CGG AIC <u>GC</u> G CTC CGC TAC TAC
B44 (Kottman)	TGG GAC CGG GAG ACA CAGA	TC TCC AAG ACC AAC ACA	TGG GAC CGG GAG ACA CAG ATC TCC AAG ACC AAG ACA CAG ACT TAC CGA GAG AAC CTG CG <u>C</u> ACC <u>GCT GC</u> C CGC TAC TAC
Bw58 (Ways)	TGG GAC GGG GAG ACA CGG	AC ATC AAG GCC TCC GCG	TGG GAC <u>G</u> GG GAG ACA C <u>G</u> G A <b>A</b> C AIC AAG GCC I <u>CC</u> GC <mark>G</mark> CAG ACT IAC CGA GAG AAC CTG CGG AIC <u>GC</u> G CTC CGC TAC TAC
B51 (Hayashi)	TGG GAC CGG AAC ACA CAGA	TC TIC AAG ACC AAG ACA	TGG GAC CGG AAC ACA CAG ATC TIC AAG ACC AAG ACA CAG ACT IAC CGA GAG AAC CTG CGG AIC GCG CTC CGC TAC TAC
Bw52 (Hayashi)	TGG GAC CGG GAG ACA CAGA	TC TCC AAG ACC AAC ACA	TGG GAC CGG GAG ACA CAG ATC T <u>C</u> C AAG ACC AA <u>C</u> ACA CAG ACT <u>T</u> AC CGA GAG <u>A</u> AC CTG CGG A <u>TC GC</u> G CTC CGC TAC TAC
A11 (Cowan)	TGG GAC CAG GAG ACA CGGA	AT GTG AAG GCC CAG TCA	TGG GAC CAG GAG ACA C <u>G</u> GAA <u>T GTG</u> AAG GCC <u>C</u> AG TCA CAG ACT GAC CGA <u>C</u> TG GA <u>G</u> CTG <u>G</u> GG ACC CTG C <u>G</u> C <u>G</u> GC TAC TAC
A3 (Cowan)	TGG GAC CAG GAG ACA CGGA	AT GTG AAG GCC CAG TCA	TGG GAC CAG GAG ACA C <u>G</u> G <mark>AAT GTG</mark> AAG GCC CAG TCA CAG ACT GAC CGA <u>CT</u> G GA <u>G</u> CTG <u>G</u> GG ACC CTG C <u>G</u> C <u>G</u> GC TAC TAC
Aw24 (N'Guyen)	TGG GAC GAG GAG ACA GGGA	AA GIG AAG GCC CAC ICA	TOG GAC G <u>A</u> G GAG ACA <u>GG</u> G <mark>AAA GIG</mark> AAG GCC CAC ICA <mark>C</mark> AG ACT GAC CGA GAG <u>AAG</u> CTG CGG A <u>IC GC</u> G CTC CGC TAC TAC

oligonucleotide probe. Nucleotides are numbered according to the system of Weiss et al (1985). The region chosen for pB27-oligo is Figure 36: Comparison of HLA class I sequences within exon 2 in order to determine a suitable sequence for a HLA-B27 specific boxed and differences within the nucleotide sequences are underlined.

Sources: N'Guyen et al (1985), Ways et al (1985), Weiss et al (1985), Coppin et al (1986), Kottman et al (1986), Seemann et al (1986), Cowan et al (1987), Choo et al (1988), Hayashi et al (1989)

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