CHARACTERISATION OF A NOVEL VARIANT IN THE HUMAN CYCLOOXYGENASE-2 GENE:

IMPLICATIONS FOR FIBROTIC LUNG DISEASE AND INFLAMMATION

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A thesis submitted to the University of London for the degree of Ph.D.

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ABSTRACT

Cyclooxygenase-2 (COX-2) is an inducible enzyme with key regulatory roles in both inflammatory and homeostatic processes. It is therefore not surprising that dysregulated expression of COX-2 often leads to disease. In pulmonary fibrosis, a decreased capacity to upregulate COX-2 occurs despite the increased presence of mediators capable of stimulating its synthesis. The cause of this failure is unknown but may partly be explained by sequence variants in the regulatory regions of COX-2 that modify gene expression. The central focus of this thesis is to identify sequence variants in the proximal promoter of the COX-2 gene, assess their functionality and test for association with interstitial lung diseases such as idiopathic pulmonary fibrosis and sarcoidosis.

This thesis describes the identification and characterisation of a novel G to a C substitution in the promoter of the COX-2 gene, at position -765 (-765G>C) upstream of the transcription start site, that is carried by 25% of a healthy UK Caucasian population. Population studies showed that carriage of the rare -765C allele associates with a significant risk for sarcoidosis subjects (OR=1.88 [95%CI 1.26-2.82], P=0.002). Transient transfection of COX-2 promoter constructs in human lung fibroblasts showed that -765G>C represses gene expression. The -765G>C variant locates within a putative binding site for the transcription factor Sp1. DNA-protein binding studies revealed that the transcription factors Sp1, Sp3 and Egr-1 bind to that region and that the -765C allele disrupts these interactions.

An investigation of -765G>C in an in vivo model of inflammation of patients undergoing coronary bypass surgery demonstrated that the magnitude of rise in levels of the acute phase protein C-reactive protein (CRP), is strongly -765G>C genotype dependent.

This thesis describes the first functional variant in the human COX-2 gene and suggests that -765G>C may impact on fibrotic lung disease as well as other inflammatory disorders.
ACKNOWLEDGEMENTS

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Last in the list, but above all in my heart. All that I am, I owe to you.

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And if you could hear the whispering of the dream
you would hear no other sound.

KAHLIL GIBRAN. THE PROPHET.

To my parents,

who never failed to hear my whispers.
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<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>15RHETE</td>
<td>15R-hydroxyeicosatetraenoic acid</td>
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<tr>
<td>5-LO</td>
<td>5-lipoxygenase</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<td>AIP</td>
<td>acute interstitial pneumonia</td>
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<tr>
<td>Amp</td>
<td>ampicillin</td>
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<td>AoXC</td>
<td>aortic cross-clamp</td>
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<tr>
<td>ARE</td>
<td>AU-rich element</td>
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<td>AT</td>
<td>annealing temperature</td>
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<tr>
<td>BAEC</td>
<td>bovine arterial endothelial cells</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BHL</td>
<td>bronchohilar lymphadenopathy</td>
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<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>BOOP</td>
<td>bronchiolitis obliterans organizing pneumonia</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CASIS</td>
<td>Coronary Artery Surgery Inflammation Study</td>
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<tr>
<td>CBP</td>
<td>cardiopulmonary bypass</td>
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<tr>
<td>CFA</td>
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<td>CLASS</td>
<td>Celecoxib Long-term Arthritis Safety Study</td>
</tr>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
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<td>cPLA₂</td>
<td>cytosolic phospholipase A₂</td>
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<td>CRP</td>
<td>C-Reactive protein</td>
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<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
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<td>CXR</td>
<td>chest X ray</td>
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<td>ddH₂O</td>
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<td>DNTP</td>
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<td>dithiothreitol</td>
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<td>ECE-1</td>
<td>endothelin-converting enzyme</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ethylene glycol-bis(2-aminoethyl)-N,N,N⁶,N⁶-tetraacetic acid</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>FAP</td>
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<td>follicle-stimulating hormone</td>
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<tr>
<td>GI</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
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<tr>
<td>HOPE</td>
<td>Heart Outcomes Prevention Evaluation study</td>
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<tr>
<td>HRCT</td>
<td>high-resolution computed tomography</td>
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<td>hrSp1</td>
<td>human recombinant Sp1 protein</td>
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<td>myocardial infarction</td>
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<td>pigeon breeder's disease</td>
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<td>protein kinase B</td>
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<tr>
<td>SSCP</td>
<td>single strand conformation polymorphism analysis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptors</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of MMP</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>tetradecanoyl phorbol acetate</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>UIP</td>
<td>usual interstitial pneumonia</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VIGOR</td>
<td>Vioxx Gastrointestinal Outcomes Research</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number tandem repeat</td>
</tr>
<tr>
<td>WHS</td>
<td>Women’s Health Study</td>
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</table>
CHAPTER I

INTRODUCTION
1.1 PULMONARY FIBROSIS

Pulmonary fibrosis is the end-stage of a heterogeneous group of disorders that are characterised by excessive deposition of extracellular matrix proteins - notably collagen. It is the result of a number of different kinds of insults to the lung. These include inhalational exposure to organic and inorganic agents (e.g. cadmium, asbestos fibres, silica), inflammatory response to infectious agents, radiation, drugs (e.g. bleomycin) and trauma. In some cases the cause of the condition is recognised whereas others are idiopathic with no specific aetiology or associated condition identified despite intensive investigation. Pulmonary fibrosis is a devastating irreversible condition and a major source of morbidity and mortality. Current treatments have had only a modest impact on the outcome of disease and are often associated with severe side effects. The pessimistic picture regarding treatment of fibrosis highlights the need for a better understanding of the mechanisms responsible for the initiation and maintenance of the fibrotic process. Our current understanding of the mechanisms involved in the pathogenesis of pulmonary fibrosis is explained in section 1.2. Two fibrotic conditions: idiopathic pulmonary fibrosis (IPF) and sarcoidosis are the focus of this thesis and will be discussed in detail in the section that follows.
1.1.1 IDIOPATHIC PULMONARY FIBROSIS (IPF)

Idiopathic pulmonary fibrosis (IPF) or cryptogenic fibrosing alveolitis (CFA), is a chronic, progressive lung disorder characterised by inflammation and fibrosis of the lung parenchyma (Crystal RG et al 1976, Turner-Warwick M et al 1980, King TE et al 1993). Although it is one of the most common forms of interstitial lung disease, it remains one of the most poorly understood. To date, despite thorough evaluation, no specific aetiology has been identified.

Although some forms of pulmonary fibrosis of known origin may have a better prognosis, IPF is a progressive condition that rarely, if ever, remits spontaneously (King T.E et al 1993). Even in the absence of complicating disease, the median survival after the diagnosis of biopsy-confirmed IPF is less than 3 years (Bjoraker JA et al 1998). In the UK, prevalence of IPF is about 5 per 100,000 and annual mortality, currently around 2000, has more than doubled since 1980. Prevalence in the USA is similar and mortality is also rising. Patients are usually over 50 years of age and typically present with symptoms of non-productive cough and breathlessness on exertion, which progresses at a variable rate. Clinical findings include fine inspiratory crackles at the lung bases, finger clubbing, and a restrictive defect on lung function testing with reduced gas transfer (Turner-Warwick M. et al 1980).

An early feature in the pathogenesis of IPF is alveolar epithelial and/or capillary cell injury. This promotes recruitment of circulating immune cells into the lung such as monocytes, neutrophils, lymphocytes and eosinophils. These effector cells, together with resident lung cells such as macrophages, alveolar epithelial and endothelial cells, release cytokines that in turn stimulate fibroblasts to proliferate and synthesise increased amounts of collagen. The increased collagen deposition seen in IPF is therefore associated with increased numbers of fibroblasts in the interstitium and in the alveolar space itself. In addition, degradation of extracellular matrix may also be inhibited, thus further contributing to the fibrotic process. These changes result in interstitial thickening and loss of alveolar architecture, leading to reduced lung volume and impaired gas exchange (Coker R.K. and Laurent G.J., 1998).

In the past, many studies included several forms of idiopathic interstitial pneumonia under the collective term IPF. Today, the clinical label 'idiopathic pulmonary
fibrosis' should be reserved for patients with a specific form of fibrosing interstitial pneumonia referred to as usual interstitial pneumonia. The previously unexplained variability in prognosis and treatment response seen with IPF can be better understood now with the awareness of distinct subgroups based on characteristic histological features. However, even today clinicians are often unsure of the correct diagnosis. The idiopathic interstitial pneumonias can be classified in pathologically distinct categories (Table 1.1): usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), desquamative interstitial pneumonia (DIP)-respiratory bronchiolitis interstitial lung disease, acute interstitial pneumonia (AIP), and cryptogenic organising pneumonia- bronchiolitis obliterans organising pneumonia (BOOP), (Katzenstein AA and Myers JL 1998). Usually, the greater the histological variability, the better the response to therapy. The pattern varies from dense septal alveolar fibrosis with little or no inflammation to a predominance of lymphocytes or plasma cells with minimal fibrosis. The degree of fibrosis is the principal determinant of the response to therapy, with severe fibrosis being resilient to treatment while mild fibrosis tends to be more responsive.

Table 1.1: Idiopathic Interstitial Pneumonias

<table>
<thead>
<tr>
<th>Clinical correlate</th>
<th>Pathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Idiopathic pulmonary fibrosis (IPF)</td>
<td>Usual interstitial pneumonia</td>
</tr>
<tr>
<td>- Non-specific interstitial pneumonia (NSIP)</td>
<td>Non-specific interstitial pneumonia</td>
</tr>
<tr>
<td>- Desquamative interstitial pneumonia</td>
<td>Desquamative interstitial pneumonia</td>
</tr>
<tr>
<td>Respiratory bronchiolitis interstitial lung disease (DIP)</td>
<td>Respiratory bronchiolitis interstitial lung disease</td>
</tr>
<tr>
<td>- Acute interstitial pneumonia (AIP)</td>
<td>Diffuse alveolar damage</td>
</tr>
<tr>
<td>- Cryptogenic organizing pneumonia-</td>
<td>Cryptogenic organizing pneumonia-</td>
</tr>
<tr>
<td>Bronchiolitis obliterans organising</td>
<td>Bronchiolitis obliterans organising pneumonia</td>
</tr>
<tr>
<td>pneumonia (BOOP)</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Katzenstein AA and Myers JL 1998)

The pathologic changes in IPF have a patchy appearance. They display marked variation in the location and age of the lesions, which are mainly found in the peripheral subpleural parenchyma. Fibrotic zones are characterised by the formation of large cystic air spaces lined by bronchiolar epithelium, known as honeycomb lung, and alternate with areas of relatively unaffected lung tissue. Fibrotic areas can vary in age and activity. Regions of chronic lung injury with scarring and honeycombing
contrast with regions of acute injury with distinctive foci of actively proliferating fibroblasts and myofibroblasts. These fibroblastic foci form at sites of recent alveolar injury and are considered to be the hallmark lesions of IPF (Gross TJ and Hunninghake GW 2001). The interstitial inflammation of IPF is mild and generally associated with fibrosis. (Katzenstein AA and Myers JL 1998).

The recognition of IPF as a distinct entity with lesions varying in age and location raised questions about the previously established view that IPF was a disease in which parenchymal fibrosis was directly caused by chronic inflammation. Based on early observations demonstrating inflammatory cells in the distal air space, many studies investigated the use of corticosteroids and cytotoxic agents in the management of IPF. The assumption was that if the inflammatory cascade was interrupted before irreversible tissue damage occurred, fibrosis might be avoided.

Early studies reported that 10-30% of patients with IPF have an initial improvement with corticosteroid therapy (Turner-Warwick MB et al 1980, Rudd RM et al 1981). However, re-evaluation of these studies has questioned whether most of the patients who responded to therapy truly had IPF (Ryu JH et al 1998). Most, if not all reports of treatment efficacy in IPF have included a mixed population, especially a proportion of patients with NSIP, a subgroup of patients, which are now known to respond well to treatment and have a better prognosis. Indeed, when anti-inflammatory agents were given only to patients with a secure diagnosis of idiopathic pulmonary fibrosis, there was no evidence of a meaningful response (Ryu JH et al 1998, Kolb M et al 1998, Zisman DA et al 2000). Furthermore, high-dose corticosteroid therapy is associated with serious side effects including hyperglycaemia requiring insulin, myopathy, exacerbation of hypertension and accelerated osteoporosis (Gross TJ and Hunninghake GW 2001).

A recent consensus among international experts has clarified the definition of IPF by providing major and minor clinical criteria (Table 1.2). The joint American Thoracic Society (ATS) European Thoracic Society (ERS) statement emphasises that IPF can only be diagnosed accurately when patients have typical clinical features of IPF and histological characteristics of UIP by surgical lung biopsy (American Thoracic Society 2000).
Table 1.2: American Thoracic Society statement for the diagnosis of IPF.

Criteria for the diagnosis of IPF

Major criteria
- Exclusion of other known causes of interstitial lung disease such as drug toxicities, environmental exposures and connective tissue disease
- Abnormal pulmonary function studies that include evidence of restriction and/or impaired gas exchange
- Bibasilar reticular abnormalities with minimal ground-glass opacities on high resolution computed tomography.
- Transbronchial lung biopsy or bronchoalveolar lavage showing no features to support an alternate diagnosis.

Minor criteria
- Age > 50 years
- Insidious onset of otherwise unexplained dyspnoea on exertion
- Duration of illness ≥ 3 months
- Bibasilar inspiratory crackles (dry or 'velcro'-type in quality)

Adapted from duBois RM and Wells A.U. 2001.

Several studies have evaluated the effects of cytotoxic agents in combination with corticosteroids. An early study suggested that combination therapy with cyclophosphamide and prednisone improves survival compared with therapy with prednisone alone (Johnson MA et al 1989). However, the same reservations regarding patient collection apply to this study. Similar observations and reservations exist regarding the clinical trials assessing the efficacy of azathioprine (Raghu G et al 1991). Moreover, cytotoxic agents also have limiting side effects including myelosuppression, secondary cancers and drug-induced interstitial pneumonia (Gross TJ and Hunninghake GW 2001).

Taken together, these observations suggest that current therapy has minimal or no beneficial effect for patients with IPF. Unfortunately, despite intensive investigation, the results of therapy for IPF have remained poor and currently, lung transplantation represents the only chance to achieve meaningful recovery of lung function in the majority of patients with advanced IPF (Trulock EP 1997).

The histological findings, combined with the observation that anti-inflammatory therapy for IPF provides no benefit, prompted investigators to formulate a new
hypothesis. It is now believed that IPF is the consequence of sequential acute lung injury produced by a still unidentified stimulus. The ensuing wound healing response to injury results in a progressive accumulation of fixed fibrosis with architectural distortion. Therefore, one effective therapeutic strategy might be to modify or control the wound healing response. As our understanding of the disease process improves (discussed in section 1.2), better therapeutic strategies can be developed. In future, therapeutic approaches should include agents that interfere directly with matrix synthesis, fibroblast proliferation or epithelial/fibroblast interactions (Mason RJ et al 1999). The predominant inflammatory phenotype (Th1 or Th2) can also modify the fibrotic response and so newer approaches may include agents that alter specific classes of inflammatory cells and their products. In addition, factors such as the genetic background of the patient, and environmental inflammatory triggers such as cigarette smoking, viral infection and respirable toxins will also need to be taken into account (Gross TJ and Hunninghake GW 2001).

1.1.2 SARCOIDOSIS
In contrast to IPF, sarcoidosis is a multisystem granulomatous disorder commonly affecting young adults. While IPF is a progressive fibroproliferative disease resulting in mortality within 3 years, sarcoidosis is characterised by a fluctuating course with exacerbations and/or spontaneous remissions that may resolve or progress to fibrosis. Similar to IPF however, it has no known aetiology. Sarcoidosis is sometimes described as “the great mimicker” because of its ability to present in a wide range of appearances (R.M du Bois 2000). Sarcoidosis has been recognised by physicians for about a century, at first as a disorder affecting the skin, but subsequently because of its tendency to involve a number of organs such as the lung (affected in >90% of cases), lymph nodes, eyes, liver, spleen, heart, kidneys and nervous system (Eklund A and Grunewald J 2000).

Although sarcoidosis has been described in the literature for many years, a definitive understanding of its aetiology, clinical course and prognosis, remains elusive. It is thought that sarcoidosis represents an imbalance of the immune system with localised hyper-reactive aggregations of effector cells (granulomas) responding to an
unknown antigen. The granulomas may resolve or eventually lead to fibrosis with organ failure as a consequence.

The incidence of sarcoidosis varies substantially world wide, partly because the disease goes unrecognised in many countries. Sarcoidosis incidence varies among different ethnic groups. The exact incidence of sarcoidosis is difficult to assess because many patients are asymptomatic and are detected only during routine screening. Estimates range between 3 (Caucasians) and 47 (African Americans) per 100,000 in North America and up to 64 per 100,000 in Scandinavia (Eklund A and Grunewald J 2000). Sarcoidosis is three times more common in the Irish living in London than in native Londoners (James DG et al 1992), while West Indians or Asians living in London have a 10-fold excess of the disease compared to those of Caucasian descent (Edmonstone WM and Wilson AR 1985). A study among 1,216,425 recruits entering the US Navy 1958-1969 showed that sarcoidosis affected African-Americans 10-17 times more than Caucasians (Sartwell PE and Edwards LB 1974). Disease presentation and prognosis also vary with ethnic origin, with those of African or Asian descent being more severely affected than the general population (McNicol MW and Luce PJ 1985). Familial sarcoidosis is also more common in African Americans (19%) than in Caucasians (5%) (Harrington DW et al 1994). Although environmental factors may vary by race, preponderance of sarcoidosis in certain ethnic groups and familial aggregation suggests that genetic susceptibility may play a role in sarcoidosis aetiology.

The general concept of the immunopathogenesis of sarcoidosis includes an initiating event that is most probably triggered by an as yet unknown sarcoidosis-associated antigen that results in disease in genetically predisposed individuals. As a result of this initial event, activated CD4+ T helper (Th cells) accumulate in the alveolar space of the lungs creating a T-cell alveolitis. Alveolar macrophages also accumulate in the lung through chemotaxis and/or local proliferation. The increase in the number of cells is thought to result from both redistribution from the peripheral blood and in situ proliferation mediated by cytokines emitted from activated macrophages and T cells (Eklund A and Grunewald J 2000, Moller DR 1999)
Cytokine profiles are of central importance to the pathogenesis of sarcoidosis. The lung accumulated T cells display an activated phenotype with increased expression of interleukin 2 receptor (IL-2R) and human leukocyte antigen-DR (HLA-DR) (Konishi K et al 1988). They also produce high levels of the Th1 cytokines IL-2 (Muller-Quernheim J et al 1986) and interferon (IFN)-γ (Robinson R et al 1985). IL-12 has also been found to be elevated in the lungs of sarcoidosis patients (Moller DR, 1996). IL-12 is produced by alveolar macrophages and induces the production of IFN-γ by T cells. The expression of pro-inflammatory cytokines such as IL-1, tumour necrosis factor (TNF)-α and IL-6 is also increased in sarcoidosis (Muller-Quernheim J 1998). In contrast, expression of cytokines that down regulate inflammation, such as IL-10, could not be detected (Zissel G et al 1996). The inflammation in sarcoidosis is considered to be to some extent a prototype for a Th1 immune response.

The accumulation of inflammatory cells in the lung may progress to form the hallmark of sarcoidosis – the non-necrotising granuloma. The granuloma is a mass or a nodule composed of chronically inflamed tissue that forms as a response to a sustained antigenic challenge. The non-caseating granuloma in sarcoidosis consists of a collection of fibroblasts and epithelioid cells, with mononuclear cells at the periphery. CD4-T cells predominate in the centre, whereas CD8-T cells occupy the periphery. Cytokines such as IL-1, TNF-α and IFN-γ are essential to induce and maintain granuloma formation (Muller-Quernheim J et al 1998).

In sarcoidosis, granulomas are usually located in the upper two-thirds of the lung fields with a predilection for perilymphatic structures, but can occur anywhere in the lung parenchyma, blood vessels, airways and pleural surfaces. Granulomas may regress spontaneously or progress to overt pulmonary fibrosis, with fibrosis focusing around the granulomas. Macrophages are considered to be of central importance through their abilities to stimulate fibroblasts to proliferate and produce extracellular matrix components. However, the pathways involved in the passage from granuloma to fibrosis are not well understood. It has also been speculated that a change from the Th1 profile towards a Th2 cytokine pattern that might occur locally around the granulomas, could promote fibrosis (Eklund A and Grunewald J 2000).
Markers for progression to fibrosis are still lacking. However there have been a number of population studies on sarcoidosis reporting HLA associations (Berlin M et al 1997). It is currently unclear what these associations represent but the most likely explanation is that certain HLA alleles confer specific immune responses by presenting antigenic peptides to T cells. A skewed repertoire of T cell receptors (TCR) found in bronchoalveolar lavage (BAL) cells of sarcoidosis patients has also been suggested to have an involvement in sarcoidosis. (Moller DR et al 1988)

**Clinical manifestations of sarcoidosis.**

The clinical picture of the disease is very variable. Sarcoidosis can present either acutely or with an insidious chronic onset. Almost every organ system can be affected. The lungs are affected in over 90% of patients. Up to one-third of patients in the early stages of sarcoidosis have few or no symptoms and are detected by chance when a chest radiograph is taken for other reasons. A further one-third present with non-specific pulmonary symptoms, such as cough or dyspnoea. The last group of patients have systemic complaints such as fever, myalgias or weight loss (Eklund A and Grunewald J 2000).

The classic chest X-ray finding is bilateral bronchohilar lymphadenopathy (BHL). Parenchymal involvement may either accompany lymphadenopathy or occur by itself. These abnormalities have been grouped into a staging system that not only is descriptive, but may also provide information regarding prognosis. These can be seen in Table 1.3.
Table 1.3: Chest radiographic stages of Sarcoidosis.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Changes</th>
<th>Frequency</th>
<th>Spontaneous remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>5-10 %</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>BHL</td>
<td>50 %</td>
<td>55-90%</td>
</tr>
<tr>
<td>II</td>
<td>BHL with parenchymal infiltrates</td>
<td>25 %</td>
<td>40-70%</td>
</tr>
<tr>
<td>III</td>
<td>Parenchymal infiltrates, no BHL</td>
<td>15 %</td>
<td>10-30%</td>
</tr>
<tr>
<td>IV</td>
<td>Signs of fibrosis</td>
<td>5-10 %</td>
<td>0-5%</td>
</tr>
</tbody>
</table>

Adapted from Costabel U 2001.

**Extrathoracic manifestations**

Skin involvement may occur in approximately 10% to 40% of patients and usually manifests itself as erythema nodosum or lupus pernio. Erythema nodosum is an immune-complex–related condition that presents as painful panniculitis with red tender lesions over the shins and calves. Lupus pernio is a chronic bluish purple elevation that affects the nose, cheeks and ears. Ocular sarcoidosis is common, occurring in up to 25% of all cases, and can lead to blindness if undetected. The major manifestations are uveitis, conjunctival granulomas and dry eyes as a result of lacrimal gland infiltration. When erythema nodosum occurs with BHL, peripheral lymphadenopathy, uveitis and constitutional upset, the picture is complete for Löfgren's syndrome (Löfgren S 1952). The latter has an acute onset and is associated with favourable prognosis. Clinically recognisable neurological sarcoidosis occurs in less than 10% of patients. Almost any area of the nervous system can be affected by sarcoidosis with cranial nerve involvement being the most common manifestation. Renal failure may also occur. Hepatomegaly or splenomegaly are seen in 20% of patients. Any part of the heart may also be involved, although rarely the valves (Eklund A and Grunewald J 2000).

**Diagnosis**

Diagnosis of sarcoidosis is made on clinical suspicion alone in an asymptomatic 20- to 40- year old patient with bilateral hilar lymphadenopathy, especially if erythema nodosum and uveitis are present. In more difficult clinical scenarios, tissue diagnosis is required. Laboratory tests are non-specific but may help support the diagnosis.
Angiotensin converting enzyme (ACE) is produced by sarcoid granuloma (Silverstein E et al 1979) and is elevated in 40% to 80% of patients with sarcoidosis. ACE levels tend to decrease with regression and treatment of the disease. Nevertheless, the enzyme is non-specific and can be elevated in other disease processes e.g. ARDS. BAL is also non-specific although the percentage of lymphocytes is usually elevated. The earliest findings in pulmonary function tests are reduction in lung volume and a reduced diffusion capacity. Obstructive patterns may also be encountered if granuloma or inflamed lymph nodes impinge on the airways. Pulmonary function tests however correlate poorly with X-ray findings and clinical findings because the pulmonary functional reserve is so large. High-resolution computed tomography (HRCT) may also be used to distinguish between fibrotic and irreversible changes in the thorax from areas of active inflammatory disease. As the disease is very variable, the most accurate way to assess its activity is by combined evaluation based on symptoms and changes in radiographic involvement and lung function (Eklund A and Grunewald J 2000).

**Treatment**

Treatment of sarcoidosis is often difficult because the clinical course is variable and unpredictable and because most of the agents used to treat the disease are associated with significant side effects. Corticosteroids have been considered as the first-line agents as they have powerful anti-inflammatory capabilities and may interrupt the heightened immune response seen in sarcoidosis. In Löfgrens syndrome there is no need for steroid therapy as the prognosis is very favourable. Cytotoxic drugs may also be used in combination with corticosteroids but in some selected cases they may be given as single therapy (Costabel U 2001). In advanced pulmonary sarcoidosis causing respiratory insufficiency, oxygen therapy or lung transplantation must be considered.
1.2 PATHOGENESIS OF PULMONARY FIBROSIS

1.2.1 CURRENT CONCEPTS ABOUT THE PATHOGENESIS OF PULMONARY FIBROSIS

The events leading to fibrosis are similar to those seen during normal wound healing (Figure 1.1) suggesting that fibrosis may be the consequence of an aberrant wound healing response. One commonly held hypothesis is that fibrosis is the result of an initial inflammatory insult to the alveolar wall with subsequent failure of resolution and repair. A universal feature of pulmonary fibrosis is alterations in the epithelial cells that define the alveolar space. Following epithelial cell injury and loss, a provisional fibrin matrix is formed within the alveolar space. As in normal wound healing, inflammatory cells accumulate within the alveolus and secrete soluble mediators that in turn attract other inflammatory cells and cause the migration and proliferation of interstitial fibroblasts. Many of these fibroblasts acquire a distinctive ‘contractile’ phenotype (myofibroblasts) in their effort to contribute to wound closure. In pulmonary fibrosis, this process differs from the normal wound healing model because of abnormalities in myofibroblast behaviour. These fibroblasts produce excess collagen that is deposited in the interstitium and the alveolar space. Actively proliferating fibroblasts/myofibroblasts aggregate and form fibroblastic foci (Katzenstein AA and Myers JL 1998). Furthermore, re-epithelialisation of the denuded areas, an essential step in normal repair, is slow and inadequate. Loss of regulated turnover as a result of an imbalance between matrix degrading (matrix metalloproteases or MMPs) and matrix enhancing (tissue inhibitors of MMPs or TIMPs) enzymes, also favours increased deposition of extracellular matrix (ECM). The progressive and disorganised deposition of collagen results in interstitial thickening and loss of alveolar architecture. The final result is an aberrant remodelling of the lung parenchyma where normal alveolar structure is completely replaced by a dense non-functional fibrotic tissue, leading to reduced lung volume and impaired gas exchange (Selman M et al 2001)
Figure 1.1. The normal wound healing model. Adapted from Selman M. et al 2001
As our understanding of the molecular pathways implicated in pulmonary fibrosis improved, considerable controversy developed over the years regarding the relevance of inflammation in the disease process. The widely held view has been that inflammation is the major driving force of the fibrotic process. Recently however, experts in the field have challenged this concept (Selman M et al 2001, Gauldie J et al 2001). As mentioned in section 1.1.1 these concerns were first raised following observations that anti-inflammatory treatment failed to improve disease outcome. Furthermore, for IPF in particular, there is very little evidence to support the concept that inflammation is a prominent feature of the disease (Selman M et al 2002).

Nevertheless, many interstitial lung diseases include an identifiable initial inflammatory response to an unknown (e.g. sarcoidosis) or known (pigeon breeder’s disease) damaging agent.

In view of that, Drs Selman and Pardo have proposed that there are at least two routes for developing diffuse pulmonary fibrosis: the ‘inflammatory route’ which is represented by almost all the non-IPF interstitial lung diseases and is characterised by an early phase of alveolitis and a late fibrotic phase, and the non-inflammatory route which is represented by IPF (Selman M and Pardo A 2002). They propose that IPF is an epithelial/fibroblastic cross-talk disorder that is associated more with intrinsic abnormal wound healing than inflammation. The ‘epithelial/fibroblast route’ postulated by Selman and Pardo in IPF is shown in Figure 1.2.
Figure 1.2: Model by Selman and Pardo, proposing that IPF is an epithelial-fibroblastic disorder. Adapted from Selman M and Pardo A 2002.
The current concepts about fibrosis progression are illustrated in Figure 1.3.

The distinction between inflammation-driven fibrosis and the 'epithelial/fibroblast' route to fibrosis may in the future prove to be highly relevant in clinical practice. Undoubtedly, appropriate therapy will rely on the understanding of the nature of the response to injury and the molecular mechanisms involved.

A number of mediators seem to be implicated in the pathogenesis of pulmonary fibrosis and it is increasingly believed that many of the defects contributing to progression to fibrosis are the result of an imbalance between stimulatory and inhibitory mediators. The remainder of this section focuses on some of the main growth factors and mediators for which there is compelling evidence for their involvement in the molecular pathways controlling fibrogenesis.
INJURY

NORMAL TISSUE

INFLAMMATION

MATRIX SYNTHESIS

REPAIR

Repeat injury or
Low level of inflammation

BYPASS OF CONTROL MECHANISMS LEADS TO EXCESS MATRIX DEPOSITION

FIBROSIS PROGRESSION

Inflammation-independent pathway

‘Chronic switch’

MATRİX DEGRADATION

MATRİX ACCUMULATION

Figure 1.3: Current concepts on pathways to fibrosis progression.
Presented at the 12th International Colloquium on Lung Fibrosis held in Glion, October 2002
1.2.2 MEDIATORS OF PULMONARY FIBROSIS

1.2.2.1 Transforming Growth Factor-β (TGF-β)

The members of the TGF-β family of peptides (TGF-β1, TGF-β2, TGF-β3) bind to the same receptors and have similar biological functions. The TGF-β1 isoform has been the most widely studied in the context of fibrogenesis. TGF-β is the most potent stimulator of collagen production yet described. TGF-β acts at various levels of collagen regulation. It stimulates procollagen gene transcription, increases mRNA stability, decreases the intracellular degradation of procollagen and limits the amount of collagen degraded extracellularly by inhibiting collagenase production and increasing the production of matrix metalloproteinase inhibitors (reviewed by Lasky JA & Brody AR 2000). In animal models of pulmonary fibrosis, enhanced TGF-β gene expression is temporally and spatially related to increased collagen gene expression and protein deposition (Hoyt DG & Lazo JS 1988). Levels of TGF-β were shown to be maximal prior to the peak in collagen synthesis (Khalil N et al 1989) and antibodies to TGF-β reduced collagen deposition in bleomycin-induced pulmonary fibrosis in mice (Giri SN et al 1993). Human fibrotic lung tissue also shows enhanced TGF-β gene and protein expression. Hybridisation studies have demonstrated the presence of TGF-β1 mRNA in areas of early active disease associated with macrophages close to fibroblasts actively expressing procollagen I mRNA (Broekelmann TJ et al 1991). Immunohistochemical studies in patients with IPF revealed increased expression of TGF-β1 in various cell types. In early disease with minimal fibrosis, TGF-β1 was found primarily in alveolar macrophages whereas in advanced fibrotic lesions TGF-β1 overexpression was localised in hyperplastic type II alveolar epithelial cells (Khalil N et al 1991). All the above demonstrate that TGF-β is an integral component of the fibrotic tissue. Importantly, TGF-β also has a role in causing the differentiation of myofibroblasts, a critical element in tissue repair and fibrosis (Desmouliere A et al 1993). Moreover, transient overexpression of active TGF-β1 results in prolonged and severe fibrosis in the rat lung (Sime PJ et al 1997). Finally, a polymorphism at codon 25 (position +915) in the signal sequence of the TGF-β1 gene results in an amino acid change that affects TGF-β production. The high-producer allele was reported to associate with allograft

1.2.2.2 Tumour Necrosis Factor-alpha (TNF-α)
There are several lines of evidence to support a key role for TNF-α in pulmonary fibrosis. TNF-α induces fibroblast proliferation and collagen synthesis in vitro, and pulmonary TNF-α gene expression is elevated in bleomycin-induced lung fibrosis (Piguet et al 1989). In patients with IPF or asbestosis, bronchoalveolar lavage fluid-derived macrophages release increased amounts of TNF-α compared with controls (Zhang Y et al, 1993). In addition, TNF-α levels were found to be increased in epithelial cells from human fibrotic lungs when compared with cells from normal lungs (Piguet et al, 1993). Mice overexpressing TNF-α develop IPF-like fibrosis whereas TNF-α-deficient or double TNF-α receptor knockout mice are resistant to bleomycin-induced fibrosis (reviewed by Lasky JA and Brody AR, 2000). Furthermore, in vivo treatment of animals with neutralising antibody to TNF-α or recombinant soluble TNF-α receptor successfully abrogates pulmonary fibrosis (Piguet PF et al 1989, Piguet PF and Vesin C, 1994). TNF-α activity promotes induction of the matrix-degrading enzymes, gelatinases, that disrupt the basement membrane and facilitate fibroblast migration (reviewed by Selman M et al, 2001). Finally, a TNF-α promoter polymorphism seems to confer increased risk of developing IPF (Whyte M et al 2000) while treatment of IPF patients with pirfenidone, a novel anti-fibrotic agent with anti-TNF-α properties, was reported to have beneficial effects (Raghu G et al, 1999).

1.2.2.3 Platelet-derived growth factor (PDGF).
PDGF mediates fibroblast proliferation and chemotaxis (Stiles CD et al 1979, Seppä H et al 1982). It is composed of two polypeptide chains, A and B, and can be active as a homodimer or a heterodimer. Two PDGF receptors (PDGF-R) have been identified which differ in their affinities for the A and B isoforms. PDGF and PDGF-R are expressed at low levels in alveolar macrophages in the normal adult lung but are upregulated in IPF (Homma S et al 1995). In alveolar macrophages obtained from IPF patients PDGF-B mRNA was present in tenfold greater abundance than PDGF-A mRNA (Nagaoka I et al 1990). Type II alveolar epithelial cells were
found to express PDGF and PDGF-R in early, but not late stage IPF (Homma S et al 1995). In addition, macrophages are also known to secrete increased amounts of PDGF in pulmonary fibrosis (Martinet Y et al 1987). Finally, overexpression of human PDGF-B in rats induced histopathologic changes similar to those seen in lung tissues from patients with pulmonary fibrosis. Specifically, PDGF-B overexpression resulted in an apparent cellular infiltration in the alveoli and significant fibroblast proliferation and collagen deposition (Yoshida M et al 1995).

1.2.2.4 Endothelin-1 (ET-1)
Endothelin-1 (ET-1) is also a profibrotic cytokine. It promotes fibroblast proliferation and chemotaxis (Peacock AJ et al 1992) and stimulates procollagen production (Dawes KE et al 1996). ET-1 is generated from its inactive precursor, big endothelin, to the mature form by the endothelin-converting enzyme (ECE-1). Expression of big endothelin, ET-1 and ECE-1 is elevated and co-localised in IPF lungs and appears to correlate with disease activity (Saleh D et al 1997). Furthermore, blocking of the endothelin receptors ET-A and ET-B by the endothelin receptor antagonist bosentan, ameliorated lung fibrosis in bleomycin-treated rats (Park S-H et al 1997).

1.2.2.5 Interleukin-1β (IL-1β)
IL-1β is a highly pro-inflammatory cytokine and justifiably most investigations to date have focused on its pro-inflammatory effects. However, there is now increasing evidence that IL-1β can also have pro-fibrotic properties. IL-1β was shown to be expressed in fibroproliferative areas in idiopathic pulmonary fibrosis (Pan LH et al 1996). Furthermore, inhibition of IL-β by administration of IL-1 receptor antagonist (IL-1ra) attenuated pulmonary fibrosis in bleomycin or silica-treated mice (Piguet PF et al 1993). More recently, it was reported that transient overexpression of human IL-β in the rodent lung induces acute lung injury and chronic repair leading to pulmonary fibrosis (Kolb M et al 2001). However, the progression to fibrosis is not due to any persistent effects of IL-β itself, since its expression was only increased transiently and had returned to near baseline values by 14 days after infection. In contrast, pulmonary fibrosis was not apparent until day 21 and dramatically increased thereafter. This suggests that inflammation may be necessary but not
sufficient to explain the pathophysiology of fibrosis as the inflammatory response had resolved by the time fibrosis became apparent and was therefore not required for subsequent progression (Sheppard D 2001).

1.2.2.6 Insulin-like growth factor-1 (IGF-1) and IGF-binding proteins (IGFBPs).
IGF-1 is another potent mitogen for fibroblasts and a stimulator of fibroblast collagen synthesis (Goldstein RH et al 1989). It has been shown to be released by macrophages from patients with pulmonary fibrosis (Rom WN et al 1988) and is present in increased amounts in the BAL fluid obtained from patients with systemic sclerosis (Harrison NK et al 1994). However, recent studies have shown that IGF-1 expression levels are actually decreased in unfractionated BAL cells from patients with IPF compared with normal controls (Bloor CA et al 2001). In addition, high levels of IGF-1 and IGF-1 receptor are only found in early-stage IPF with minimal fibrosis and are localised to a number of cell types including alveolar macrophages and in particular type II alveolar epithelial cells. By contrast, in normal controls and late-stage IPF, only alveolar macrophages were found to express IGF-1 and its receptor. This suggests that IGF-1 might play a role in the initiation of IPF (Homma S et al 1995). The activity of IGF-1 is regulated by the IGFBPs, that can both stimulate and inhibit IGF-mediated events, as well as exert IGF-independent effects. IGFBP-2 and IGFBP-3 levels were found to be increased in BAL from patients with IPF (Allen JT et al 1998, Chadelat K et al 1998). Moreover, IGFBP-3 can be induced by TGF-β1 (Parker E et al 2000).

1.2.2.7 Connective tissue growth factor (CTGF)
CTGF belongs to a family of conserved and modular proteins (known as the CCN family) with diverse biological functions involved in the regulation of cell growth and differentiation. It was originally isolated from human umbilical vein endothelial cells (Bradham DM et al 1991) but it is also highly expressed by fibroblasts (Igarashi A et al 1993). CTGF may play an important role in promoting connective tissue formation after tissue injury. Apart from being a potent fibroblast mitogen and chemoattractant, CTGF also stimulates fibroblast procollagen and fibronectin production and affects α5 integrin mRNA levels in vitro (Frazier K et al 1996). CTGF is also increased in skin and internal organ fibrosis (Igarashi A et al 1996, Ito Y et al 1998, Allen JT et al 1999) and the fibrotic areas in atherosclerotic lesions
(Oemar BS et al 1997), suggesting an involvement for CTGF in the development of tissue fibrosis. There is now increasing evidence supporting a role for CTGF as a downstream mediator of TGF-β actions. TGF-β1 induces CTGF expression in cultured fibroblasts while other fibrotic mediators such as PDGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and IGF-1 have no effect (Igarashi A et al 1996, Ricupero DA et al 1999). This finding is in agreement with the recent characterisation of a novel TGF-β-response element within the CTGF promoter (Grotendorst GR et al 1996). Increased expression of CTGF has been reported in IPF and sarcoidosis patients (Allen JT et al 1999). Furthermore, downregulation of CTGF expression seems to offer protection from fibrosis. In a preliminary trial of IFN-γ co-therapy in IPF patients, clinical improvement was associated with inhibition of CTGF expression (Ziesche R et al 1999).

1.2.2.8 Thrombin
Thrombin is a multifunctional serine protease that has a central role in hemostasis following tissue injury through its ability to convert soluble plasma fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation. The activation of the coagulation cascade has been implicated in the pathogenesis of pulmonary fibrosis. Intra-alveolar accumulation of fibrin has been described for patients with pulmonary fibrosis (Chapman HA et al 1986, Kotani I et al 1995) and acute respiratory distress syndrome (Bachofen M et al 1982, Idell S et al 1987). Increased thrombin levels were found in BAL fluid obtained from patients with pulmonary fibrosis associated with systemic sclerosis (Hernandez-Rodriquez NA et al 1995). BAL fluid thrombin levels were also shown to be elevated in bleomycin-induced fibrosis in rats (Tani K et al 1991). In addition to its procoagulant effects, thrombin can also influence a number of other biological responses that play important roles in subsequent inflammatory and tissue repair processes. Thrombin is a potent mitogen for a number of cell types including endothelial cells, fibroblasts and smooth muscle cells and can also affect the recruitment and trafficking of inflammatory cells (reviewed in Dery O et al 1998). Thrombin has been shown to stimulate fibroblast procollagen production via proteolytic activation of protease-activated receptor 1 (Chambers RC et al 1998). In addition, thrombin can modulate the secretion of extracellular matrix proteins (Papadimitriou E et al 1997) and its effects on connective tissue remodelling.
processes have also been reported (Duhamel-Clerin E et al 1997). Interestingly, thrombin has also been shown to be a potent inducer of connective tissue growth factor production (Chambers RC et al 2000). Finally, direct thrombin inhibition is known to reduce lung collagen and CTGF mRNA levels in bleomycin-induced pulmonary fibrosis (Howell DCJ et al 2001).

1.2.2.9 Prostaglandin E2 (PGE2)

PGE2 is implicated in the pathogenesis of pulmonary fibrosis through its effects on collagen production and fibroblast proliferation. Saltzman and co-workers first demonstrated that PGE2 suppressed collagen production by human foetal lung fibroblasts (Saltzman LE et al 1982). PGE2 inhibits type I collagen formation in TGF-β-stimulated lung fibroblasts by decreasing collagen gene expression (Fine A et al 1989). In adult human lung fibroblasts, stimulation of PGE2 by TNF-α and IL-1β resulted in inhibition of type I collagen production (Diaz A et al 1993). Furthermore, inhibition of IL-1-stimulated PGE2 synthesis by indomethacin, enhances type I collagen synthesis in synovial fibroblasts and chondrocytes (Goldring MB & Krane SM 1987). PGE2 is also able to act as a feedback mechanism dampening the effects of bradykinin on collagen production in human foetal lung fibroblasts (Goldstein RH & Polgar P 1982). PGE2 is also a known suppressor of fibroblast proliferation (Korn JH et al 1980, Bitterman PB et al 1986, Elias JA et al 1988). In an early study of bleomycin-induced pulmonary fibrosis in hamsters, increases in PGE2 and cyclic adenosine monophosphate (cAMP) were associated with suppression of fibroblast proliferation and collagen production (Clark JG et al 1982). In addition, PGE1 (another member of the E series of prostaglandins derived from dihomo-γ-linoleic acid) increases intracellular levels of cAMP and causes a decrease in collagen production by foetal lung fibroblasts. This was shown to be due to an increase in the proportion of newly synthesised collagen degraded intracellularly prior to secretion (Baum BJ et al 1980). PGE2 also downregulates the expression of lysyl oxidase, an enzyme that crosslinks fibrillar collagen, and inhibits the enhancing effects of TGF-β and IL-1β on the expression of this gene (Roy R et al 1996, Choung J et al 1998). In addition, PGE2 inhibits the TGF-β-stimulated transcription of the CTGF gene and thereby regulates α(I) collagen mRNA levels (Ricupero DA et al 1999).
It is interesting to note that many of the profibrotic mediators that are known to stimulate procollagen synthesis e.g PDGF, TGF-β, TNF-α, EGF and IL-1β, also have the ability to induce fibroblasts to synthesise PGE₂ (Habenicht AJ et al 1985, McAnulty RJ et al 1997, Mauviel A et al 1988, Diaz A et al 1992). This indicates the presence of tightly regulated feedback loops and suggests that an imbalance in these processes may favour a more fibrotic environment. In fact, inhibition of cyclooxygenase (COX)-2, the inducible enzyme that generates PGE₂, completely abolishes the anti-proliferative effects of TGF-β and restores the stimulatory effects observed at low TGF-β concentrations. This suggests that the inhibition of fibroblast proliferation seen at higher concentrations of TGF-β, may be mediated by autocrine stimulation of PGE₂ synthesis (McAnulty RJ et al 1997).

In this context, reduced levels of PGE₂ could de-repress fibroblast proliferation and collagen synthesis and thereby contribute to the development of fibrosis. A PGE₂ ‘deficiency’ has been reported in pulmonary fibrosis. Epithelial lining fluid from the lungs of IPF patients contains 50% less PGE₂ than normal (Borok Z et al 1991) and lung fibroblasts isolated from patients with IPF have a diminished capacity to upregulate COX-2 and therefore synthesise PGE₂ (Wilborn J et al 1995, Keerthisingam CB et al 2001). Vancheri C et al (2000) also demonstrated that fibrotic fibroblasts spontaneously produce less PGE₂ compared with normal fibroblasts. They also observed that the ability of TNF-α to induce PGE₂ was impaired in fibrotic fibroblasts and that this was related to a reduced expression of COX-2. PGE₂ production by macrophages obtained from patients with pulmonary fibrosis is also reduced (Ozaki T et al 1990). Recently, reduced COX-2 expression was also demonstrated in sarcoidosis (Petkova DK et al 2003). Furthermore, COX-2 deficient mice exhibit a pro-fibrotic phenotype (Morham SG et al 1995, Dinchuk JE et al 1995) and an enhanced fibroproliferative reaction in the lungs in response to bleomycin (Keerthisingam CB et al 2001).
1.3 GENETIC SUSCEPTIBILITY TO PULMONARY FIBROSIS

Evidence for a genetic influence in the development of pulmonary fibrosis derives from a number of observations. In pulmonary fibrosis, a broad spectrum of responses to the same aetiological factor is often observed. For example, only a small group of patients out of many who receive similar doses of a drug such as bleomycin or amioradone, go on to develop pulmonary fibrosis. In addition, there is a marked variation in response to profibrotic agents such as asbestos observed in humans, even when the level of exposure is similar. (Tisdale JE et al 1995). Certain animal strains also show differences in susceptibility to fibrotic agents, which suggests an inheritable predisposition. For example, the C57BL/6 mouse strain is sensitive to bleomycin-induced lung fibrosis whereas the BALB/c mouse strain is fibrosis-resistant (Schrier DJ et al 1993). All the above suggest that the combination of a genetic predisposition to disease together with an appropriate trigger may be required for a particular pathological process to occur. (Marshall RP et al 1997).

Sarcoidosis and idiopathic pulmonary fibrosis (IPF) represent the most frequently occurring diffuse lung diseases with multifactorial aetiology which likely develop in genetically predisposed individuals in response to environmental triggers. Because sarcoidosis and IPF are not inherited in a simple Mendelian pattern, multiple genetic loci are likely to be involved. (Luisetti M et al 2000, Marshall RP et al 1997). Reports of familial clustering in both IPF and sarcoidosis and the racial and ethnic variation observed in sarcoidosis incidence strongly support a role for genetic susceptibility. However, the understanding of the genetic boundaries of this supporting evidence is limited by the fact that the aetiology of both diseases remains unknown. One cannot therefore exclude that some racial variation might be attributed to varying environmental risk. Furthermore, it can be difficult to differentiate true inheritance of disease from the mere clustering of cases within a family exposed to a common environmental factor. (Luisetti M et al 2000, Marshall RP et al 2000). However, shared environmental factors alone are unlikely to account for familial clustering, unless the presumed environmental factors confer relative risks of 100 or more. If environmental factors of this magnitude existed, it is likely that previous case-control studies would have detected them (Rybicki BA et al 1997). Moreover, cases reported in twins and family members separated from an

1.3.1 STUDIES IN SARCOIDOSIS
Good gene targets in sarcoidosis are those that favour granuloma formation and fibrosis. Candidate genes predisposing an individual to develop sarcoidosis would therefore be expected to reside in loci that influence T-cell function, the regulation of antigen recognition, processing and presentation, and/or the regulation of matrix deposition (American Thoracic Society 1999). The majority of studies to date have focused on the role of the HLA genes of the major histocompatibility complex (MHC). The role of HLA polymorphisms has been extensively investigated in sarcoidosis although results between groups are often conflicting.

1.3.1.1 HLA and associated genes
HLA DR5 was significantly associated with disease in 73 German patients (odds ratio [OR] = 6.56) (Nowack D et al 1987) whereas no associations were found with DR antigens in 107 Italian patients (Pasturenzi L et al 1993). In another study of 107 patients from Italy and 126 patients from the Czech Republic, an association was found between the presence of HLA-A1, B8 and DR3 and certain manifestations of the disease. There was no association with HLA B12 and DR4 (Martinetti M et al 1995). In the Italian group, an association was found between HLA B22 and disseminated systemic disease that was not present in the patients from Czech Republic (Martinetti M et al 1995). In a study of a Scandinavian group of 122 patients with sarcoidosis, the presence of HLA DR17 associated with a favourable prognosis, whereas DR14 and DR15 were found to associate with a more protracted disease course (Berlin M et al 1997). Two Japanese studies, one by Kunikane et al including 53 subjects and one by Ina et al including 114 patients, showed that the frequency of HLA DRw52 was higher in patients with sarcoidosis than in controls (Kunikane H et al 1987, Ina Y et al 1989). Another Japanese study of 58 subjects showed that HLA DR5J-positive patients often experienced a poorly resolving disease (Abe S et al 1987). A study of 41 Danish patients showed a significant association with DRw6 (relative risk [RR] = 3.2) but no association with DQ or DP antigens (Odum N et al 1991). Despite the amount of data accumulated on HLA
genes in sarcoidosis, it remains unclear as to what these results really mean. The heterogeneity of the populations studied, both affected and unaffected, make comparisons difficult and the small numbers involved in these studies may partly explain the conflicting results. Nevertheless, the rationale for investigating HLA genes is valid and therefore further work is clearly needed. HLA frequencies differ between ethnic groups and geographical location. The disparity amongst investigations may be the result of population stratification or lack of power to detect an effect, but it may also suggest that the genetic predisposition is more complex and that multiple genes and external stimuli are likely to be involved (Verleden GM et al 2001).

A recent study has identified an association between berylliosis and the presence of a glutamine (Glu) residue at position 69 of the β-chain of the HLA-DP gene (HLA-DPGlu69) (Richeldi L et al 1993). Beryllium inhalation produces granulomata in the lung, which are pathologically indistinguishable from those found in sarcoidosis (Saltini C et al 1998). In fact, berylliosis is often referred to as a form of sarcoidosis of known cause. This prompted investigators to search for a similar association between HLA-DPGlu69 and sarcoidosis. In an early study, a significant increase in the frequency of HLA-DPGlu69 was found in British patients with sarcoidosis compared to controls (Lympany PA et al 1996), which was however not confirmed in a follow up study (Foley PJ et al 1999). More recently Maliarik et al reported that HLA-DPGlu69 was not associated with sarcoidosis in African-Americans while Valine (Val)36 and Aspartate (Asp)55 were associated with an increased risk for sarcoidosis (Maliarik MJ et al 1998).

Polymorphisms of the transporter associated with antigen processing (TAP) have also been investigated. Foley et al (1999) examined several polymorphisms in the TAP1 and TAP2 genes in British sarcoid patients. The TAP2 Ala565/Thr565 and Thr665/Ala665 genotypes were significantly less represented in the patient group than in controls. (Foley PJ et al 1999).

1.3.1.2 Angiotensin converting enzyme (ACE) gene
The ACE polymorphism has also been investigated in sarcoidosis. Serum levels of ACE were shown to have potential value in confirming the diagnosis and estimating
the activity of sarcoidosis (Lieberman J et al 1979). Approximately 50-60% of patients with sarcoidosis have increased levels of ACE which tend to be higher in patients with stage II and stage III disease (see Table 1.3), and are thought to reflect the whole body granuloma mass (Allen RKA 1994).

Rigat B et al (1990) described an insertion (I) / deletion (D) polymorphism in intron 16 of the ACE gene that accounts for approximately 50% of the total phenotypic variation in plasma ACE levels. In a gene-dose-dependent manner, DD genotype associates with higher levels of plasma ACE, II with lower levels and ID with intermediate levels (Rigat B et al 1990). This polymorphism is probably the best characterised in sarcoidosis but there is still no clear consensus. The DD genotype was shown to be a significant risk factor for African-American sarcoid patients (OR=3.17), in particular those that had a family history of the disorder (Maliarik MJ et al 1998). By contrast, ACE genotype distribution does not differ between patients and controls in studies of Italian (Arbustini E et al 1996), American (Maliarik MJ et al 1998) and Finnish (Pietinalho A et al 1999) groups. Contrasting results were also obtained in the Japanese. An early report found an excess of ID or DD genotypes among female patients (OR=2.18) (Furuya K et al 1996), but no association was found in a large series of subjects from central Japan (Tomita H et al 1997). When investigating the relationship between ACE polymorphisms and clinical manifestations, Tomita et al found no significant differences in the percentage of eye, skin or heart involvement between the three genotypes and no correlation with the prognosis of sarcoidosis (Tomita H et al 1997). In a study of UK and Czech cases, no significant differences were seen in the distribution of the ACE I/D genotypes and there was no association between the ACE polymorphism and pulmonary disease severity, fibrosis and progression (McGrath DS et al 2001). No correlation was found between ACE genotype and sarcoidosis parameters such as chest radiograph stage, extra-pulmonary involvement and progression in Italian and Japanese patients whereas a modest correlation was observed between the II genotype and radiographic progression in the African-American patients (Maliarik MJ et al 1998). By contrast, the DD genotype was associated with poor prognosis in Finnish patients (Pietinalho A et al 1999). In another study, Papadopoulos et al found that the DD genotype was significantly increased in sarcoidosis patients with autoimmune manifestations and major granuloma mass, suggesting that the DD
genotype may confer susceptibility for autoimmune manifestations in sarcoidosis (Papadopoulous KI et al 2000). Finally, in a paper by Takemoto et al (1998), the AGTR1 allele C of the polymorphism in an untranslated region of the angiotensin II type I receptor was reported to associate with higher levels of ACE in sarcoidosis, thus suggesting a regulatory factor for ACE elevation in sarcoidosis that is independent from allele D.

1.3.1.3 Other studies

Several other polymorphisms have been examined in sarcoidosis. TNF-α is considered to be an important mediator in granuloma formation and in the immunopathogenesis of progressive sarcoid disease (Muller-Quernheim J 1998). Two polymorphisms have been described in the TNF gene complex: LtaNc0l in the first intron of the lymphotoxin α gene, and TNF-308 in the promoter of the TNF-α gene. LtaNc0l*1 and TNF-308*2 alleles are associated with increased levels of TNF production. Neither polymorphism was associated with sarcoidosis in 101 German sarcoid patients (Seitzer U et al 1997). Similar negative findings were reported in Italians (Richeldi L et al 1997) and Japanese (Ishihara M et al 1995).

IL-1 is a cytokine with well-known roles in inflammation and innate immunity. The most important members of the family are the agonists IL-1α, IL-1β and their naturally occurring inhibitor, IL-1 receptor antagonist (IL-1Ra), which counteracts the pro-inflammatory functions of IL-1. In a study of a 95 sarcoidosis patients from the Czech Republic, the IL-1α -889 1.1 genotype was found to be over-represented in patients with sarcoidosis compared with controls but there were no significant differences in the distribution of IL-1β -511, IL1-β +3953 and IL-1Ra variable number tandem repeat (VNTR) genotypes (Hutyrova B et al 2002). Similarly, Niimi T et al (2000) found no significant differences in IL-1-Ra and IL-β genotypes between 108 Japanese sarcoidosis patients and 113 controls.

Rybicky BA et al (1999) found a significant association with the IL-1α marker on 2q13 and with the F13A marker on 6p23-25 in African Americans. Both genes code for mediators relevant to the pathogenesis of sarcoidosis. The role of IL-1α in induction and maintenance of granuloma is well recognised and the F13A marker is
located close to the interferon regulatory factor protein (IRF-4) gene coding for a member of the IRF family of transcription factors which is thought to contribute to granuloma formation by attracting T-cells. Rybicki BA et al found that individuals with both IL-1α*137 and F13A*188 alleles have a six-fold increased risk of sarcoidosis and that the risk is even higher if the analysis is restricted to subjects with a family history of sarcoidosis (Rybicki BA et al 1999).

The 64Val→Ile variant in monocyte chemotactic protein-1 (MCP-1), an important chemotactic factor for inflammatory cells in sarcoidosis, was found to be less frequent in 100 Japanese patients with sarcoidosis than in 122 controls (OR=0.369). This would suggest a protective role for this variant in sarcoidosis (Hizawa N et al 1999).

A polymorphism in the Vitamin D receptor has also been shown to have a role in sarcoidosis. 1,23-Dihydroxy-vitamin D3 (1,25(OH)₂D₃) is one of many sarcoid granuloma products. It has modulating effects on several immune reactions and it has been postulated that its high levels are in part responsible for the increased bone resorption often observed in sarcoidosis (Rizzato G et al 1994). (1,25(OH)₂D₃) binds to nuclear vitamin D receptors (VDR). A polymorphism in intron 8 of the VDR gene accounts for three genotypes bb, bB and BB. The bb genotype is related to reduced VDR mRNA expression. Investigation of this polymorphism in Japanese patients with sarcoidosis revealed that the genotype distribution was significantly different between patients and controls with the frequency of allele B being increased in sarcoidosis (Niimi T et al 1999).

Despite the lack of a rationale linking the cystic fibrosis gene with sarcoidosis the possibility of such a relationship has been raised. During gene screening in different respiratory diseases, 5 out of 8 sarcoid patients were found to have at least one mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Bombieri C et al 1998). This finding was subsequently confirmed in a larger series of patients in a case-control study (Bombieri C et al 2000). A possible explanation is that a modified CFTR protein may facilitate entry of an infectious agent into the airways and trigger an immune-mediated response. Further studies are however necessary in order to confirm a relationship between CFTR and sarcoidosis.
1.3.2 STUDIES IN IPF

Studies in IPF have yielded very few results to date. An early study reported an increase in the frequency of the MZ phenotype of the α1 antitrypsin deficiency in IPF patients compared to controls (Geddes DM et al 1977). Associations between IPF and major histocompatibility genes have also been suggested, including HLA-B15, -B8, -B12, -DR2 and -Dw6, but clear evidence for these associations is still lacking (Lympany PA and duBois RM 1997).

Whyte M et al (2000) studied the +2018 polymorphism in the IL-1ra gene and the −308 variant of the TNF-α gene in 88 IPF cases and controls from England and 61 cases and 103 controls from Italy. They found that in the English group, the +2018 allele 2 of the IL-1ra gene conferred an increased risk of developing IPF. In homozygous subjects, the relative odds of IPF were increased by an OR of 10.2 (95% confidence intervals [CI] = 1.26 to 81.4; P=0.03) while for carriers the results were not significant (OR = 1.85; 95% CI = 0.94 to 3.63; P=0.075). In the Italian group however, risk of IPF was significantly increased in carriers by an OR of 2.40 (95% CI = 1.26 to 4.60; P=0.008) but not in the homozygous subjects (OR = 2.54; 95% CI = 0.68 to 9.50; P=0.2). Carriage of the -308 allele 2 in the TNF-α gene was associated with increased risk of IPF in the Italian population (OR = 2.50; 95% CI = 1.14 to 5.47; P=0.022) but the results were not significant in the English group (OR = 1.85; 95% CI = 0.94 to 3.63; P=0.075) (Whyte M et al 2000).

Pantelidis P et al (2001) found no significant differences between 74 white UK IPF cases and 100 controls in the frequencies of the following polymorphisms: TNF-α +488 G/A, -238 G/A, -308 G/A, lymphotoxin (LT)-α +720 C/A, +365 C/G, +249 A/G, TNF—receptor 2 (TNF-RII) 676 T/G, 1633 A/G, 1668 T/G, 1690 C/T and IL-6 -174 G/C, 4A/G. They observed however an increase in the frequency of cocarriage of the IL-6 intron 4G and the TNF-RII 1690C allele in the IPF population but not in the control group (Pantelidis P et al 2001).

Transforming growth factor (TGF)-β1 is a critical mediator of fibrogenesis with direct effects on cells that stimulate ECM production, fibroblast proliferation and induction of a myofibroblast phenotype (Sime PJ et al 2001). In view of that, functional alterations in the TGF-β gene could influence the pathogenesis of
pulmonary fibrosis. There are two genetic polymorphisms in the DNA sequence encoding the leader sequence of TGF-β1, located at codon 10 (leucine to proline substitution) and at codon 25 (arginine to proline substitution). The arginine/arginine homozygous genotype at codon 25 and the presence of the leucine allele at codon 10 have been associated with increased TGF-β1 production (Awad MR et al 1998). It has been demonstrated that the production of TGF-β1 varies between individuals and partly depends on these two polymorphisms (Grainger DJ et al 1999). Xaubet A et al (2003) examined the codon 10 and codon 25 polymorphisms in 128 IPF subjects from Spain and 140 healthy subjects. These polymorphisms did not appear to predispose to the development of IPF. However, the authors found that the presence of the proline allele in codon 10 of the TGF-β1 gene was independently associated with increased deterioration in gas exchange in patients with IPF, after controlling for the effect of treatment, suggesting that TGF-β1 polymorphisms may be linked with the progression of IPF.

1.3.3 OTHER GENETIC STUDIES IN PULMONARY FIBROSIS

1.3.3.1 Lung allograft fibrosis
The codon 10 and codon 25 TGF-β gene polymorphisms have also been examined in the context of lung allograft fibrosis. El-Gamel A et al (1999) compared 91 UK pulmonary transplants with 96 normal healthy volunteers and showed that homozygosity for arginine at codon 25, which correlates with higher TGF-β production in vitro, associated with fibrotic lung pathology before lung transplantation and with the development of fibrosis in the graft. In addition, homozygosity for the codon 25 arginine allele in combination with the codon 10 leucine allele was shown to be a marker for poor post-transplant prognosis and recipient survival (El-Gamel A et al 1999). A CA repeat allele polymorphism in the first intron of the human IFN-γ gene was also shown to associate with lung allograft fibrosis (Awad M et al 1999).
1.3.3.2 Silicosis
Polymorphisms of the IL-1 gene complex have been examined in silicosis, a disease characterised by fibrosing nodular lesions that eventually develop into progressive pulmonary fibrosis. Yucesoy B et al (2001) examined 318 Caucasian cases of silicosis and compared them with 163 controls with no apparent inflammation or other pulmonary disease. An increase in the proportion of the IL-1Ra (+2018) allele 2 genotype was observed in coal miners with silicosis compared with controls, with those carrying at least one copy of allele 2 being 2.15 times more likely to have silicosis (CI = 1.4-3.3), suggesting that this polymorphism may confer increased risk for the development of the disease (Yucesoy B et al 2001).

1.3.3.3 Fibrosing alveolitis with systemic sclerosis
Polymorphisms in the fibronectin gene were investigated in the context of pulmonary fibrosis associated with systemic sclerosis. Avila JJ et al investigated whether polymorphisms of the fibronectin gene are associated with lung fibrosis in systemic sclerosis. They examined four restriction fragment length polymorphisms (RFLP) with the restriction enzymes HaellI, MspI, HindIII and TaqI in 161 UK patients with systemic sclerosis and 253 healthy controls. Avila et al found that the coassociation of the genotypes AB (HaellI RFLP) and CD (MspI RFLP) was present in 45% of systemic sclerosis patients with fibrosing alveolitis (P=0.0059), with an increased relative risk of developing fibrosis of 1.988. The authors concluded that genotypes of the fibronectin gene are useful prognostic factors in systemic sclerosis, helping to predict individuals likely to develop pulmonary fibrosis (Avila JJ et al 1999).
1.4 THE CYCLOOXYGENASE PATHWAY

Although the molecular pathways leading to fibrosis are at present poorly understood, it is becoming increasingly clear that progression to fibrosis is the result of an imbalance between stimulatory and inhibitory mediators. A deficiency in one of the inhibitory mediators, PGE$_2$, is well documented in patients with IPF, and a number of studies have shown that this 'PGE$_2$ deficiency' is the result of a diminished capacity to upregulate COX-2 (Wilborn J et al 1995, Vancheri C et al 2000, Keerthisingam CB et al 2001).

The observation that patients with IPF fail to upregulate the COX-2 gene is central to the work presented in this thesis. Consequently, the following three sections (1.4, 1.5 and 1.6) that follow concentrate on COX-2. A detailed overview of the COX pathway is given with later emphasis on the many roles of COX-2 in health and disease. The last section (section 1.6) introduces the complex regulation of COX-2 gene expression.

Cyclooxygenase is the key enzyme required for the conversion of arachidonic acid to prostanoids (prostaglandins, prostacyclins and thromboxanes). Prostanoids are lipid mediators with diverse and potent biological actions. They are involved in numerous homeostatic biological functions and inflammation. This section addresses the regulatory steps implicated in the cyclooxygenase pathway and introduces the concept that COX-2 has both pro-inflammatory and homeostatic actions.

1.4.1 EICOSANOIDS

The term eicosanoids comes from the Greek eicose = twenty. It describes a class of lipid mediators derived from twenty carbon fatty acids. The term is now used to refer collectively to compounds derived from free arachidonic acid. The first step in eicosanoid biosynthesis is the liberation of arachidonic acid from membrane phospholipid by phospholipase. A host of enzymes regulate cellular levels of arachidonic acid, keeping it esterified until mobilised by phospholipase in response to various cellular activation processes. Several phospholipases have been characterised but type IV cytosolic phospholipase A2 (cPLA$_2$) is thought to be the
main player for eicosanoid production because cells lacking cPLA\textsubscript{2} are generally devoid of eicosanoid synthesis (Funk CD 2001). The eicosanoids are divided into two major subsets: the leukotrienes and the prostanoids.

Leukotrienes (LTs) are lipid mediators that are synthesised from free arachidonic acid in a multistep pathway initiated by the enzyme 5-lipoxygenase (5-LO). 5-LO converts free arachidonic acid to LTA\textsubscript{4} with the concerted efforts of 5-lipoxygenase-activating protein (FLAP). LTA\textsubscript{4} can then be metabolised by other enzymes to a variety of bioactive compounds such as LTB\textsubscript{4} and LTC\textsubscript{4} (Funk CD 2001). LTs are thought to play important roles in normal host defence as well as in a variety of inflammatory disorders (Lewis RA et al 1990, Henderson W Jr 1987).

Prostanoids are formed by the initial conversion of released arachidonic acid to a common precursor, PGH\textsubscript{2}, by the cyclooxygenase (COX) enzyme (also known as prostaglandin H synthase). Cyclooxygenase is a bifunctional enzyme that can carry out two distinct, sequential biochemical reactions. It catalyses a cyclooxygenase (bis-oxygenase) reaction in which arachidonic acid is converted to an unstable intermediate PGG\textsubscript{2}, and a peroxidase reaction in which PGG\textsubscript{2} is reduced to PGH\textsubscript{2}. PGH\textsubscript{2} is then further metabolised by tissue and cell type specific isomerases or synthases to various bioactive compounds including PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, PGD\textsubscript{2}, prostacyclin (PGI\textsubscript{2}), and thromboxane A\textsubscript{2} (TXA\textsubscript{2}) (Herschman HR 1996).

1.4.2 MECHANISMS OF PROSTANOID ACTION
Prostanoids are formed by most cells in our bodies. Because they are either chemically or metabolically unstable (thromboxane and prostacyclin have half-lives of the order of seconds to a few minutes), it is believed that they act as autocrine and paracrine lipid mediators i.e. they signal at or immediately adjacent to their site of synthesis. As a consequence, they are not stored but are synthesised \textit{de novo} when cells are stimulated by a specific cytokine, growth factor and other stimuli (Funk CD 2001). Immediately after their synthesis, prostanoids are released outside of the cell. Although generally regarded as hydrophobic compounds, prostanoids do not permeate the cell membrane freely. They are released from cells predominantly by facilitated transport through a known prostaglandin transporter (PGT) and also
potentially by other as yet uncharacterised transporters (Schuster VL 1998). The only prostanoid that is not a substrate for PGT is PGI\textsubscript{2}. Although PGT is found in a limited range of cells, cyclopentenone prostaglandins are efficiently transported in most cells, suggesting that other transport systems mediate the uptake of at least some prostanoids (Narumiya S and FitzGerald GA 2001).

Prostanoids exert their biological effects by binding to specific cell-surface receptors that are linked to a range of signal transduction pathways. There are at least 9 known prostanoid receptor forms in mouse and man: the PGD receptors, DP\textsubscript{1} and DP\textsubscript{2}; the PGE\textsubscript{2} receptors, EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3} and EP\textsubscript{4}; the PGF receptor, FP; the PGI receptor, IP; and the TXA receptor, TP. In addition, there are several splice variants of the EP\textsubscript{3}, FP and TP receptors that differ only in the C-terminal tails. All the prostanoid receptors belong to the G-protein coupled receptor (GPCR) superfamily of seven transmembrane spanning proteins, with the exception of DP\textsubscript{2}, which is a member of the chemoattractant receptor subgrouping (Funk CD 2001). The IP, DP\textsubscript{1}, EP\textsubscript{2}, and EP\textsubscript{4} receptors signal through G\textsubscript{q}-mediated increases in intracellular cAMP and have been termed “relaxant” receptors. The EP\textsubscript{1}, FP and TP receptors induce calcium mobilisation through G\textsubscript{q} and are known as the “contractile” receptors. The EP\textsubscript{3} receptor couples to G\textsubscript{i} to induce a decline in cAMP levels and is consequently regarded as an “inhibitory” receptor. Although most of the prostanoid receptors are localised at the plasma membrane, some are situated at the nuclear envelope (Bhattacharya M et al 1998). In recent years, considerable interest has focused on the role of the nuclear hormone receptors in mediating the actions of prostanoids. This interest stems from several reports proposing that prostanoids work as endogenous ligands for peroxisome proliferator-activated receptors (PPARs) (Forman BN et al 1995, Kliewer SA et al 1995, Reginato MJ et al 1998).

**1.4.3 THE ‘CONSTITUTIVE’ COX**

Purification and characterisation of the cyclooxygenase enzyme was accomplished in the 1970s. Cyclooxygenase was identified and purified using classical biochemical techniques from bovine (Miyamoto T et al 1976) and sheep (Hemler M and Lands WE 1976) vesicular glands and found to be a membrane bound homodimer of 70 kDa. The cDNA for ovine cyclooxygenase (EC 1.14.99.1) was cloned in 1988.
(DeWitt DL and Smith WL 1988, Merlie JP et al 1988, Yokayama C et al 1988). The murine (DeWitt DL and Smith WL 1990) and human (Funk CD et al 1991) COX cDNAs were then cloned using sequence information from the ovine cDNA. The murine (Kraemer SA et al 1992) and human (Yokoyama C et al 1989) COX genes were subsequently cloned and characterised. Until the early 1990s it was assumed that only one COX gene and protein existed.

For many years, the conventional view of prostanoid synthesis was that the rate-limiting step for prostaglandin production is activation of phospholipase(s) and release of arachidonic acid. According to this model, constitutive COX is readily available for the conversion of free arachidonic acid to PGH₂. The general concept was that COX is present in excess and that the limiting factor is availability of arachidonic acid. Constitutive COX activity is found in the endoplasmic reticulum of essentially all tissues (Herschman HR 1996 & 1998).

1.4.4 EVIDENCE FOR AN INDUCIBLE FORM OF COX

Studies of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulation of prostaglandin production in rat ovarian granulosa cells, generated biochemical and immunological evidence for a distinct COX isoform, that is induced by hormonal stimulation (Wong WL and Richards JS 1991). Needleman and colleagues demonstrated that mitogen stimulation of fibroblasts (Raz A et al 1988 and 1989) and endotoxin stimulation of monocyte/macrophages (Fu JY et al 1990), lead to an increase in COX enzyme activity. Initially it was thought that the treatments induced the known COX gene but no increase in EC 1.14.99.1 message was found. Han JW et al (1990) identified a 72/74 kDa protein complex that was induced in murine fibroblast cell lines in response to activation of v-src oncogene, and by treatment with the tumour promoter tetradecanoyl phorbol acetate (TPA) or PDGF. Furthermore, the complex could be immunoprecipitated with antiserum to COX EC 1.14.99.1 (Han JW et al 1990). Reports of inhibition of prostanoid production, COX enzyme activity and mitogen-induced COX synthesis by glucocorticoids, suggested the existence of two distinct COX pools: a constitutive pool that is unaffected by inducers or steroid hormones and a second inducible, glucocorticoid-inhibited COX pool (Masferrer JL et al 1990). Finally, the
identification of a mitogen-inducible mRNA species in mitogen-treated sheep tracheal epithelial cells that cross-hybridised with COX EC 1.14.99.1 cDNA further supported the concept of an inducible COX gene (Rosen GD et al 1989).

1.4.5 THE DISCOVERY OF AN INDUCIBLE COX GENE

In the late 1980's differential cloning techniques were used to identify genes induced in quiescent, non-dividing fibroblasts by mitogenic stimulation with TPA. One cDNA was cloned, the murine TISIO (for TPA-induced sequence 10), that had substantial homology to the first COX message cloned in 1988 (Kujubu DA et al 1991). Moreover, when expressed from a heterologous promoter, the TISIO protein had both the cyclooxygenase and the peroxidase activities demonstrating that the TISIO gene encoded an alternative inducible form of COX (Fletcher BS et al 1992). This inducible COX gene is now referred to as COX-2 while the constitutive isoform is known as COX-1. At around the same time the chicken COX-2 was cloned (Xie WL et al 1991) followed by the cloning of the murine COX-2 cDNA as a growth factor and oncogene-induced gene (O' Banion MK et al 1991, Ryseck RP et al 1992). The human (Hla T and Neilson K 1992, Jones DA et al 1993) and rat (DuBois RN et al 1994) COX-2 cDNAs were later cloned using sequence information from the murine message.

1.4.6 COX STRUCTURE AND LOCALISATION

Mature, processed COX-1 has 576 amino acids and the mature form of COX-2 contains 587 amino acids. The primary sequences of COX-1 and COX-2 are 60-65% identical within a species and there is a 85-90% identity among individual isoforms from different species (Smith WL et al 2000). The most significant sequence differences between the COX isoforms occur in the membrane binding domains (Otto JC and Smith WL 1996, Spencer AG et al 1999). Particularly notable, is the 18 amino acid insert that is located 6 residues in from the C terminus of COX-2 and is absent in COX-1. COXs are homodimers both structurally and functionally (Xiao GS et al 1998). Each monomer is made up of three domains: a small N terminal domain composed of an EGF module, a membrane binding domain (MBD), and a large C-terminal globular catalytic domain. COX-1 and COX-2 contain KDEL-like...
sequences that target COXs to the endoplasmic reticulum (ER) and the associated nuclear envelope (Song I et al 1996). Both enzymes are located on the luminal surfaces of the ER and of the inner and outer membranes of the nuclear envelope, although COX-2 appears to be more concentrated within the nuclear envelope (Otto JC and Smith WL 1994, Morita I et al 1995, Spencer AG et al 1998). This raises the possibility that products formed via COX-2 may have greater access to the nucleoplasm to modulate nuclear events (e.g. transcription of target genes) perhaps via nuclear receptors (Lim H et al 1999). Both COX-1 and COX-2 are N-glycosylated. COX-1 N-glycosylation occurs at three sites and is necessary for enzyme folding. COX-2 is variably glycosylated at two to four sites. (Reviewed by Smith WL and DeWitt DL 1996, Smith WL et al 2000).

1.4.7 WHY THERE ARE TWO COX ENZYMES: EVIDENCE FOR SEGREGATED BIOSYNTHETIC AND SIGNALLING PATHWAYS

Since the discovery of a second cyclooxygenase isoform, there has been considerable interest in why there are two isoforms of this enzyme and what roles they might play. It is now clear that there are processes in which each isoform is uniquely involved, others in which both COX-1 and COX-2 are involved, and events where the lack of one isoform is compensated by the presence of the other. For each isoform to have unique physiological functions, COX-1 and COX-2 must be able to function independently, even when co-expressed in the same cells. The independent functioning of the COX isoforms appears to involve metabolic rather than physical segregation of these enzymes (Smith WL and Langenbach R 2001). A rapidly expanding body of evidence suggests that the two COXs play distinct roles in regulating arachidonic acid metabolism. When COX-1 and COX-2 are both expressed in the same cell, arachidonic acid is always directed to the COX-2 pathway. COX-1 can only function independently when COX-2 is absent, but because COX-1 is constitutive while COX-2 is inducible, this is actually the usual situation (Smith WL and Langenbach R 2001). Furthermore, when the substrate is present at low concentrations, the bulk of the available arachidonic acid is metabolised by COX-2 (Smith WL et al 2000). This may account for the biphasic production of prostanoids observed after treating cells with growth factors, hormones, cytokines or lipopolysaccharide (LPS). Stimulation of cells results in an

In addition, several studies have suggested that preferential coupling between the COX enzymes and distinct phospholipases, may account for the differential regulation of the immediate and delayed responses. Three phospholipase A₂ (PLA₂) forms are primarily involved in agonist-stimulated arachidonic acid release: type IV cytoplasmic cPLA₂, that can be activated by increases in intracellular calcium and activation of mitogen-activated protein kinases (MAPKs), and types IIa and V secreted (s) PLA₂ enzymes (Smith WL et al 2000). There seems to be no general pattern of coupling between specific PLA₂ and specific COX isoforms and results yielded by different experimental systems have so far been controversial. In theory, each PLA₂ could provide arachidonic acid for both COX-1 and COX-2. Moreover, neither enzyme appears to interact directly with any of the PLA₂s.

Studies with cPLA₂ knockout mice showed that an initial activation of cPLA₂ is required for both the acute and delayed phases of arachidonic acid release (Uozumi N et al 1997, Bonventre JV et al 1997). This initial cPLA₂-mediated step might either provide the bulk of arachidonic acid, or act as a trigger for the activation of sPLA₂ required for early or late prostanoid synthesis. In fibroblasts (Reddy ST and Herschman HR 1996) and mast cells (Reddy ST and Herschman HR 1997), acute prostanoid synthesis requires sPLA₂. In isolated bone marrow-derived mast cells (Bingham CO III et al 1996), cPLA₂ mediates acute prostanoid synthesis whereas sPLA₂ plays a role in the delayed phase synthesis. In the MC3T3-E1 cell line and in primary cultures of mouse osteoblasts, cPLA₂ mediates both the acute and delayed phase prostanoid synthesis (Chen QR et al 1997, Murakami M et al 1997).

Finally, another way in which COX-1 and COX-2 biosynthetic pathways may be segregated is by differential coupling of the isoforms to various downstream synthases. Preferential coupling of synthases to COX-2 could be achieved by coordinating their expression with that of COX-2. Alternatively, restricted expression
of the COXs with specific isomerases (e.g. COX-1 with TXA₂ in platelets) might account for specific coupling. However, to date, there is no concrete evidence for these mechanisms (Smith WL et al 2000, Fitzpatrick FA and Soberman R 2001).

1.4.8 THE HOMEOSTATIC VS PROINFLAMMATORY THEORY OF COX ACTIONS

Studies of COX-1 expression revealed that this isoform is expressed constitutively in most cells and tissues. Importantly, COX-1 is the only isoform expressed in the normal gastric mucosa and the platelet where it has a protective role. In the gastric antrum, COX-1 products such as PGE₂ and endothelial cell-derived prostacyclin promote vasodilation and maintain mucosal integrity. In platelets, COX-1 is vital for the production of TXA₂, an eicosanoid that promotes platelet aggregation. By contrast, COX-2 expression is undetectable in most normal tissues and cells, but rapidly induced when cells are challenged with inflammatory mediators (DuBois R et al 1998). Moreover, COX-2 expression is dramatically up-regulated at sites of inflammation such as the rheumatoid synovium (Crofford LJ et al 1994, Kang RY et al 1996). These observations suggested that COX-1 is involved in cellular housekeeping functions necessary for normal physiologic activity whereas COX-2 acts primarily at sites of inflammation. With accumulating knowledge of the biology COX-1 and COX-2 in the last decade, it has become clear that the homeostatic versus inflammatory paradigm is an oversimplification.

COX-1 levels have been shown to change during development (Brannon T et al 1994). COX-1 expression can be downregulated in endothelial cells in response to acidic fibroblast growth factor (Hla T and Maciag T 1991) and upregulated in mast cells treated with stem cell factor plus dexamethasone (Samet JM et al 1995). In addition, COX-1 has been shown to contribute to inflammatory responses as mice lacking the gene for COX-1 exhibit diminished inflammatory responses compared with wild type controls (Langenbach R et al 1995). By contrast, mice with a disrupted gene for COX-2 exhibit inflammatory responses of similar magnitude to those observed with wild type controls (Morham SG et al 1995).
There is considerable evidence that COX-2 is expressed constitutively in many tissues including the brain (Yamagata K et al 1993), tracheal epithelia (Walenga RW et al 1996) and the macula densa in the kidney (Harris RC et al 1994). It is also clear that COX-2 performs important physiological functions. Reports that COX-2 deficient mice exhibit severe renal abnormalities (Dinchuk JE et al 1995, Morham SG et al 1995) suggest that COX-2 plays an important role in renal homeostasis. A physiologic role for COX-2 is also very well established in the ovaries and the uterus and multiple reproductive failures have been reported in female COX-2 deficient mice (Lim H et al 1997). In addition, COX-2 appears to play an important role in promoting the healing of ulcers in the stomach. Treatment of mice with the COX-2 selective-inhibitor NS-398 results in significant inhibition of ulcer healing (Mizuno H et al 1997). Moreover, Gilroy DW et al (1999) have recently shown that COX-2 is induced in the resolution of an inflammatory response and that inhibition of COX-2 results in persistence of inflammation due to the prevention of synthesis of the anti-inflammatory cyclopentenone prostaglandins. The cyclopentenone prostaglandins have recently been shown to exert their anti-inflammatory effects by directly inhibiting the activation of IkB Kinase (IKK), the enzyme responsible for the activation of NFkB by proinflammatory stimuli (Rossi A et al 2000). Finally, it has also been suggested that a third isoform COX-3 may be generated during resolution and may be responsible for the first step in the synthesis of anti-inflammatory prostaglandins (Willoughby DA et al 2000).

1.4.9 NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND COX-2 INHIBITORS

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, are amongst the most widely prescribed drugs worldwide. They are used to treat pain, fever, chronic disease, acute infection and a variety of other illnesses. Although they have been available for over a century, their mode of action only became clear in 1971 when Vane published his seminal observations proposing that the ability of NSAIDs to suppress inflammation rests primarily on their ability to inhibit COX activity (Vane JR 1971).
NSAIDs inhibit both COX-1 and COX-2 by competing with arachidonic acid for binding to the cyclooxygenase active site. NSAIDs can be grouped into three classes (I-III) with different kinetic modes of inhibition. Class I inhibitors, such as ibuprofen, are competitive inhibitors that exhibit rapid but reversible binding. Class II inhibitors, such as indomethacin, are competitive, time-dependent inhibitors with slow reversible binding. Class III inhibitors like aspirin, are competitive inhibitors that exhibit an initial rapid reversible binding, followed by irreversible covalent modification (acetylation) of the protein. A cell treated with a class III inhibitor must synthesise new COX to regain cyclooxygenase activity (reviewed in Smith WL and DeWitt DL 1996, Smith WL et al 2000). Acetylation of COX-1 by aspirin completely inhibits the cyclooxygenase activity. Acetylation of COX-2 converts it to a form that can still oxygenate arachidonic acid, but at C-15 instead of C-11, yielding 15R-hydroxyeicosatetraenoic acid (15RHETE) instead of PGG2. At this stage it should be noted that 15RHETE can be further metabolised by 5'LO to form lipoxins (Claria J and Sherhan CN 1995), which may have effects on inflammation. This raises the intriguing possibility that the beneficial effects of aspirin may in part be accounted for by the production of anti-inflammatory eicosanoids (Mitchell JA and Warner TD 1999).

Despite their analgesic and anti-inflammatory properties, NSAIDs can also cause significant side effects, leading to stomach ulcers and renal failure in susceptible individuals. The homeostatic (COX-1) versus proinflammatory (COX-2) theory was so compelling, that before it was rigorously tested, it served as the rationale for the development of COX-2 specific inhibitors. The idea was that COX-2 specific inhibitors would have all of the anti-inflammatory and analgesic properties of NSAIDs, but lack the well-documented toxicity associated with COX-1 inhibition (Lipsky PE et al 2000). The first highly selective COX-2 inhibitor celecoxib was introduced into the market place in the United States early in 1999 and a second inhibitor, rofecoxib, was introduced in June 1999. Clinical trials showed that these inhibitors had potent anti-inflammatory and analgesic properties without the severe side-effects that accompany classical NSAID and led to the use of these agents for the treatment of osteoarthritis (celecoxib/rofecoxib), rheumatoid arthritis (celecoxib) and acute pain (rofecoxib) (Ehrich E et al 1999, Simon LS et al 1998). However, as the homeostatic versus proinflammatory theory proved to be an oversimplification,
suppression of COX-2 with selective inhibitors should not be expected to come without some adverse consequences. Moreover, when discussing the role of COX-2 in inflammation, it should not be forgotten that the inflammatory response is in itself a defence mechanism to protect the body. With accumulating evidence that COX-2 is involved in normal physiologic processes and that COX-1 also contributes to inflammation and pain, COX-2 selective inhibitors might not be after all be the final answer in the search for a 'better aspirin'.

The actions of NSAIDs and COX-2 selective inhibitors in the context of the roles of COX-1 and COX-2 in various pathophysiologic states are discussed in section 1.5.

1.4.10 ACETAMINOPHEN AND COX-3?

Acetaminophen, known as paracetamol in the UK, is one of the most popular analgesic and anti-pyretic medicines. It is often classified as an NSAID, yet it appears to have very little anti-inflammatory activity. Acetaminophen is a very weak inhibitor of both COX-1 and COX-2 in vitro and lacks the other typical actions of other NSAIDs, such as anti-platelet activity and gastrototoxicity (Botting RM 2000). As a result, acetaminophen’s potent analgesic and anti-pyretic actions have long been a mystery. In the past, investigators speculated on the existence of a third COX isoform in the brain that is mainly responsible for pain and high temperature.

In 2002, Chandrasekharan and colleagues reported the identification of a new COX isoform, COX-3 and two smaller forms of COX-1 (partial COX-1 or PCOX-1), all of which are derived by alternative splicing of the COX-1 mRNA. COX-3 and one of the PCOX-1 proteins (PCOX-1a) retain intron 1 in their mRNAs. It was suggested that the retention of intron 1 might change the way the enzyme is folded or the conformation of its active site. PCOX-1 proteins also contain an in-frame deletion of exons 5-8 of the COX-1 mRNA (Chandrasekharan NV et al 2002).

COX-3 and PCOX mRNAs are expressed in canine cerebral cortex and in lesser amounts in other tissues. In humans, COX-3 is most abundant in the heart and the cerebral cortex.
Chandrasekharan NV et al found that only canine COX-3 has a glycosylation-dependent COX activity. They also found that in vitro, COX-3 is more sensitive than COX-1 or COX-2 to inhibition by acetaminophen, and is also much more sensitive to diclofenac, indomethacin, ibuprofen and aspirin. However, it is the most established COX inhibitors, such as ibuprofen, diclofenac and indomethacin, that show the most powerful COX-3 inhibition. Also worth noting is the observation that COX-2 selective inhibitors have no effect on COX-3 (data not shown by Chandrasekharan and colleagues but mentioned in the text).

The results by Chandrasekharan NV et al do not explain how acetaminophen can be analgesic and anti-pyretic without being anti-inflammatory and their findings open up the search for further variants. The observation that COX-3 is derived from COX-1 argues against a central role for COX-3 in pyresis as studies in mice have shown that it is deletion of COX-2 and not COX-1 that blunts the febrile response (Li S et al 1999). Similarly, pain is the result of prostanoids produced by COX-2 at sites of inflammation (see section 1.5.5) and this would support a role for a COX-2 variant rather than a COX-1 variant. At this point it should be noted that Simmons DL et al (1999) have previously proposed the existence of an isoform of COX-2 that is particularly sensitive to acetaminophen.

The most significant implication of the study by Chandrasekharan and colleagues is that multiple COX-1 isoforms can be derived from just one gene, providing a range of COX enzymes. However, much more work needs to be done to determine whether COX-3 plays a role in the human brain. If the finding holds up in humans, it may suggest the presence of other variants of COX-1 and COX-2. The presence of multiple isoforms of COX-1 and COX-2 may explain why there are so many different NSAIDs on the market and why different patients appear to benefit from different types of NSAIDs. In the future, this could lead to pain and fever relievers that better tailored to individual patients.
1.5 COX IN HEALTH AND DISEASE

The purpose of this section is to discuss some of the important physiological and pathophysiological functions of the COX enzymes. First, emphasis is placed on the roles of COX-1 and COX-2 in maintaining homeostasis and the ways in which they interplay to mediate a variety of physiological responses. Cancer and pulmonary fibrosis are then presented as two examples where dysregulated expression of COX-2 can lead to pathology. The effects of NSAIDs and COX-2 selective inhibitors on homeostatic and disease processes are also considered.

1.5.1 RENAL FUNCTION

COX-1 has long been recognised to be involved in normal kidney function by producing vasodilating prostaglandins that maintain renal plasma flow and glomerular filtration rate, especially during conditions of angiotensin-stimulated systemic vasoconstriction. The clinical effects of NSAIDs on kidney function were thought to be the consequence of the inhibition of this COX-1 protective response, leading to renal ischemia and functional damage in some individuals (Zambraski EJ 1995, Breyer MD et al 1996). However, during the past years, evidence has accumulated suggesting that COX-2 may also play a role in physiological renal functions. COX-2 null mice show severe disruption of kidney development (Dinchuk JE et al 1995, Morham SG et al 1995). Furthermore, constitutive expression of COX-2 was reported in the rat kidney, particularly in the macula densa, the site of regulation of glomerular blood flow and renin release (Harris RC et al 1994). Chronic sodium deprivation or experimental hyperfiltration states increase COX-2 expression in the rat kidney suggesting that prostaglandins produced by COX-2 may function to increase sodium reabsorption in response to volume contraction or hyperfiltration that may occur with progressive renal failure. Moreover, administration of ACE inhibitors or angiotensin II receptor subtype 1 blockers was shown to increase COX-2 expression in both control and salt-restricted animals, suggesting feedback inhibition of COX-2 expression by the renin-angiotensin system (Harris RC et al 2000). Conversely, COX-2 specific inhibitors prevent renin secretion stimulated by a reduction in luminal sodium chloride concentration at the macula densa, suggesting that COX-2 plays a role in the regulation of renin production (Traynor TR et al 1999). The involvement of COX-2 in human renal

1.5.2 GASTROINTESTINAL TRACT INTEGRITY
COX-1 is the only isoform identified in the gastric mucosa of normal animals and humans. Prostaglandins derived from COX-1 are considered to confer cytoprotection in the gastrointestinal (GI) tract. Inhibition of COX-1 by NSAIDs has for many years been deemed responsible for gastric ulcer development and the major complications of gastrointestinal bleeding, perforation and obstruction. It is therefore surprising that COX-1 deficient mice had a 99% reduction in gastric PGE$_2$ levels but did not spontaneously develop ulcers (Langenbach R et al 1995). GI bleeding caused by NSAIDs seems to result from the combined inhibition of platelet COX-1 activity (increases tendency to bleed) and gastric COX-1 activity (increases the likelihood of ulceration). As COX-2 is not detectable in the normal gastric mucosa nor the platelet, selective inhibition of COX-2 would be expected to be ‘Gl-safe’. Consistent with this concept, celecoxib and rofecoxib were shown to cause significantly lower incidence of upper GI adverse effects (Bombardier C et al 2000, Silverstein FE et al 2000). However, COX-2 has been shown to be markedly induced in the mouse stomach in which an ulcer had been induced. Treatment of these mice with the COX-2 inhibitor NS-398 resulted in reduction of mucosal prostaglandin synthesis and significant inhibition of ulcer healing (Mizuno H et al 1997). Similar results were obtained using another COX-2 inhibitor (L-745,337) (Schmassmann A et al 1998), suggesting that COX-2 may play a role in promoting ulcer healing. Furthermore, indomethacin-induced ulcers heal poorly in COX-2 null mice compared with wild type mice and ultimately prove fatal (Wallace JL et al 1998).

Another important finding is that COX-2 influences angiogenesis. Angiogenesis, the formation of new capillary blood vessels, is essential for ulcer healing because without the restoration of blood flow, oxygen and nutrients cannot be delivered to the healing site. COX-2 selective inhibitors and classical NSAIDs were shown to inhibit

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angiogenesis through direct effects on endothelial cells, involving inhibition of MAPK activity and interference with extracellular signal-regulated kinase (ERK) nuclear translocation (Jones MK et al 1999).

COX-2 may also play an important physiological role in other parts of the GI tract. In response to infection or invasion, COX-2 expression is induced in epithelial cells leading to increased PG production. The PGs then stimulate chloride and fluid secretion from the mucosa, which flushes bacteria from the intestine (Eckmann L et al 1997).

1.5.3 CARDIOVASCULAR SYSTEM

Maintenance of normal blood flow and the appropriate thrombogenic response to injury relies on a fine balance between the vasoconstrictive action of platelet-produced TXA$_2$ and the vasodilating properties of endothelial cell-derived PGI$_2$ (Lipsky PE et al 2000). COX-1 is the only isoform found in platelets, as these cells do not have nuclei and cannot produce an inducible enzyme in response to activating conditions. Following activation, platelets produce TXA$_2$ via COX-1. The release of eicosanoids by activated platelets is thought to provide both a substrate and a stimulus for the generation of PGI$_2$ by the endothelium (Schaffer Al 1995). By stimulating vasodilation, prostacyclin counteracts the vasoconstrictive effects of TXA$_2$. This has been the basis for the therapeutic aim of the “half an aspirin a day” prophylaxis against thromboembolic disease. The protective effects of aspirin are achieved through inhibition of COX-1 and the resulting decrease in TXA$_2$. Prostacyclin synthesis by endothelial cells is also decreased, but COX-1 can be regenerated so that PGI$_2$ activity is re-established. By contrast, platelets cannot form new enzyme and TXA$_2$ synthesis is irreversibly inhibited (Vane JR et al 1998).

A substantial proportion of PGI$_2$ production is mediated by COX-2. Endothelial COX-2 expression is up-regulated by shear stress and substantial amounts of eicosanoid production have been shown to be COX-2 derived (Topper JN et al 1996). Furthermore, COX-2 specific inhibitors have been reported to decrease systemic PGI$_2$ production in healthy volunteers (Catella-Lawson F et al 1999, McAdam BF et al 1999). In this context, it is interesting to note that specific COX-2
inhibitors may limit production of PGI₂ by endothelial cells while having no effect on the production of TXA₂ by platelets. An alteration in PGI₂ / TXA₂ balance through the use of COX-2 specific inhibitors may therefore favour platelet aggregation and result in increased tendency for vascular occlusion and tissue ischemia.

1.5.4 REPRODUCTIVE FUNCTIONS

The important role of COX-2 in female reproduction is emphasised by the observation that COX-2 knockout mice have multiple failures in reproductive function, including ovulation, fertilisation, and decidualisation (Lim H et al 1997). During ovulation, LH surge leads to induction of PG synthesis. This marked response led to the first observation of COX-2 induction during a normal physiological event. In COX-2 deficient mice, although follicular development is observed, ovulation is greatly reduced, and of the eggs released, very few are fertilised (Lim H et al 1997). The observation that ovarian PGE₂ production is increased by pituitary gonadotrophins in wild-type and COX-1 null mice but not in COX-2 null mice suggests that COX-2 mediates the increase in ovarian PGE₂ synthesis. PGE₂ is considered to be the product that is responsible for ovulation as ovulation in COX-2 deficient mice can be restored by PGE₂ administration (Davis BJ et al 1999). COX-2 induction also appears to be necessary for the production of proteolytic enzymes that rupture the follicles (Tsafiriri A 1995). COX-2 induction may be triggered by the gonadotrophins LH and FSH, and by TGF-α, IL-1 and other cellular signalling pathways (Morris JK and Richards JS 1996). Following fertilisation in the mouse, COX-2 also plays a role in the implantation of the embryo in the uterine endometrium, while COX-1 and specific PG receptors prepare the wall for interaction with the embryo (Chakraborty I et al 1996, Yang ZM et al 1997). More recently, upregulation of COX-2 expression was shown to mediate increased PG synthesis in the human myometrium and within the foetal membrane (Slater DM et al 1999a and b).

At the completion of pregnancy, PGs help mediate the delivery process. COX-1 seems to be the main isoform involved in parturition. Early studies showed that COX-1- knockout female mice produced litters of normal size but had difficulty with parturition (Langenbach R et al 1995) while other aspects of the reproductive process
were normal. The onset of parturition was also found to be delayed in COX-1 knockout mice. Administration of PGF$_{2\alpha}$ to these mice resulted in the onset of labour, suggesting that COX-1 is the source of PGF$_{2\alpha}$. However, in the absence of COX-1 and under certain pathological conditions, COX-2 can be induced and produce the PGF$_{2\alpha}$ required to initiate parturition, thereby playing a compensatory role (Gross G et al 2000). Given the roles of COX activity in the induction of labour, NSAIDs and COX-2 inhibitors have received considerable interest as potential agents for the treatment of premature labour (Zuckerman H et al 1974, Hammerman C 1995, Sawdy et al 2000). However, inhibition of COX activity appears to be associated with premature closure of the ductus arteriosus, a circulatory shunt in the foetus that allows the output of the left ventricle to bypass the foetal lungs. Furthermore, COX-2 has emerged as the major isoform involved in the initiation of ductus arteriosus closure, suggesting that maternal use of COX-2 inhibitors near the time of delivery might increase the risk of patent ductus arteriosus (failure of the ductus to close) after birth (Loftin CD et al 2001).

1.5.5 BRAIN AND NERVE FUNCTIONS

**Fever.** Prostaglandins play a central role in the fever response. The mechanism of fever induction seems to involve the COX-2 enzyme. Intra-peritoneal injection with LPS causes a marked fever response in rats that temporally parallels COX-2 induction in brain endothelial cells (Cao C et al 1997, Matsumura K et al 1997). The resulting PGs can then act on temperature-sensitive neurons in the preoptic area to induce fever, a response that can be effectively blocked with a COX-2 specific inhibitor (Taniguchi Y et al 1997). Moreover, the febrile response to LPS is blocked in COX-2-deficient mice but not in mice lacking the COX-1 gene (Li S et al 1999).

**Pain.** Another effect of PGs is pain. During inflammation, increased levels of COX-2-derived PGs sensitise peripheral nociceptor terminals (pain receptors) and produce localised pain hypersensitivity. This explains the pain-relieving action of COX inhibitors at the local site of injury or inflammation. In addition, PGs may also act in the central nervous system to cause hyperalgesia. Following sensitisation, nociceptor signals are transferred to secondary neurons that propagate the signals to the higher
centres of the central nervous system and the sensation of pain is finally assembled in the cortex (Hinz B and Brune K 2002). COX-2 is expressed constitutively in the dorsal horn of the spinal cord and becomes upregulated in response to trauma. This induction of spinal cord COX-2 expression is thought to facilitate the transmission of pain responses (Beiche F et al 1996). The specific COX-2 inhibitor celecoxib, but not the COX-1 inhibitor SC-560, was shown to suppress inflammation-induced PG levels in cerebrospinal fluid, thereby further supporting a role for COX-2 in central pain perception (Smith CJ et al 1998). Samad TA et al (2001) also showed a widespread induction of COX-2 expression in spinal cord neurons and other regions of the central nervous system following peripheral inflammation, and found IL-1 β to be the major inducer of COX-2 upregulation in the central nervous system. Taken together, these observations suggest that COX-2 can act both locally and centrally to mediate pain.

1.5.6 CANCER

COX-2 upregulation and the ensuing increase in prostaglandin synthesis in cancerous tissues have been shown to enhance tumour growth. The precise mechanism by which COX-2 expression contributes to tumour formation is not understood. One possibility is that the COX-2 pathway is involved in the signalling of mitogens. Prostaglandins have been shown to be important for signalling of the EGF and EGF-related pathways, and blocking of both the COX-2 and EGF-like pathways
synergistically reduced the growth of a cancer cell line (Mann M et al 2001). In another study of colorectal carcinoma cells, PGE$_2$ promoted cell growth and motility via the EP$_4$ receptor by activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt/PKB) signalling pathway (Sheng H et al 2001).

COX-2 may also promote tumour growth by preventing apoptosis (programmed cell death). COX-2 expression contributes significantly to the tumourigenic potential of epithelial cells by increasing their adhesion to extracellular matrix and decreasing their response to apoptotic stimuli (Tsujii M and DuBois RN 1995). In addition, high concentrations of arachidonic acid can promote apoptosis suggesting that COX-2 catalysis may serve to lower intracellular levels of free arachidonic acid thereby depleting an apoptotic signal (Surette ME et al 1996 & 1999, Chan TA et al 1998). Moreover, accumulation of free arachidonic acid has been shown to elevate the level of ceramide, a small lipid messenger that is a potent inducer of apoptosis (Chan TA et al 1998). Consequently, removal of arachidonic acid by COX-2 catalysis might prevent apoptosis by lowering the concentration of intracellular ceramide. Furthermore, PGE$_2$ has been shown to inhibit programmed cell death by inducing expression of the Bcl-2 protooncogene (Sheng H et al 1998). In addition, PGE$_2$ and other prostaglandins often elevate cAMP concentrations which can also suppress apoptosis (Orlov SN et al 1999). PGE$_2$ production has also been reported to prevent apoptosis by inhibiting nitric oxide signalling in some cells (Chang YW et al 2000).

Studies with NSAIDs and COX-2 specific inhibitors further support a key role for the COX-2 pathway in the prevention of apoptosis. COX-2 selective inhibitors and classical NSAIDs induce apoptosis in a variety of cancer cells including colon, gastric, glioma, pancreatic and lung cancer cells (Ding XZ et al 2000, Hida T et al 2000, Joki T et al 2000, Uefuji K et al 2000, Li M et al 2001). However, NSAIDs have been shown to trigger apoptosis in COX-2 deficient cells, and NSAID metabolites that do not inhibit COX, such as sulindac sulfone, can also cause apoptosis, suggesting that other COX-2-independent pathways may also be involved (Piazza GA et al 1995).

Importantly, COX-2 seems to play a role in tumour angiogenesis. COX-2-derived PGs may modulate the production of angiogenic factors thereby promoting the
formation of blood vessels that sustain tumour cell viability and growth (Jones MK et al 1999, Masferrer JL et al 2000). Furthermore, COX-1 activity in endothelial cells has also been shown to play an important role in the modulation of angiogenesis (Tsujii M et al 1998).

Accumulated evidence from genetic studies also supports a role for COX-2 in tumorigenesis, particularly in colon cancer. Oshima et al (1996) reported that in APCΔ716 knockout mice (a model in which a targeted truncation deletion in the tumour suppressor gene APC causes intestinal polyposis), a COX-2 null mutation dramatically reduced the number and size of the intestinal polyps. COX-2 induction in colon cancer is thought to occur after the loss of the second APC allele but the events leading to COX-2 upregulation are unknown (Prescott SM and White RL 1996). COX-1 is also implicated in colon carcinogenesis. A recent study in mice with intestinal neoplasia showed that deficiency for either COX-1 or COX-2 reduces polyp formation about equally (Chulada PC et al 2000). Whether COX-1 and COX-2 contribute to tumourigenesis through common or different mechanisms remains to be elucidated.

The natural outcome of all these findings was the use of NSAIDs and COX-2 specific inhibitors in cancer clinical practice. The NSAID sulindac has been used to treat patients with familial adenomatous polyposis (FAP). Several studies show that sulindac significantly reduces the number and size of established polyps (Wadell WR et al 1983 & 1989, Giardiello FM et al 1993, Nugent KP et al 1993, Labayle D et al 1991), but this is associated with severe side effects in some patients. Treatment of FAP patients with the COX-2 selective inhibitor celecoxib also results in a significant reduction in the number of colorectal polyps (Steinbach G et al 2000). However, standard doses of sulindac do not prevent the development of adenomas in subjects who are genotypically affected with FAP but who were phenotypically unaffected (Giardiello FM et al 2002). Furthermore, a recent study aroused concern about the risk of cardiovascular events and the prothrombotic potential of COX-2 inhibitors (Mukherjee D et al 2001). Taken together, these observations suggest that the use of NSAIDs and COX-2 selective inhibitors as chemopreventive and chemotherapeutic agents requires further evaluation.
1.5.7 PULMONARY FIBROSIS

Cancer is an example where increased expression of COX-2 leads to pathology. However, as COX-2 also has important homeostatic functions, a pathogenic role for decreased expression of COX-2 should not be surprising. In pulmonary fibrosis, COX-2 is protective and therefore a decreased capacity to upregulate the COX-2 gene is detrimental. The 'COX-2 deficiency' in pulmonary fibrosis is well documented. Fibroblasts cultured from patients with IPF fail to induce COX-2 in response to IL-1β, TNF-α or LPS (Wilborn J et al 1995, Vancheri C et al 2000). COX-2 is also decreased in macrophages in sarcoidosis (Petkova DK et al 2003). This failure to upregulate COX-2 occurs despite the increased presence of mediators capable of stimulating its synthesis, and results in decreased levels of the anti-fibrotic PGE$_2$ being produced (the anti-fibrotic effects of PGE$_2$ are discussed in section 1.2.1.9). PGE$_2$ levels in BAL fluid from patients with IPF have been shown to be 50% lower than in normal individuals (Borok Z et al 1991). Reduced levels of PGE$_2$ have also been found in conditioned medium obtained from alveolar macrophages from patients with IPF (Ozaki T et al 1990).

Furthermore, mice deficient in COX-2 exhibit fibroproliferative disorders of the heart and the kidneys (Morham SG et al 1995, Dinchuk JE et al 1995). In human fibrotic lung fibroblasts, COX-2 deficiency results in a loss of the anti-proliferative response to TGF-β while in mice, COX-2 deficiency promotes bleomycin-induced pulmonary fibrosis (Keerthisingam CB et al 2001).
1.6 REGULATION OF COX-2 GENE EXPRESSION

Despite the wealth of available information, it is not yet clear how the COX-2 gene is regulated transcriptionally by external stimuli. Transcriptional regulation of COX-2 expression has to date been investigated in many cell types with numerous stimuli but there does not appear to be a general consensus. The differential regulation of COX-2 in different cells and the redundancy in signalling pathways and promoter elements ensure a higher level of complexity where the same signal can elicit different responses. Ten years after the discovery of COX-2, the growing body of evidence implicating COX-2 in both physiological and pathophysiological processes stresses the need to elucidate the molecular mechanisms underlying the differential regulation of COX-2 expression.

1.6.1 COX-2 CAN BE REGULATED BY A VARIETY OF STIMULI

The COX-2 gene can be rapidly induced by tumour promoters, cytokines and mitogens in many cell systems. In NIH 3T3 fibroblasts COX-2 expression can be upregulated by v-src transformation, PDGF and serum (Xie W and Herschman HR 1995 & 1996). LPS and TPA can upregulate COX-2 in vascular endothelial cells (Inoue H et al 1995). LPS has also been shown to stimulate COX-2 in RAW 264.7 macrophages (Wadleigh DJ et al 2000). TNF-α can induce COX-2 in the osteogenic MC3T3-E1 cell line (Yamamoto K et al 1995) and in human lung fibroblasts (Diaz A et al 1998). IL-β upregulates COX-2 in rat renal mesangial cells (Guan Z et al 1998), pulmonary type II epithelial cells (Newton R et al 1997) and human lung fibroblasts (Diaz A et al 1998, Endo T et al 1995), while a combination of IL-β and TNF-α results in rapid induction of COX-2 in MC3T3-E1 cells (Wadleigh DJ and Herschman HR 1999). IFN-γ and TGF-α were shown to induce COX-2 expression in normal human epidermal keratinocytes (Matsuura H et al 1999).

In addition, COX-2 expression can be negatively regulated in a cell type-dependent manner. The anti-inflammatory steroid dexamethasone suppresses COX-2 expression in murine 3T3 fibroblasts (Xie W et al 1994) and macrophage-like differentiated U937 cells (Inoue H and Tanabe T 1998) but not in bovine arterial endothelial cells. 

1.6.2 SIGNALING PATHWAYS LEADING TO ACTIVATION OF COX-2 TRANSCRIPTION

Several signal transduction pathways have been linked to stimulation of COX-2 transcription. COX-2 induction by v-src, PDGF and serum in murine fibroblasts is mediated through both the Ras/MEKK1/JNK/c-jun and Ras/Raf-1/MAP kinase/ERK signalling pathways (Xie W et al 1994, Xie W and Herschman HR 1995 & 1996). The IL-13 induction of COX-2 expression in primary rat mesangial cells involves the activation of both JNK/stress activated protein kinase (SAPK) and p38 MAP kinase pathways (Guan Z et al 1998). In macrophages, COX-2 induction in response to LPS can be mediated through both MAPK and protein kinase C (PKC) pathways (Mestre JR et al 2000).

1.6.3 PROMOTER ELEMENTS IMPLICATED IN TRANSCRIPTIONAL REGULATION OF COX-2

Sequence analysis of the 5' flanking region of the mouse (Fletcher BS et al 1992), rat (Sirois J et al 1993) and human (Appleby SB et al 1994, Tazawa R et al 1994, Kosaka T et al 1994) genes has shown several potential transcriptional control elements. Regardless of the species, these promoter regions include a TATA box and various putative regulatory elements such as CRE, NF-IL6 (C/EBP) motif, Sp1, NFκB sites, AP-2 sites and Ets, suggesting that the regulation of the COX-2 gene involves a complex array of regulatory factors. The genomic organisation of the COX-2 gene and 5' flanking region are illustrated in Figure 1.4.

The nucleotide sequence of the COX-2 5' flanking region is very dissimilar to that of the COX-1 gene. Notably, like many housekeeping genes, COX-1 lacks a TATA box (Wang LH et al 1993). Differences in the regulatory elements in the 5' flanking

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regions of the COX1 and COX-2 genes reflect, at least in part, the differential expression patterns of the two genes.
Figure 1.4: Genomic organisation of the COX-2 gene and 5′flanking region. The COX-2 gene consists of 10 exons and nine introns and is contained within an 8.5Kb region located on chromosome 1q25. The 5′ flanking region is also shown at a greater scale to show schematic representation of putative transcription factor response elements. The arrow and +1 denotes the transcriptional start site. Untranslated regions of exon 1 and 10 are unshaded key.
1.6.3.1 CRE
The COX-2 cyclic AMP response element (CRE) plays a pivotal role in COX-2 gene expression in a wide range of cells and in response to a wide variety of stimuli. In the murine COX-2 promoter, CRE is found to be essential for COX-2 expression induced by the v-src oncogene, PDGF and Serum in NIH3T3 cells (Xie W and Herschman HR 1995 and 1996). The CRE site in the murine COX-2 promoter is essential for both basal and induced gene expression in activated mast cells (Reddy ST et al 2000). In BAEC, induction of the human COX-2 promoter by LPS and Phorbol Ester is mediated in part by the CRE (Inoue H et al 1995). Similarly, in the murine COX-2 promoter, the CRE is involved in the induction of COX-2 in response to serum, bFGF, PDGF, PGE2 or the combination of TNF-α + IL1β in MC3T3-E1 cells (Wadleigh DJ and Herschman HR 1999). CRE is also required for optimal endotoxin-dependent induction in RAW 264.7 macrophages (Wadleigh DJ et al 2000).

1.6.3.2 NF-IL6 (C/EBP)
The NF-IL6 sites cooperate with CRE for the induction of the human COX-2 promoter by LPS and Phorbol Ester in BAEC (Inoue H et al 1995) and for optimal endotoxin-dependent induction in RAW 264.7 macrophages (Wadleigh DJ et al 2000). As with CRE, the two NF-IL6 sites (C/EBP) are involved in the induction of murine COX-2 promoter in response to serum, bFGF, PDGF, PGE2 or the combination of TNF-α + IL1β in MC3T3-E1 cells (Wadleigh DJ and Herschman HR 1999). NF-IL6 (C/EBP) is involved in the TNF-α-dependent induction of the mouse COX-2 promoter in MC3T3-E1 cells (Yamamoto K et al 1995). Involvement of NF-IL6 (C/EBP) activity has also been suggested in the aberrant overexpression of COX-2 in mouse skin carcinoma cells (Kim Y & Fischer SM 1998). NF-IL6 elements also facilitate the induction of the murine COX-2 promoter in activated mast cells (Reddy ST et al 2000).
1.6.3.3 NFκB
NFκB plays a role in the induction of the mouse COX-2 promoter by TNF-α in MC3T3-E1 cells (Yamamoto K et al 1995). NFκB has been reported to mediate human COX-2 gene induction by LPS in differentiated U937 monocytic cells (Inoue H & Tanabe T 1998). A role for NFκB in the activation of a human COX-2 promoter has also been reported in pulmonary type II epithelial cells (Newton R et al 1997). Comparison of the COX-2 5' flanking regions from human (Kosaka T et al 1994, Appleby SB et al 1994) and mouse (Fletcher BS et al 1992) reveals that the NFκB sites are conserved and therefore suggests an important role for NFκB in COX-2 transcription. However, it is now clear that the relative contribution of the different promoter elements in mediating COX-2 transcription varies between cell types and inducers. For example, there is no requirement for the NFκB site in MC3T3-E1 cells (Wadleigh DJ and Herschman HR 1999). Similarly, NFκB is not required for the endotoxin-dependent induction of COX-2 in RAW 264.7 macrophages (Wadleigh DJ et al 2000). NFκB elements are not required for the induction of the murine COX-2 promoter in either unstimulated or activated mast cells (Reddy ST et al 2000).

1.6.3.4 E-box
In the murine and human COX-2 promoters, the CRE site overlaps an E-box (Xie W et al 1994, Inoue H et al 1994). Consequently, this proximal element is bound by different factors depending on the species and on the cell. In the rat COX-2 promoter, the E box does not contain the CRE activity found in the human and mouse promoters. In rat ovarian granulosa cells, the E box-region binds the upstream stimulatory factor (USF) and is critical for basal transcription of the COX-2 gene (Morris JK & Richards JS 1996). The E-box has also been identified as a positive regulatory element in the murine COX-2 promoter in mouse skin carcinoma cells (Kim Y & Fischer SM 1998).

1.6.3.5 Sp1
The proximal Sp1 site in the human promoter was shown to be involved in the induction of COX-2 in hypoxic vascular endothelium (Xu Q et al 2000). See Chapter 5, section 5.4 for detail on Sp1.
1.6.4 POST-TRANSCRIPTIONAL REGULATION OF THE COX-2 GENE

The human COX-2 gene is located on chromosome 1 and contains 10 exons (see Figure 1.4). The entire 3’untranslated region (UTR) of the gene (2550bp) is contained within exon 10 which also includes 410 bp of the coding region. The 3’UTR has three potential polyadenylation motifs, approximately 280 bp apart from each other, and contains 22 copies of the AUUUA motif (Appleby SB et al 1994). The presence of this motif, also known as Shaw Kamen sequence, has been associated with rapidly degraded RNA messages (Shaw G & Kamen R 1986) and suggests a high turnover of COX-2 mRNA. Indeed, the steady-state mRNA levels for COX-2 have been shown to be less stable than the COX-1 transcript in Cos-7 cells (Hla T and Neilson K 1992).

Alternative polyadenylation at the 3’end results in the formation of two distinct mRNA isoforms of 4.6 and 2.8 kb. This is consistent with the general view that short size is a common feature among the primary response genes (Herschman HR 1991). The COX-2 4.6 mRNA isoform is more unstable than the 2.8 kb isoform suggesting that multiple elements in the 3’UTR cooperate to destabilize mRNA (Ristimaki A et al 1996). The presence of such elements at the 3’UTR indicates that post-transcriptional mechanisms may be important in the regulation of COX-2 gene expression. For example, post-transcriptional regulation contributes to the sustained induction of COX-2 by IL-1α in vascular endothelial cells (Ristimaki A et al 1994), while dexamethasone rapidly downregulates COX-2 by destabilising the COX-2 mRNA in human lung and synovial fibroblasts (Ristimaki A et al 1996). The 3’ UTR of COX-2 influences mRNA stability along with controlling translation efficiency. A study in human synovial fibroblasts showed that IL-1β increases reporter gene mRNA stability and translation via AU-rich containing distal regions of the COX-2 mRNA 3’ UTR. This response is mediated by a PGE2/p38 MAPK dependent process (Faour WH et al 2001). AU-rich elements (AREs) were shown to play a critical role in IL-1β-mediated induction of the murine COX-2 in messangial cells (Srivastava SK et al 1994). It has been suggested that IL-1β induces phosphorylation of cytosolic proteins that bind to the 3’ UTR and COX-2 and stabilize the message (Srivastava
SK et al 1994). Dixon DA et al (2000) also reported the specific binding of a complex of cytoplasmic proteins to the AREs. Distinct regions of the 3’ UTR can have different influences on COX-2 expression. Gou Q et al (1998) reported that deletion of the distal region of the 3’ UTR strongly inhibited basal mRNA turnover while deletion of the proximal highly conserved region resulted in increased basal turnover. In this study, the 3’UTR conferred IL-1-induced stabilization but not dexamethasone-induced downregulation (Gou Q et al 1998). Newton R et al (1998) reported a mechanism for dexamethasone-dependent repression of COX-2 that involves shortening of the COX-2 poly (A) tail and requires determinants other than just the 3’ UTR for specificity (Newton R et al 1998). A unique post-transcriptional regulatory mechanism has been reported in a metastatic cell line (MDA-MB-231) derived from a human mammary tumor. In these cells, COX-2 mRNA, protein and enzyme activity were induced by serum withdrawal and potently inhibited by the addition of serum. This regulation was shown to be primarily at the level of mRNA stability and required signal transduction pathways involving p38 (Jang BC et al 2000).

In summary, the regulation of COX-2 expression is achieved by fine-tuning mechanisms of gene transcription with post-transcriptional events. Cell-specific differences add yet a further level of complexity to the regulation of the COX-2 gene. This high level of complexity is in keeping with the need for tight control of COX-2, which can have pathogenic effects when its expression is deregulated.
1.7 SUMMARY AND AIMS OF THESIS

1.7.1 SUMMARY
Pulmonary fibrosis is a debilitating condition for which there is currently no effective treatment. Intensive research in the past years has established that fibrosis results from an imbalance between profibrotic and antifibrotic mediators. However, although defective collagen regulation and fibroblast proliferation are recognised as central points in the development and maintenance of fibrosis, the exact molecular pathways driving these processes have yet to be elucidated.

There is now considerable evidence supporting a key role for COX-2 in pulmonary fibrosis. Importantly, patients with pulmonary fibrosis fail to upregulate COX-2 (Wilborn J et al 1995, Keerthisingam CB et al 2001). This finding suggested a defect in the regulation of COX-2 gene expression.

1.7.2 HYPOTHESIS
The work presented in this thesis stems from the hypothesis that:

Failure to upregulate the COX-2 gene in patients with pulmonary fibrosis is due to sequence changes in the regulatory regions of the gene modifying gene expression.

1.7.3 AIMS
This thesis aims to investigate the contribution of COX-2 polymorphisms to genetic susceptibility to pulmonary fibrosis. In particular, it focuses on a novel promoter variant, identified during the course of this work, using population and functional studies.

This thesis has the following overall aims:
• To investigate the proximal promoter region of the COX-2 gene and identify novel sequence changes.

• To investigate novel variants in population studies, by genotyping them in healthy subjects and patients with disease, specifically IPF and sarcoidosis, and test for association with disease.

• To assess the functionality of the novel promoter variant -765G>C using in vitro experiments.

• To investigate a phenotypical consequence the -765G>C variant in an in vivo model of inflammation.
CHAPTER II

MATERIALS AND METHODS
2.1 SOURCE OF REAGENTS AND CONSUMABLES

Chemicals used were of analytical or molecular biology grade, supplied by Sigma-Aldrich Company Ltd, UK, unless otherwise stated. Distilled and de-ionised water (ddH₂O) was used for the preparation of buffers and solutions (Millipore Water Purification System, Millipore R010 followed by Milli-Q Plus, Millipore, Hertfordshire, UK). Barrier pipette tips were purchased from Greiner Bio-One, UK.

Polymere Chain Reaction (PCR)

Oligonucleotides used for PCR were purchased from Invitrogen Ltd, UK. Stock reagents of dNTPs and Taq DNA polymerase were purchased from Amersham Pharmacia Biotech UK Limited. PCR was performed in polycarbonate microtitre plates supplied by Hybaid Ltd, UK. QIAquick PCR Purification Kits were purchased from QIAGEN Ltd, UK.

Gel electrophoresis

DNA molecular weight standards for gel electrophoresis were purchased from Roche Diagnostics Ltd, UK. For the gel matrix, agarose was supplied by Anachem Ltd, UK, and acrylamide was supplied by Severn Biotech Ltd, UK. For agarose gel electrophoresis, the Wide Mini-Sub Cell GT Electrophoresis cell and the Sub-Cell GT Electrophoresis cell were used for 10 and 25cm gels respectively, both from Bio-Rad Laboratories Ltd, UK. Novex 6% DNA retardation gels were purchased from Invitrogen Ltd, UK and electrophoresed in the NOVEX E19001-XCELL II™ Mini Cell, NOVEX, USA. SSCP and EMSA PAGE were performed using the Bio-Rad Protean® II Xi cell apparatus purchased from Bio-Rad Laboratories Ltd, UK. SYBRGold nucleic acid gel stain for SSCP gels was obtained from Molecular Probes Inc., Oregon, USA. Sequencing gel reagents were purchased from national diagnostics, UK.

Sequencing

Reagents for sequencing using fluorescent chemistry were supplied by Amersham Pharmacia Biotech UK Limited.

Enzyme digests

Restriction endonuclease enzymes were supplied by New England Biolabs (UK).
**Tissue Culture**

Tissue culture disposable plastics were purchased from Corning Inc., NY, USA and Bibby Sterilin Ltd, UK. Dulbecco's Modified Eagles Medium (DMEM), trypsin, penicillin, streptomycin and L-glutamine were all purchased from Invitrogen Ltd, UK. Foetal Calf serum was purchased from Autogen Bioclear UK Ltd.

**Electrophoretic Mobility Shift Assays (EMSAs)**

EMSAs kits were supplied by Roche Diagnostics Ltd, UK. For radioactive EMSAs, Gel Shift Assay System was supplied by Promega, UK. Gel shift oligonucleotides were purchased from Santa Cruz Biotechnology Inc., UK, and Promega UK. Gel Shift antibodies were purchased by Santa Cruz Biotechnology Inc., UK.

**Plasmid preparations**

The COX-2 clone 973JM2 from the library RPC15, was used to generate a 1933bp product of the COX-2 gene (-1811+108) (kindly supplied by the Sanger Centre Clone Resource Group, Cambridge, UK). Purification of plasmid from bacterial cell culture was performed using the WIZARD® Plus SV Miniprep DNA Purification System from Promega, UK. The DNA fragments used to generate the COX-2 promoter constructs were isolated using a Gel Extraction Kit from, QIAGEN, Ltd, UK. Endonuclease-free purification of plasmid constructs prior to transfection was performed using an Endo-free maxi prep kit supplied by QIAGEN, Ltd, UK.

**Transfections and Reporter Gene Assays**

Promoter activities were measured using the Dual-Luciferase® Reporter Assay (Promega, UK).
2.2 DNA EXTRACTION FROM WHOLE BLOOD SAMPLES

Blood samples were taken in the anticoagulant ethylenediaminetetraacetic acid (EDTA) and maintained at 4°C for up to 20 hours prior to storage at 70°C, while awaiting DNA extraction. 20ml samples were collected in 50ml polypropylene tubes.

*White cell extraction.* Blood was thawed at ambient temperature or 37°C in a waterbath with constant monitoring. An equal volume (20ml) of phosphate buffered saline (PBS) was added, the sample mixed by inversion and then centrifuged (1800 g, 30 min). Lysed red cells were decanted to leave the pelleted white cells. The cell pellet was washed by addition of 10-20ml 0.2% NP40, 0.9% NaCl. This step significantly reduces the presence of any remaining red blood cells or plasma products, which may cause an excess presence of protein in the extracted DNA that could interfere with PCR. The sample was centrifuged (1800 g, 15 min) and the supernatant decanted.

*Digestion.* The white cell pellet was resuspended in 10ml lysis buffer [10mM TrisHCl (pH8.0), 10mM NaCl, 100mM EDTA (pH8.0)] and then 0.5ml of 10% SDS was added (final concentration of SDS approximately 0.5%). RNAase was added [20µg/ml of starting volume of blood] and the sample was incubated for 1 hour in a 37°C waterbath. Proteinase K was then added (100µg per ml of original blood volume) and the sample was incubated overnight in a 37°C waterbath.

*Phenol/chloroform extraction.* Tris-Saturated phenol equal to half the digest volume (5ml) and Chloroform/isoamyl alcohol (24:1 mix) equal to half the digest volume (5ml) were added, and the sample was mixed by inversion and vortexing before being centrifuged (1800 g, 10 min). The aqueous layer (upper layer) was transferred to a clean 50ml polypropylene tube and the phenol/chloroform treatment repeated. Chloroform/isoamyl alcohol (24:1 mix) equal to the digest volume (10ml) was then added, and again the sample was vortexed and then centrifuged (1800 g, 10 min). This time the lower organic layer was aspirated through the aqueous layer using a bulbous Pasteur pipette and discarded. The chloroform treatment was then repeated.
The chloroform wash removes residual phenol and protein in addition to the following precipitation and re-suspension steps.

**Precipitation and re-suspension.** 2ml of 4 M Sodium Chloride and 2 volumes of 100% ethanol were added at ambient temperature and mixed by inversion. If a precipitate was clearly seen, the sample was centrifuged (1800 g, 30 min), the supernatant discarded and the pellet washed by carefully pouring 20ml of 70% ethanol into the tube and decanting it without disturbing the pellet. The pellet was then rescued using a sterile inoculating loop and transferred to a screw-capped 2ml tube where it was allowed to air-dry before being resuspended in 1ml ddH2O. The tube was then placed on a rotating incubator at 4°C overnight to a few days to allow complete re-suspension. If the precipitate was not clearly visible following the precipitation reaction, the tube was placed at -70°C for 1-2 hours before being centrifuged and washed in 70% ethanol as described, but instead of rescuing the DNA pellet, the 50ml tube was allowed to air-dry and the 1ml ddH2O used to resuspend the DNA was added directly to the 50ml tube. Following incubation at 4°C overnight, the contents were transferred to a screw-capped 2ml tube.

Samples had their concentration determined using a spectrophotometer (Ultrospec®3000, Amersham Pharmacia biotech). In brief, 5μl of the extracted DNA was transferred to a 1.5ml tube containing 495μl ddH2O. The tube was inverted several times and the contents transferred to a UV compatible glass cuvette which then had light absorbance read through a 1cm cell path at A_{260}, A_{280} and A_{320} nm. The DNA concentration was calculated by the following formula: \( A_{260} \) reading x 100 (dilution factor) x 50 μg/ml (Absorbance of 1.0 through a 1cm cell path at \( A_{260} \) nm is equal to approximately 50 μg/ml double stranded DNA concentration). The reading at \( A_{280} \) measures protein. The \( A_{260}/A_{280} \) ratio should be approximately 1.8 (acceptable range 1.5-2.0) indicating the DNA extraction procedure has produced good quality DNA for PCR. The reading of \( A_{320} \) is outside the range of protein and DNA and should read an absorbance of 0.00 indicating that there are no erroneous contaminating products present. This DNA extraction method usually yields 10-50μg DNA per ml of whole blood starting material.
2.3 MUTATION DETECTION

Mutation detection of sequence changes in the COX-2 5’flanking region was carried out by PCR based methods. First the region -1122 to 54 bp was PCR amplified by overlapping primer sets. PCR product was the investigated for changes by SSCP analysis. Any band pattern differences were investigated by sequencing.

2.3.1 Polymerase Chain Reaction

PCR was performed in a total volume of 25µl with a 1x PCR reaction mix consisting of 1x KCl (50mM KCl, 10mM Tris-HCl pH 8.0, 0.001% gelatin), 0.5-2.5mM MgCl₂, 200µM dNTPs, 0.2 µM of each oligonucleotide primer and 50ng of genomic DNA. Barrier pipette tips were used for PCR to avoid contamination. The PCR reagents were stored at -20°C as a 10x KCl buffer, 10x 2’deoxynucleoside 5’-triphosphate (dNTPs) (2mM dNTPs), 25mM MgCl₂ and 20µM working stocks of each primer and thawed at ambient temperature prior to use. Once defrosted, reagents were kept on wet ice.

Five µl of 10ng/µl working stock DNA was pipetted into the walls of a microtitre plate and each well was overlaid with a drop of mineral oil. DNA was omitted from negative ddH₂O controls. The PCR reaction mix was prepared and Taq polymerase (5U/µl) was added last with a final concentration of 0.02U/µl. The enzyme was mixed by gentle pipetting and 20µl of PCR reaction mix was added to each well, through the mineral oil, to make a 1x PCR reaction mix.

The plate was pulsed in a centrifuge (13000rpm, 30 sec) to remove any air bubbles and placed in a PTC-225 Peltier thermal cycler (MJ Research). PCR reaction conditions were titrated for each primer set, altering the MgCl₂ concentration and annealing temperature, in order to obtain optimal amplification of PCR product. A typical PCR cycle consists of denaturing at 94°C for 4 min and then 35 cycles of [denaturation (94°C for 1 min), annealing (50-60°C for 1 min), elongation (72°C for 1 min)] followed by 1 cycle at 72 °C for 5 min before holding at 10°C.
The PCR primers were designed so that a series of overlapping fragments was obtained, spanning the COX-2 promoter. Primers were designed to be between 19 and 22bp in length, aiming for a melting temperature (Tm) between 60-62°C and a GC content between 47-53%. An equal distribution of bases in the primer was also preferred. When possible, primers were designed to have one or more G or C bases in the last 3 bases at the 3’ end and sequences containing repeats were avoided. Ten sets of primers were designed to cover the COX-2 promoter region from -1122 upstream of the transcription start site to 54 bp. These primers and their optimal MgCl₂ concentrations and annealing temperatures (AT) are shown in table 2.1. Figure 2.1 is a schematic representation of the COX-2 promoter showing the position of the primer sets.
Figure 2.1 The COX-2 promoter
This is a schematic representation of the promoter of the human COX-2 gene showing the transcription start site and the putative response elements. The positions of the overlapping primer sets are illustrated.
Table 2.1 Primers used for PCR
Table showing primer sets used to amplify the 5’ flanking region of the COX-2 gene and optimised PCR conditions for each set.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Label</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Annealing position*</th>
<th>[Mg$^{2+}$]</th>
<th>AT (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF1</td>
<td>CR1</td>
<td>GCGGAAAGAAACAGTCTAT</td>
<td>131</td>
<td>'77-'60</td>
<td>1.5</td>
<td>55</td>
</tr>
<tr>
<td>CR2</td>
<td>CTCGAGGAATGCTGCCGAGT</td>
<td>201</td>
<td>'193-'174</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR3</td>
<td>TCGGGCAAAGACTGCGAAG</td>
<td>264</td>
<td>'323-'305</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR4</td>
<td>ATGACTGTTTCTCTCTCAGGCC</td>
<td>54-36</td>
<td>'54-'36</td>
<td>1.5</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR5</td>
<td>TCGGGCAAGACTGCGAAG</td>
<td>201</td>
<td>'193-'174</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR6</td>
<td>ATGACTGTTTCTCTCTCAGGCC</td>
<td>264</td>
<td>'323-'305</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR7</td>
<td>ATGACTGTTTCTCTCTCAGGCC</td>
<td>54-36</td>
<td>'54-'36</td>
<td>1.5</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR8</td>
<td>CTCGAGGAATGCTGCCGAGT</td>
<td>201</td>
<td>'193-'174</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR9</td>
<td>ATGACTGTTTCTCTCTCAGGCC</td>
<td>264</td>
<td>'323-'305</td>
<td>2.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>CR10</td>
<td>ATGACTGTTTCTCTCTCAGGCC</td>
<td>54-36</td>
<td>'54-'36</td>
<td>1.5</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

* relative to transcription start site, base 1 of exon 1.
2.3.2 DNA agarose gel electrophoresis

A 2% agarose gel mix (2g agarose/100ml 1x Tris-borate/EDTA (TBE) buffer [0.09M Tris borate, 0.002M EDTA pH 8.0] was heated in a 850W microwave until the agarose was completely dissolved. The melted agarose was cooled by rotating the flask under cold running tap water for uniform cooling. Ethidium bromide to a final concentration of 0.5µg/ml was added and the gel solution swirled and poured into a casting tray. Combs for making the loading wells were placed in position and the gel was allowed to solidify at ambient temperature for 30 min. The gel tank was filled with 1x TBE buffer, the combs were removed, and the gel was immersed in the buffer. DNA molecular weight Marker VIII (0.25 µg/ml) was loaded into the first well of each lane of wells on the gel. 5µl of PCR product was mixed with 2 µl of 5x loading buffer (50% glycerol, 0.01% xylenol orange) before loading to the wells. The gel was run at 5V/cm for 30 min. The Wide Mini-Sub Cell GT Electrophoresis cell and Sub-Cell GT Electrophoresis cell were used for 10 and 25cm gels respectively, both from Bio-Rad Laboratories Ltd, UK. Gels were visualised on a Fujifilm PhosphorImager using a wavelength of 532 nm and the 0580 filter.

2.3.3 Single Strand Conformation Polymorphism (SSCP)

SSCP is a simple and sensitive method for the detection of unknown mutations. The detection of sequence changes such as point mutations is believed to be due to an alteration in the structure of single-stranded (ss) DNA. In SSCP, ssDNA (i.e. denatured PCR product) is electrophoresed through a non-denaturing gel. This allows for intramolecular interactions to occur so that the ssDNA is partially able to bind to itself. As the DNA is not running as a linear molecule, the mobility of the DNA is determined by its size and conformation (secondary structure formation). As the conformation of a ssDNA fragment is dependent on the sequence, any sequence change can potentially alter the conformation of the fragment and result in an abnormal mobility on polyacrylamide gels. The non-denaturing nature of the SSCP gel also allows for some re-annealing of the ssDNA into double stranded (ds). The faster migrating band of dsDNA can also give valuable information as some mutations can be detected as heteroduplex mobility shifts.
The conformation of ssDNA is however dependent on gel electrophoresis conditions. To maximise the ability to detect a conformational change it is therefore usual practise to run denatured PCR product under different gel electrophoresis conditions. The sensitivity of mutation detection by SSCP depends on conditions such as gel temperature, cross-linker concentration, acrylamide concentration, presence of glycerol and type of gel matrix. Another critical parameter is the length of the DNA fragment being analysed. PCR product within the range of 250-600bp is more likely to show band pattern changes than PCR product > 600bp. SSCP has an overall detection rate greater than 90% (Sheffield V.C et al 1993) and the advantage of being a non-isotopic method.

SSCP gel electrophoresis was performed using the Bio-Rad Protean® II Xi cell apparatus (Bio-Rad Laboratories Ltd, UK). Glass plates were cleaned with ethanol, assembled, clamped and placed upright on the casting stand. The gel mix was prepared as stated in table 2.2.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH$_2$O</td>
<td>11ml</td>
</tr>
<tr>
<td>50% glycerol</td>
<td>6ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>3ml</td>
</tr>
<tr>
<td>30%, 19:1 acrylamide/bisacrylamide solution</td>
<td>10ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30ml</strong></td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>93.75µl</td>
</tr>
<tr>
<td>fresh 10% Ammonium Persulphate (APS)</td>
<td>37.5µl</td>
</tr>
</tbody>
</table>
* for a 10% acrylamide, 10% glycerol mix

The gel was then cast with combs and allowed to set for 2 hours at ambient temperature. When set, the combs were removed and the wells washed with ddH$_2$O. The gel was then placed in the electrophoresis tank and pre-cooled to 4°C in a walk-in cold room prior to electrophoresis. Electrophoresis was also carried out at 4°C. Pre-chilled 1x TBE buffer was applied to the upper chamber and after checking for leaks, the lower chamber was filled until the bottom of glass plates was submerged.
under 1cm of buffer. Wells were flushed with TBE using a disposable syringe and 19G needle and the gel electrophoresed for 30 min at 10W prior to sample loading. Samples were prepared 5 min before the end of this electrophoresis and loaded immediately. To prepare the samples 4μl of PCR product was mixed with 2μl loading buffer (95% formamide, 10mM EDTA, 0.1% SDS, 0.05% xylene orange). Samples were denatured (95°C, 5 min) and maintained on wet ice prior to loading. Gels were electrophoresed at 10W per gel for between 16-24 hours depending on the size of the PCR product.

2.3.4 SSCP gel imaging
Following electrophoresis, the plates were removed from the gel tank, laid flat and the top glass plate removed. A corner was cut from each gel to assist orientation and the plates were agitated in 1xTBE to lift the gel off the glass plate. The gel was then carefully transferred to a tray containing 1x SYBR Gold nucleic acid gel stain (Molecular Probes Inc. USA), a highly sensitive fluorescent stain for detecting ds or ss DNA, the tray was covered with foil (SYBR Gold is light sensitive) and placed on a rotating shaker at sufficient speed to maintain movement of buffer over the gel for 15 min. Gels were subsequently visualised on a Fujifilm PhosphorImager using a wavelength of 473nm and the Y520 filter.

2.4 AUTOMATED DNA SEQUENCING
2.4.1 PCR for sequencing
Regions of interest as determined by SSCP were PCR amplified for sequencing. All band patterns from a specific PCR product were PCR-amplified and sequenced. In cases where a sequence change was not detected primers were moved further away from the region of interest in order to obtain high quality sequence close to the original primer position, in case a change occurred close to the primer site.

PCR reactions with a total volume of 50μl were set up in duplicate and PCR was performed using the appropriate conditions for the primers involved. The two reactions were then combined and 5μl of the PCR product was checked for quality on a 2% agarose gel.
2.4.2 Purification of the PCR product for sequencing

PCR product was purified from the PCR mix (primers, nucleotides, polymerases and salts) using the QIAquick PCR Purification kit, QIAGEN Ltd, UK, according to the manufacturer's instructions. In brief, 5 volumes of binding buffer PB were added to 1 volume of the PCR reaction. To bind the DNA, the sample was applied to the QIAquick column. The column was centrifuged (13,000 rpm, 60sec) and the flow-through discarded. To wash, 0.75 ml of wash buffer PE were added to the QIAquick column which was then centrifuged for 30-60sec. The flow-through was discarded and the column spun for an additional 2 min at maximum speed. To elute the DNA, 30μl of elution buffer (10mM TrisHCl, pH 8.5) was added to the centre of the QIAquick membrane. The column was let to stand for 1 min and then centrifuged for 2 minutes at maximum speed. The purified PCR product was then run on a 2% agarose gel.

2.4.3 Preparation of sequencing reaction

Amersham sequence chemistry (ThermoSequenase™II dye terminator cycle sequencing premix kit) was used in conjunction with an automated Applied Biosystems Inc (ABI) PRISM sequencer for sequencing. A modified version of Amersham’s sequencing protocol was used. For the sequencing reaction, ddH2O, ThermoSequenase II reagent mix A, ThermoSequenase II reagent mix B, dilution buffer, primer and template DNA were combined in this order in a 0.2ml thin-wall microtube to a total volume of 20μl. The recipe is shown in table 2.3. Each reaction mix was overlaid with mineral oil and vials were placed in a GeneAmp PCR system 9700 (PE Applied Biosystems, UK). Thermal cycling conditions were 25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 1 min.
Table 2.3: Sample mix for 1 sequencing reaction

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>12.5</td>
</tr>
<tr>
<td>ThermoSequenase II reagent mix A</td>
<td>0.5</td>
</tr>
<tr>
<td>ThermoSequenase II reagent mix B</td>
<td>0.5</td>
</tr>
<tr>
<td>dilution buffer</td>
<td>3</td>
</tr>
<tr>
<td>primer [5µM]</td>
<td>2.5</td>
</tr>
<tr>
<td>template DNA</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

2.4.4 Preparation of the sequencing gel matrix

Table 2.4 shows the constituents of the sequencing gel used. To prepare the sequencing gel, urea was dissolved in acrylamide and ddH₂O in the presence of a mixed-bed ion exchange resin. The solution was stirred in a beaker using a magnetic flea until all the urea crystals were dissolved. Five ml of 10x TBE was filtered (0.2µM) under vacuum using tap water pressure using a Buchner filter flask. The gel solution was added afterwards and the mix de-gased for 2-5 min. Fresh 10% APS was then added to the mixture followed by the addition of TEMED.

Table 2.4: The sequencing gel.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Mass/volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>18g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>27.5ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>5.2ml</td>
</tr>
<tr>
<td>Mixed-bed ion exchange resin</td>
<td>0.5g</td>
</tr>
<tr>
<td>10x TBE</td>
<td>5ml</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>35µl</td>
</tr>
<tr>
<td>fresh 10% Ammonium Persulphate (APS)</td>
<td>250µl</td>
</tr>
</tbody>
</table>

The ABI gel plates were cleaned and mounted in an ABI casting cassette. The gel solution was poured under gravity and a sharkstooth comb inserted using the straight
edge first. The gels were always left for 2 hours at ambient temperature to ensure consistency between gels with respect to polymerisation. When the gel was set, the comb was removed. Any excess acrylamide from the wells was flushed away with ddH2O. The comb was then re-inserted with the sharksteeth penetrating the gel by 1.5mm.

2.4.5 Gel loading and electrophoresis
Avoiding mineral oil, the contents of each sequence tube were transferred to a fresh 1.5 ml eppendorf containing 2μl of 1.5 M sodium acetate. Three volumes of 100% ethanol (66μl) were added to each reaction. Samples were mixed by vortexing, placed on ice for a maximum of 15 minutes to precipitate the DNA and pelleted at ambient temperature by centrifugation (11,000g, 20 min). The supernatant was removed and 250μl of cold 70% ethanol was added to wash the pellet. Samples were briefly centrifuged again (11,000g, 2 min) before the 70% wash was removed and the pellet allowed to air dry. Pellets were either stored at ~20°C for up to one month or used immediately. Each pellet was resuspended in 4μl of 95% formamide loading dye (Amersham). Care was taken to redissolve the DNA completely. Tubes were vortexed vigorously for 10-20 seconds to ensure complete resuspension and pulse centrifuged (11,000g, 30 sec) to collect the contents at the bottom of the tube. Samples were then denaturated at 80°C for 3 minutes to denature and immediately placed on ice prior to loading. Two μl of each sample was loaded to each well of the sequencing gel that was pre-electrophoresed without samples for 30 min in 1xTBE. The gel was then electrophoresed for 7 hours at 48°C (1.68kV, 50mA, 150W). Sequence data was processed by ABI Factura software for base calling and aligned using ABI Navigator Software (ABI Inc.).
2.5 POPULATION GENOTYPING

2.5.1 Restriction Endonuclease Enzyme digests
Sequence changes were genotyped in population samples by restriction endonuclease enzyme digest of PCR product containing the variant. The appropriate region of the promoter was PCR amplified and 5μl of the PCR product were incorporated into the digest mix. In the case of -765G>C, an AcI I site already present due to the common G allele, is removed by the presence of the C allele.

Primers CF8 and CR7 were used to amplify a 305bp product. Digestion of this product with AcI I generates two fragments of 117 and 188bp that are easily resolved in a MADGE polyacrylamide gel (see section 2.5.2). Digests were set up in standard 96-well microtiter plates. Each reaction contained 5μl of PCR product, 6U of AcI I and 1xNEBuffer3 [100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol (pH 7.9 at 25°C)] in a total volume of 20μl. Samples were overlaid with mineral oil and placed at 37°C for either 3 hours or 16 hours overnight.

2.5.2 Microtiter Array Diagonal Gel Electrophoresis (MADGE)
Digests were run on an 8% MAGE polyacrylamide gel to obtain highest resolution (Day IN and Humphries SE 1994). The constituents of the MADGE gel are listed in table 2.5.

Table 2.5: The MADGE gel

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>25.3ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>4ml</td>
</tr>
<tr>
<td>19:1 acrylamide/bisacrylamide solution</td>
<td>10.7ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40ml</strong></td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>50μl</td>
</tr>
<tr>
<td>fresh 25% Ammonium Persulphate (APS)</td>
<td>100μl</td>
</tr>
</tbody>
</table>
The gel was cast on a MADGE casting tray where the comb teeth of the tray preserve
the exact configuration of an 8 x 12 microarray and enable horizontal electrophoresis
in tracks following a 71.6° diagonal between wells. The MADGE comb was laid flat
in a tray with teeth facing upwards, the gel mix poured and a suitably sized glass
plate, which was previously silanised with a silanizing agent [0.5% γ-
methacryloxypropyltrimethoxysilane/0.5% glacial acetic acid/ ethanol (v/v)], placed
on top with care so that no air bubbles were trapped between the glass and the
casting tray. This was achieved by resting the glass plate in the gel at one end while
smoothly lowering the other end until the gel/air front was driven out and the plate
contacted all teeth. The gel was left to polymerise at room temperature for 45 min.
Samples were prepared by mixing 5μl of the digest reaction with 2μl of formamide
dye and loaded onto the MADGE gel using a multichannel pipette. Gels were run in
a small electrophoresis tank (Wide Mini Sub-Cell GT Electrophoresis Cell, BioRad
Laboratories, Ltd, UK) in 1x TBE buffer for 45 min, post-stained for 20 min 1x TBE
containing ethidium bromide (0.5μg/ml) and visualised on a Fujifilm
PhosphorImager using a wavelength of 532nm and the O580 filter.

2.6 PREPARATION OF NUCLEAR EXTRACTS FROM FIBROBLASTS

2.6.1 Routine Cell Culture
Cells were routinely grown in 75 cm² tissue culture flasks in DMEM containing 10%
foetal calf serum (FCS) (v/v), 4mM L-glutamine, penicillin (100U/ml) and
streptomycin (100μl/ml). Flasks were incubated in a humidified atmosphere of air
containing 10% CO₂ at 37 °C. Culture media was removed and replaced every 3-4
days. Cells were passaged when confluent with a 1: 4 split ratio.

2.6.2 Cell passage
Cells were passaged upon reaching visual confluence. Culture media was removed
and discarded and the cell monolayer was washed with 4ml of trypsin/EDTA (trypsin
0.05% w/v; EDTA 0.02% w/v). 1ml of trypsin EDTA was added to coat the cell
monolayer and the cells were incubated for 5 min at 37°C. Rounding and detachment
of cells from the plasticware was observed using an inverted-phase contrast light
microscope (Olympus TCK-2, Olympus Optical Company Ltd, London, UK). 11 ml DMEM with 10% FCS was added to the flask to neutralise the trypsin and resuspend the cells. The cell suspension was passed through a 10ml pipette several times to ensure a single cell suspension and then 3ml (1:4 passage) placed in a new tissue culture flask and the volume made up to 15ml by further addition of DMEM with 10% FCS. Experiments were conducted on cells between passages 15 and 17.

2.6.3 Isolation of nuclear extracts

For the purpose of preparing nuclear extracts from HFL-1, cells were seeded into large (150mm x 20mm) dishes. Upon reaching visual confluence, the medium was removed and cells were washed twice with PBS. 30ml of serum free medium (SFM) or DMEM with 10% FCS, including penicillin (100U/ml) and streptomycin (100μl/ml), was added to each dish and cells were left to quiesce for 16 hours at 37°C. After 16 hours the media was removed and cells were processed as described below. Following removal of the media plates were kept on ice at all times.

Plates were washed twice with ice-cold tris buffered saline (TBS) 25mM Tris (To prepare 1L of TBS: 3gr Tris Base, 8gr NaCl, and 0.2gr KCl were dissolved in ddH2O). For each plate, cells were collected using a cell scraper into 1ml of TBS, resuspended and transferred to a 1.5ml eppendorf. Tubes were centrifuged (13,000 rpm 15 sec) and supernatant was removed. Cells were resuspended in 200μl of cold Buffer A [10mM HEPES pH 7.9; 10mM KCl; 0.1mM EDTA; 0.1mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA); 1mM dithiothreitol (DTT); 0.5mM phenylmethylsulfonyl fluoride (PMSF)] containing 1x complete protease inhibitors (Roche, UK) and samples were left on ice for 15 min. Following addition of 25μl of 10% NP-40, samples were vortexed vigorously and pelleted by centrifugation (11,000g 30 sec). The supernatant was removed and the nuclear pellet resuspended in 100μl of ice-cold Buffer C (20mM HEPES pH 7.9; 0.4M NaCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 1mM PMSF) containing 1x complete protease inhibitors (Roche, UK). The tubes were rocked vigorously in a mixer (the Coulter mixer, Coulter Electronics Limited, UK) in the cold room for 15 min and then the samples were pelleted by centrifugating at 4 °C (11,000g, 5 min). Supernatants were aliquoted in 5x 20μl aliquots and stored at −70 °C.
2.6.4 Measurement of protein concentration

The protein content of each sample was measured using the BCA Protein Assay Kit according to the manufacturers instructions. This assay is based on the oxidation of copper ions (Cu$^{2+}$ to Cu$^{1+}$) in the presence of protein. The Cu$^{2+}$ ions react in a dose-dependent manner with bicinechonic acid (BCA) to form a purple precipitate that exhibits a strong absorbance at 562nm, thus allowing the spectrophotometric quantitation of protein in aqueous solution.

A standard curve from which unknown samples could be quantified was generated from a range of standard dilutions (2000μg/ml-50μg/ml) of BSA that was prepared in the same diluent as the sample buffer (buffer C, section 2.6.3). A mixture of the BCA reagent A and BCA reagent B was prepared at a 1:50 dilution respectively. Twenty-five μl of each standard or sample was pipetted into separate wells of a 96-microwell plate (Gibco-BRL) and 200μl of solution A/B was added to each well. The plate was agitated on a shaker (Luckham R100, Luckham Ltd., Sussex, UK) for 30 seconds and incubated at 37°C for 30 minutes. Absorbance at 562nm was measured on a plate reader (ICN Flow, High Wycombe, Bucks, UK). A standard curve was generated, and the protein concentration of each sample was determined.

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA is a powerful tool for detection of proteins binding to specific DNA sequences. It works on the principle that complexes of DNA and protein will migrate more slowly through a gel during electrophoresis than unbound DNA. The assay was performed using the DIG Gel Shift Kit from Roche Molecular Biochemicals (UK) and also the radioactive method.

2.7.1 DIG Gel Shift method

The Dig Gel shift kit uses a nonisotopic technique for detecting sequence-specific DNA binding proteins where probes are labelled at the 3’end with digoxigenin-11-ddUTP. The labelled oligonucleotides are detected by an enzyme immunoasay. The following solutions were prepared as required: TEN buffer (10mM TrisHCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0), 4 M LiCl, 100% ethanol at -20°C, 70% ethanol at -20°C, 10x TBE buffer, 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0),
Buffer 1 (0.1M Maleic, 0.15M NaCl, adjusted with solid NaOH to pH 7.5 and autoclaved), Buffer 2 (Blocking reagent provided by kit diluted 1:10 in buffer 1, autoclaved and stored at 4°C), Buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 autoclaved), Washing buffer (buffer 1 + Tween 20, 0.3% v/v)

2.7.1.1 Designing the oligonucleotides
Oligonucleotides across the region containing the −765G>C variant were designed taking into account general rules for oligonucleotide design (see section 2.3.1) and the manufacturer’s recommendations. Fragments should ideally be between 30 and 100bp. The shorter the DNA fragments, the smaller the risk of non-specific interactions occurring between proteins and sequences that flank the specific binding site. The oligonucleotides used in this study were designed to be 30bp long and had an equal distribution of bases at both sides of the −765 position. Probes were composed of two complementary oligonucleotides annealed together (table 2.6). Probe G contains the common -765G allele and probe C the-765C allele.

<table>
<thead>
<tr>
<th>Label</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe G</td>
<td>GAATTTCACCTTTCCCGCCCTCTCTTCCTTCCAAAG</td>
</tr>
<tr>
<td></td>
<td>CTAAATGGAAAGGGGGAGAGAGGTTC</td>
</tr>
<tr>
<td>Probe C</td>
<td>GAATTTCACCTTTCCCGCCCTCTCTTCTTCTTCCAAAG</td>
</tr>
<tr>
<td></td>
<td>CTAAATGGAAAGGGGGAGAGAGGTTC</td>
</tr>
</tbody>
</table>

2.7.1.2 Annealing of oligonucleotides
The single-stranded complementary oligonucleotides [100µM] were mixed in a molar ratio of 1:1 in TEN buffer in a 1.5ml eppendorf that was then incubated for 10 min in a heated beaker with boiling ddH2O. The beaker was then removed from the heat source and placed on the bench to allow for the oligonucleotides to anneal as the temperature slowly decreased to ambient temperature. Finally, the annealed oligonucleotides were diluted with TEN buffer to 4pmol/µl.
2.7.1.3 Labelling of oligonucleotides

To label the double stranded oligonucleotides the following were mixed on ice and made up with ddH₂O to a final volume of 20 µl: 4 µl of 5x labelling buffer (1M potassium cacodylate, 0.125M TrisHCL, 1.25mg/ml bovine serum albumin, pH 6.6), 4 µl of CoCl₂ [25mM], 1µl of double stranded oligonucleotide (4pmol/µl), 1 µl DIG-11-ddUTP solution and 1µl of terminal transferase. The mix was incubated in a 37°C waterbath for 15 min and the tube was placed on ice. The labelled double stranded oligonucleotides were then precipitated with 2µl of 4 M LiCl and 60µl of chilled 100% ethanol. Subsequently, the samples were incubated for 30 minutes at -70°C and centrifuged at 4°C (10,500g, 15 min). Pellets were washed 3 times with 500µl of 70% chilled ethanol and left to air dry. Finally, the labelled oligonucleotide probes were resuspended in 25µl of TEN buffer.

2.7.1.4 Control of labelling efficiency

In order to assess the efficiency of labelling, 1µl of serial dilutions of labelled oligonucleotides in TEN buffer was spotted onto a dry, positively charged BM nylon membrane (Amersham Pharmacia Biotech, UK). Included on each membrane was also a control labelled oligonucleotide (provided in the DIG Gel Shift Kit). The membrane was soaked in 10x SSC and the oligonucleotides fixed by UV crosslinking (UV Stratalinker™ 2400, Stratagene).

2.7.1.5 Chemilluminescent Detection

All washes and incubations were carried out using a shaking incubator at ambient temperature. The membrane was washed for 5 min in washing buffer and incubated for 30 min in buffer 2. The membrane was then incubated for 30 min with 20ml of diluted anti-digoxygenin-AP, Fab fragments (75mU/ml, 1:10000 dilution in buffer 2). Unbound conjugate was removed by washing twice for 15 min with 20ml of washing buffer and the membrane was equilibrated for 5 min in buffer 3.

The membrane was incubated with 10ml of the diluted chemilluminescent substrate CSPD (10mg/ml, 1:100 dilution in buffer 3) for 5 min with DNA side down and subsequently blotted for a few seconds on a dry Whatman 3MM paper to remove excess liquid. The damp membrane was then sealed in a hybridisation bag and
incubated for 15 min at 37°C before exposing to an X-ray film for 15-40 min. The film was subsequently developed using a developer (RGII Fuji X-Ray Film Processor).

2.7.1.6 Preparation of the polyacrylamide gel

A 6% acrylamide gel in 0.25x TBE buffer was prepared 16-20 hours before electrophoresis to help standardise experiments with respect to gel polymerisation. The gel constituents are shown in table 2.7. The Bio-Rad Protean® II Xi cell apparatus (Bio-Rad Laboratories Ltd, UK) was used.

Table 2.7: The EMSA gel mix

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>46 ml</td>
</tr>
<tr>
<td>19:1 acrylamide/bisacrylamide solution</td>
<td>12 ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>100 µl</td>
</tr>
<tr>
<td>fresh 10% Ammonium Persulphate (APS)</td>
<td>400 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>60ml</strong></td>
</tr>
</tbody>
</table>

2.7.1.7 Binding reaction

A master mix for all samples was prepared at the same time. The constituents of a 1x reaction were: 4µl of 5x binding buffer [100mM HEPES, pH 7.6, 5mM EDTA, 50mM (NH₄)₂SO₄, 5mM DTT, Tween 20, 10% w/v, 150mM KCl], 1µl of poly (A-T) [1µg/µl], 1µl of poly-L [1µg/µl] and 1µl of Zinc (final concentration 1mM). For each binding reaction, 7µl of the master mix was therefore combined with a sufficient volume of ddH₂O to make a final volume of 20µl and the following ingredients were added in this order: 5µg of nuclear extract, 4pmol unlabelled oligonucleotide and 15-30fmol of labelled oligonucleotide. Samples were kept on wet ice until all samples were prepared. The binding reaction constituents were mixed carefully and samples were incubated for 15 min at ambient temperature. Following incubation samples were placed back on ice, 5µl of loading buffer without bromophenol blue (0.25x
TBE, 60%; glycerol, 40%, provided in the kit) was added and the samples were used immediately for PAGE electrophoresis.

2.7.1.8 Polyacrylamide Gel Electrophoresis (PAGE)
Combs forming the sample wells were removed and the wells flushed with a syringe to remove excess acrylamide that may interfere with loading. Gels were pre-electrophoresed for 2 hours at 70V at 4°C in a walk-in cold room using 0.25x TBE as running buffer. The first lane was used to load bromophenol blue dye (0.25x TBE, 60%; glycerol, 40%; bromophenol blue, 0.2% w/v) provided in the kit) to follow the progress of the electrophoresis. Loading buffers containing bromophenol blue are known to interfere with the protein-DNA interactions and so in subsequent lanes, 25µl of sample was applied in loading buffer without this dye. The gel was run in 0.25x TBE buffer for 4 hours at 180V at 4°C.

2.7.1.9 Transfer onto a nylon membrane (electroblotting)
The electrophoresed products were transferred onto a positively charged membrane by means of electroblotting. First, a sheet of nylon membrane (Amersham Pharmacia Biotech, UK), trimmed to the size of the gel, was equilibrated for 15 min in transfer buffer (0.25x TBE). One glass plate was removed carefully from the gel and the equilibrated nylon membrane placed carefully onto the gel, avoiding air bubbles between gel and filter. Four layers of gel-sized Whatman 3MM papers, pre-soaked in transfer buffer, were then placed on the filter. A 10ml stripette was used to gently roll over the 4 layers of Whatman 3MM paper to remove air bubbles. The pad of Whatman 3MM paper/nylon membrane/gel was then peeled away from the remaining glass plate and another 4 layers of presoaked Whatman 3MM papers were added to the exposed side of the gel. The resulting sandwich was placed between the electrodes of an electroblotting device (NovaBlot, Pharmacia LKB). Transfer was performed for 1 hour at 400mA. The membrane was then removed and soaked in 10x SSC, the oligonucleotides were fixed by UV crosslinking and the chemilluminescent signal detected as described in section 2.7.5.
2.7.2 RADIOACTIVE EMSA

Radioactive EMSAs were performed using the Promega Core Gel Shift Assay System (Promega, UK).

2.7.2.1 Phosphorylation Reaction

Oligonucleotides were radiolabelled in a 10μl phosphorylation reaction consisting of 4pmol of double stranded oligonucleotide, 1x T4 Polynucleotide Kinase buffer (700mM Tris-HCl pH 7.6, 100mM MgCl₂, 50mM DTT), 1μl [γ-³²P] ATP (3,000Ci/mmol at 10mCi/ml) (Amersham), 1μl of T4 Polynucleotide Kinase (5U/μl) and filtered ddH₂O to the final reaction volume. The labeling reaction was allowed to proceed in a waterbath (37°C for 10 min) and stopped by the addition of 1 μl of 0.5M EDTA. The labeling reaction was made up to a 50μl volume with TE (10mM TrisHCl, 1mM EDTA, pH 8.0). Removal of unincorporated label was achieved using ProbeQuant G-50 spin columns (Amersham Pharmacia). Prior to sample application the contents of the column were mixed by inversion, the moulded endbit was snapped off, and the column centrifuged at 700g for 1 min. The column was placed in a clean eppendorf tube and the sample was loaded carefully onto the matrix. The column was centrifuged 700g for 2 min and then discarded. Of the eluted radiolabelled probe, 2μl was combined with 3 ml scintillation fluid (EcoScint, National Diagnostics) and counts were measured (Minax β Tri-Carb® 4000 series liquid scintillation counter, United Technologies, Packard). The radiolabelled oligonucleotides were then diluted with TE to a working solution of 50000 CPM/μl.

2.7.2.2 Binding Reaction

Binding reactions were set up containing 2μl of 5x Gel Shift Binding Buffer (10mM Tris-HCl pH 7.5, 1mM MgCl₂, 0.5mM EDTA, 4% glycerol, 50mM NaCl, 0.5mM DTT, 0.5μg polydI-dC; Promega) and 5μg of nuclear extract in a total volume of 9μl made up with ddH₂O. Samples were mixed carefully and the reactions were incubated first at 4°C for 30min followed by a 10-min incubation at ambient temperature. 1μl of 32-P labelled oligonucleotide was then added and the reactions were incubated for an additional 30 min at ambient temperature. In competition assays, 100x excess unlabelled oligonucleotide probe was added to the binding reaction 10 min prior to the addition of labelled oligonucleotide probe. For antibody
supershift and blocking assays 2μl of antibody (2μg/μl) was added to the binding mixture containing the nuclear extracts and pre-incubated on ice for 1.5 hours prior to the addition of labelled oligonucleotides. In control samples 2μl of non-immune IgG was added (purchased lyophilised from R&D and resuspended at 2μg/μl). For experiments with human recombinant (hr) Sp1 protein, 1.5-3μl of hrSp1 [100ng/μl] (Promega) was added and incubated for 20 min at ambient temperature prior to addition of labelled oligonucleotides.

2.7.2.3 Electrophoresis of DNA-Protein Complexes
Samples were loaded immediately on a Novex 6% DNA retardation gel. The progress of the electrophoresis was monitored by adding 1μl of bromophenol blue dye (0.25x TBE, 60%; glycerol, 40%; bromophenol blue, 0.2% w/v) to the first well of the gel. Samples were loaded in subsequent lanes in loading buffer without dye (0.25x TBE, 60%; glycerol, 40%). Gels were run at ambient temperature in 0.5x TBE buffer at 250V for 17 min. The gel plates were opened carefully with the gel remaining on one plate. The gel was then placed in a tray containing enough fixing solution (10% acetic acid, 20% methanol) to cover it and left to fix for 10 min. A sheet of Whatman 3MM filter paper was placed on the gel and used to peel the gel of the plate. The gel was then covered with plastic wrap and dried on a gel dryer (Model 583 Gel Dryer, Biorad) for 20 min at 80°C, before exposing to an X-ray film overnight at -80°C with an intensifying screen. The Bio-Rad Protean® II Xi cell apparatus (Bio-Rad Laboratories Ltd, UK) was also used for radiaoactive EMSAs as described in section 2.7.1.6). Gels were run at ambient temperature in 0.25x TBE (180V, 2 hours) and dried for 1 hour at 80°C.

2.7.2.4 Isolation of radiolabelled probe from polyacrylamide gels
One of the problems encountered with EMSAs was the presence of additional bands in lanes where free probe (with no protein) was loaded. This was thought to be the result of secondary structure formation due to the high GC content of the oligonucleotide probes. For probe C in particular, the substitution of a G for a C resulted in a run of C which might have caused the oligonucleotide to lose its integrity and form secondary structures, such as hairpin loops. Such structures are easily distinguished as they migrate differently through the polyacrylamide gel. The
presence of annealed probe in different conformations could interfere with protein binding and in result in artefactual binding patterns being observed. To overcome this problem, the labelled free probe was isolated away from secondary structures by gel extraction and the purified labelled product used in subsequent binding assays.

In order to isolate the free probe, both wild type and mutant probes were radiolabelled and the entire reaction loaded on an EMSA gel (as described in section 2.7.1.6). The gel was electrophoresed for 2hrs and then dismantled carefully so that the gel remained stuck on one glass plate. A transparent plastic membrane was used to cover the surface of the gel and an individually wrapped X-ray film (X-Omat-AR film, KODAK) was carefully positioned on top and exposed for 15 min. In order to be able to reposition the film on the gel following the development, a sharp syringe was used to inject a small amount of dye through the film and into the gel in three corners of the gel. Following exposure, the film was developed and the bands of interest located. A scalpel was used to cut the area of interest out of the film thereby leaving a hole at the exact position of the desired band. The film was then repositioned back on the gel by aligning the dye marks left on the film with those on the gel. A sterile scalpel was then used to excise the area of interest from the polyacrylamide gel by cutting around the edges of the hole made in the X-ray film. The excised gel slices was then placed in sterile 1.5ml eppendorf tubes (taking care to avoid any pieces of plastic membrane) containing 1ml of elution buffer (0.5M NH₄Oac, 0.5% SDS, 1mM EDTA). To each sample was added 10μg of carrier t-RNA in order to enhance DNA precipitation and maximise recovery. The samples were left shaking overnight in a 37°C incubator prior to phenol/chloroform extracting the DNA. Each sample was split into 3 tubes and 500μl of phenol/chloroform/isoamyl alcohol mix (25:24:1) was added to each tube. Samples were vortexed briefly and centrifuged (11,000g, 5 min). The aqueous (top) phase was transferred to a fresh eppendorf and 1ml of 100% ethanol was added to each tube. Samples were mixed and left at -70°C for 30min to precipitate the DNA. Samples were then centrifuged at 4°C (11,500g, 20min) and the supernatant was removed carefully. To wash the pellets, 100μl of 80% ethanol was added to each tube and the samples were centrifuged (11,000g, 5 min). The ethanol was removed and pellets allowed to air dry prior to dissolving in 100μl of ddH₂O.
2.8 PREPARATION OF CONSTRUCTS FOR TRANSFECTIONS

2.8.1 Verification of clone and preparation of glycerol stocks

The COX-2 clone 973JM2 from the library RPC15 was kindly supplied by the Sanger Centre Clone Resource Group, Cambridge, UK. Serial dilutions of the clone were made in 100μl Luria Broth (LB) containing 25μg/ml of Kanamycin [Kan25] and streaked onto Petri plates containing solidified LB agar [Kan25] medium. A sterile inoculating loop was used to streak the bacteria over the surface of the medium in an overlapping criss-cross fashion, thereby diluting the sample, with the aim of achieving single colonies at some point on the plate. The lid of the Petri plate was removed for the least time possible to reduce the risk of aerial contamination. Streaked plates were left at 37°C to grow overnight.

Single colonies were picked from the plates and their position was marked. These colonies were used to inoculate 10ml cultures in LB [Kan25], which were incubated at 37°C, shaking at 220rpm. The marked plates were placed back at 37°C and allowed to grow for a further 2-3 hours. The same colonies were picked again and diluted into 10μl ddH2O for use in PCR. A simple PCR using primers CF1CR1 was carried out to confirm that the colony contained the plasmid with the COX-2 gene.

Two 10ml cultures that had been PCR-confirmed to contain the plasmid with the COX-2 gene were selected and left at 37°C shaking for a total of 8 hours. The others were discarded. 850μl of the cultures were mixed with 150μl glycerol in order to prepare glycerol stocks for long-term storage at -80°C. Samples were snap frozen in liquid nitrogen and stored in a -80°C freezer. From the glycerol stocks, LB agar plates [Kan25] were streaked using a sterile loop and left at 37°C overnight. Single colonies were picked for subsequent culture in LB broth [Kan25] by inoculating a 1ml starter culture for 1 hour and using 100μl of this to ‘spike’ a 10ml culture which was then incubated at 37°C, in a 220rpm shaking incubator for 16 hours.
2.8.2 Purification of the COX-2 clone

The 973JM2 clone was purified from E.coli cultures using the WIZARD® Plus SV Miniprep DNA Purification System (Promega, UK). Following a 16-hour incubation overnight (37°C, 220rpm), bacteria were pelleted by centrifugating 5ml of each culture at ambient temperature (10,000g, 5 min). Supernatants were discarded and 250μl of Cell Resuspension solution was added. The tubes were vortexed to resuspend the pellets and the contents transferred to a 1.5ml eppendorf. 250 μl of Cell Lysis solution was added and mixing was achieved by inverting the tubes 4 times. After a 5 min incubation at RT, 10μl of Alkaline Protease solution was added and the tubes were inverted 4 times. The samples were incubated for a maximum of 5 min at RT, 350μl of Neutralisation solution was then added and contents were mixed by inversion. Samples were centrifuged (10 min, 14,000g) and the supernatant was carefully removed, without disturbing the pellet, and poured into the spin column. The tubes were centrifuged (1 min, 14,000g) and the flow-through discarded. Next, 750 μl of Column wash solution was added, samples were centrifuged (1 min, 14,000g) and the flow-through discarded. The wash was repeated with 250μl of Column wash solution (2 min, 14,000g). The column was then transferred to a fresh 1.5ml eppendorf and the DNA was eluted with 30μl of Nuclease-Free H₂O. Columns were left to stand for 1 min and then centrifuged (1 min, 14,000g) to collect the isolated clones.

2.8.3 PCR amplification of insert

Primers CF11 and CR11 were designed for PCR amplification of the insert (see table 2.8 for sequences). The CF11 and CR11 primers had extended 5’ends that introduced an Nhe I and Hind III restriction endonuclease site respectively to allow restriction enzyme digest for subsequent insertion of the fragment into the vector (the bases of the tagged ends are indicated in lower case, table 2.8). Primers CF11CR11 failed to PCR-amplify a product directly from the purified plasmid following multiple attempts using several combinations of PCR conditions (0.5-5mM Mg Cl₂; 45-65°C annealing temperature). Several commercially available PCR kits designed for difficult PCR, due to GC-rich regions and secondary structure, also failed to amplify a product. It did however prove possible to amplify a product using primers CF12
(table 2.8) and CR11 and use a 1:100 dilution of this product as template for a CF11CR11 PCR.

A commercially available kit, GC-RICH PCR System (Roche Diagnostics Ltd, UK), was used for the CF12CR11 PCR according to the manufacturers instructions. The conditions resulting in a product were as follows: Master Mix 1: 200 μM dNTPs, 200nM of each primer, 50ng of template DNA, 0 M resolution solution and PCR grade water (supplied) up to a volume of 35μl. Master Mix 2: 1.5M MgCl$_2$, 2u/50μl GC-RICH PCR System enzyme mix and PCR grade water up to a volume of 15μl. The two master mixes were combined in a 0.2μl PCR tube, the mix overlaid with mineral oil and placed in a thermal cycler. The PCR thermal cycle conditions were as follows: 95°C for 3 min, 10 cycles of 95°C for 30 sec, 45°C for 30 sec, 72°C for 2 min, 20 cycles of 95°C for 30 sec, 45°C for 30 sec, 72°C for 2 min plus 5 sec for each additional cycle, 72°C for 1 min.

Two μl of a 1:100 dilution of the PCR product was then successfully used as a template for the CF11CR11 primers using the commercially available kit, Advantage® Genomic PCR (CLONTECH Laboratories Inc., UK). The reaction mix consisted of 1.1mM Mg(OAc)$_2$, 2μM CF10, 2μM CR9, 0.1U of Advantage Genomic Polymerase Mix, 1x genomic PCR buffer and ddH$_2$O up to 50μl. The cycle conditions were as follows: 95°C for 1 min, 35 cycles of 95°C for 30s, 68°C for 2 min, 68°C for 2 min. The product was then purified using the QIAquick PCR Purification kit (section 2.4.2).

Table 2.8: Primers used to amplify 1.9kb insert.

<table>
<thead>
<tr>
<th>Label</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF12</td>
<td>CCACTATGGGATAGATGGAG</td>
</tr>
<tr>
<td>CF11</td>
<td>acgattgctagCTCTCTCACAGATGGATTC</td>
</tr>
<tr>
<td>CR11</td>
<td>tatGGGTAAGCTTTGCTGTCTGAG</td>
</tr>
</tbody>
</table>

* bases in lower case indicate tagged ends
2.8.4 pGL3basic vector and insert digests

Double digests of the vector and insert DNA were carried out with an excess of enzyme. Specifically, 5 units of Nhe I and Hind III were used to digest 4050ng of pGL3basic vector (Promega, UK) and 1500ng of PCR-generated insert DNA in 1x NEBuffer2 [50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol (pH 7.9 at 25°C)] in separate reactions. Reactions were supplemented with 100μg/ml bovine serum albumin (BSA) (supplied with the Nhe I enzyme by New England Biolabs, UK) and the volume was adjusted by addition of ddH₂O making up a final volume of 50 and 30μl for vector and insert digests respectively. Digestion of the insert was carried out in duplicate. Digests were overlaid with mineral oil and incubated at 37°C for 2 hours and 30 min before adding 5μl of 0.2M EDTA to stop the reaction. Both vector and insert were purified using the QIAquick PCR Purification kit (section 2.4.2).

2.8.5 Vector dephosphorylation

To reduce background in transfection due to self re-ligation, the cut vector was dephosphorylated at the 5' end. 0.5 units of Calf Intestinal Alkaline Phosphatase (CIP) were used to treat 1pmol DNA ends (equivalent to 6.3μg of vector). The DNA was first suspended [0.5μg/10μl] in 1x NEBuffer 3 [100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol (pH 7.9 at 25°C)] and enzyme was added. The volume was adjusted to 126μl by addition of ddH₂O. The reaction was overlaid with mineral oil and incubated at 37°C for 1 hour. The dephosphorylated vector DNA was purified as described (section 2.4.2).

2.8.6 Ligation

To maximise the efficiency of the ligation reaction, ligations were set up as 1:2, 1:5 and 1:10 vector (50ng) to insert ratio. Reactions containing vector alone, phosphorylated vector alone and dephosphorylated vector with T4 polynucleotide kinase (PNK) were also included as controls. Ligation reactions in a final volume of 20μl contained 400U of T4 DNA ligase in 1x T4 ligation buffer. Dephosphorylated vector with PNK contained 10U of T4 PNK. Reaction volumes were adjusted accordingly with ddH₂O. Reactions were overlaid with mineral oil and incubated for 3 hours at 16°C followed by a 10-min incubation at 20°C. Each ligation reaction was diluted to 40μl with ddH₂O.
2.8.7 Transformation

Epicurian Coli® XL1-Blue supercompetent cells (Stratagene Ltd., Cambridge) were thawed on ice and 50μl aliquots were transferred to a pre-chilled 15ml Falcon® 2059 polypropylene tube. For each transformation, 5μl of diluted ligation product and 1μl of β-Mercaptoethanol (1.42M) were added to a 50μl aliquot of competent cells and the tubes were swirled gently. To control for the transformation efficiency of the cells, 1μl of pUC19 was added instead of ligation product to a 50μl aliquot of supercompetent cells. Tubes were incubated on ice for 20 min before being heat-pulsed in a 42°C waterbath for 45 sec and rested on ice for 2 min. To each tube, 900μl of pre-warmed (42°C) SOC medium (see note below for recipe) was added and tubes were incubated at 37°C for 1 hour shaking at 220 rpm. A 200μl sample of each culture was then plated onto LB agar plates containing 100μg/ml ampicillin [Amp100], by means of an L-shaped glass spreader. Standard sterile technique was used throughout. The spreader was sterilised by dipping the end in a beaker containing 70% alcohol, allowing the excess to drain from the spreader and then igniting the remainder in a Bunsen flame. After cooling, the spreader was used to distribute the cell suspension across the plate. For the pUC19 control, 5μl of the culture was diluted 1:40 in SOC medium and then plated out. The plates were then left for 16 hours in a 37°C incubator.

Note: Preparation of SOC Medium. To prepare 1L of SOB Medium: 20g tryptone, 5g yeast extract, 0.5g NaCl were dissolved in ddH₂O and autoclaved. Prior to use, 10ml of 1M MgCl₂ and 10ml of 1M MgSO₄ were added per liter of SOB medium and the solution was filter-sterilized. To make up 100ml of SOC medium, SOB medium was combined with 1ml of a 2M filter-sterilised glucose solution and the solution was filter-sterilised prior to use.

2.8.8 Isolation of construct

Plates were checked for the presence of colonies and single clones were picked with a sterile loop. Half of each clone was introduced into a PCR reaction using primers CF9CR9 (table 2.1) to confirm presence of insert and the other half was used to inoculate a 10ml LB culture [Amp100]. PCR positive cultures for the insert were used for preparation of glycerol stocks as described in section 2.8.1. From glycerol
stocks, agar plates were streaked and 10ml LB cultures [Amp100] were set up overnight in shaking incubator (220rpm) at 37°C. 7ml of the culture was used in a miniprep to isolate the construct away from bacteria (for details see section 2.8.2)

2.8.9 Clone Sequencing
Primers pGL3F, CF11, CF10, CF13, CF3, CF1 and CR13, CR9, CR4, CR11, pGL3R were used for sequencing in order to cover the entire sequence of the 1.8kb insert in both the sense and antisense directions (see table 2.9 for additional primer sequences).

<table>
<thead>
<tr>
<th>Label</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3F</td>
<td>Purchased from Promega, UK</td>
</tr>
<tr>
<td>pGL3R</td>
<td>Purchased from Promega, UK</td>
</tr>
<tr>
<td>CF13</td>
<td>GTATGTGCTAGCATATAGAGCAGATATAGAGCC</td>
</tr>
<tr>
<td>CR13</td>
<td>TGGAACATAGTTGGATGAGG</td>
</tr>
</tbody>
</table>

Of ten clones that were sequenced none, matched the wild type consensus sequence for the region derived from sequences U44805, AF044206, U04636, D28235, U20548, L34209). However, 3 clones (clones 2, 5 and 6) contained large segments of sequence that matched the consensus sequence. These three clones were used to generate the final -765G and -765C constructs through a combination of site-directed mutagenesis, restriction enzyme digestion and re-ligation.

2.8.10 Site-directed Mutagenesis
A site-directed mutagenesis reaction was carried out on clone 6 in order to introduce a C base at position -765 upstream of the transcription start site of the COX-2 gene. Two complimentary oligonucleotides were designed containing the desired mutation, flanked by unmodified nucleotide sequence (see Table 2.10 for sequences). The oligonucleotide primers were PAGE purified prior to use.
Table 2.10: Primers used in site-directed mutagenesis

<table>
<thead>
<tr>
<th>Label</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF14</td>
<td>GAATTACCTTTCCTCTCTTTCC</td>
</tr>
<tr>
<td>CR14</td>
<td>GAAAGAGAGGGGGAAGGTAATTC</td>
</tr>
</tbody>
</table>

Note: underlied base indicates position of the −765 variant

The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Ltd., Cambridge) was used to perform the mutagenesis reaction. Following the manufacturer’s recommendations, two different concentrations of ds DNA template were used while keeping the primer concentration constant. Reactions were set up using 25 or 50ng DNA template (clone 6), 125ng of each primer, 0.2mM dNTP mix, 1x pfu buffer and 1μl pfu polymerase at 2.5U/μl and ddH2O to a final volume of 50μl. The cycle conditions were 95°C for 30 sec followed by 12 cycles each consisting of 95°C for 30 sec, 55°C for 1 min and 68°C for 14 min (i.e. 2 min per kb of plasmid length). To check for presence of product, 5μl of the reaction mix was removed and run on a 1% agarose gel for 50 min. The remainder of the reaction was incubated with 20U of Dpn I enzyme for 1 hour at 37°C. This step removes the E.coli-derived methylated DNA (i.e. the non-mutated sequence) by digestion while leaving the new PCR-synthesized non-methylated product intact. Following digestion, 1μl of the mix was added to 50μl aliquots of XL-1 Blue supercompetent cells and transformations were performed as described in section 2.8.7. The reactions were plated onto LB agar [Amp 100] and left at 37°C for 16 hours. Clones were then picked and grown in a 1ml starter culture for 6 hours. The starter culture was used to inoculate a 10ml culture that was allowed to grow for 16 hours at 37°C in a shaking incubator (220rpm). The plasmid was isolated using the WIZARD® Plus SV Miniprep DNA Purification System (Promega, UK) as described in section 2.8.2.

2.8.11 Restriction endonuclease digests of purified clones

The −765C clone 6 was sequenced to confirm that no further changes had been incorporated by the mutagenesis reaction. Errors in sequence in clones 2, 5 and 6 could be avoided if a 645bp fragment from clone 2 was combined with an 843bp fragment from clone 6 or −765C clone 6 and a 436bp fragment from clone 5 (see Chapter 5, figure 5.6 for details). The enzymes Nhe I and Ase I were used to cut out
the 645bp fragment from clone 2. Clone 6 and \(-765C\) clone 6 were cut with \textit{Ase} I and \textit{Afe} I in order to obtain the common/variant 843bp fragment. Finally, clone 5 was digested with \textit{Afe} I and \textit{Hind} III to generate the 436bp fragment. All digests were performed with 5\mu g of DNA and 25U of enzyme (5U enzyme/\mu g of DNA). Single digests were performed as controls for enzyme activity using 1\mu g of DNA and 5 units of enzyme. All reactions were incubated at 37°C for 2 hours. Clone 2 and clone 5 digests were run on a 1.2% low melting point agarose gel (5V/cm, 1hr in 1xTBE). Clone 6 digests were run on 0.7% low melting agarose gel (5V/cm, 2hr in 1xTBE). All gels were run at 4°C. The bands of interest were cut out of each gel and extracted using the QIAGEN gel extraction kit section 2.8.12).

2.8.12 Gel extraction

Gel extraction was performed using the QIAquick Gel Extraction Kit QIAGEN Ltd, UK, according to the manufacturer's instructions. Gels were visualised, and bands of interest located, under long-wavelength UV to minimise radiation damage to the DNA. The desired DNA fragments were excised from the agarose gel with a clean sharp scalpel. The slice was minimised by removing extra agarose and weighed in an eppendorf tube. One volume of buffer QG was added for each volume of gel (100mg ~ 100\mu l). The sample was incubated at 50°C until the gel slice had completely dissolved (approximately 10 min), with the tube vortexed every 2-3 min during the incubation to help dissolve the gel. After the gel slice had completely dissolved the sample was checked for the presence of yellow colour indicating appropriate pH. One gel volume of isopropanol was added and the sample was mixed by pipetting. A QIAquick spin column was placed in a 2ml collection tube and the sample was applied to the column to bind DNA. The column was centrifuged (11,000g, 1 min) and the flow-through discarded. To remove all traces of agarose, a further 0.5ml of buffer QG was added, the sample was centrifuged (11,000g, 1 min) and the flow-through was discarded. To wash, 0.75ml of buffer PE was added, the column was left to stand for 5 min, and then centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min to ensure that all residual ethanol from buffer PE was removed. The column was then placed in a clean eppendorf tube. To elute DNA, 30\mu l of buffer EB (10mM TrisCl, pH 8.5) was added
to the centre of the membrane and the column was centrifuged (11,000g, 1 min). A 2μl sample of the elute was checked on a 10cm, 2% agarose gel (80V, 30min).

2.8.13 Generation of constructs

Ligation reactions were set up containing the three size fragments (654, common –765G or variant –765C allele 843, and 436bp) and the digested, dephosphorylated pGL3 vector. Ligations were performed in a final volume of 20μl with 1:2 and 1:5 vector (50ng) to insert ratio and 2.000U of T4 ligase. The reactions were incubated at 25°C and the ligation time was extended to 2 hours, as one of the ligations was blunt end.

A 5μl sample from each ligation reaction was then added to 50μl of supercompetent cells and transformation was performed as described in section 2.8.7.

A total of 96 clones, 48 -765G and 48 -765C, were chosen from the plates. Half of each clone was incorporated into 150μl cultures set up in a 96 well plate (37°C for 8 hours) and the other half was used as template in a PCR reaction using the pGL3 primers. PCR was performed in a final reaction volume of 10μl that consisted of 200μM dNTPs, 50mM KCl, 1.5 mM MgCl2, 0.2 μM of pGL3 Forward and pGL3 Reverse primer, 0.02U Taq (5U/μl) and ddH2O. The cycle conditions were as follows: 4min 94°C, then 35x (1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C) and 5 min at 72°C.

Only 4 clones (three –765G and one –765C) generated a product of the expected size (1.9kb). The minicultures in the 96-well plates that corresponded to these clones were then used to inoculate a 15ml culture overnight. Glycerol stocks were prepared the following day as described in section 2.8.1.

Minipreps for these four clones were carried out using the WIZARD® Plus SV Miniprep DNA Purification System (Promega, UK) as described in section 2.8.2. The products were then checked on a 10cm, 1% agarose gel (80V, 30 min). One clone was contaminated with additional bands and so three of the four clones were
sequenced. Two of these were consensus −765G and one was consensus wild type with −765C. These constructs were used in all subsequent transfection experiments.

2.8.14 Endonuclease-free maxi preparation of constructs

Endonuclease-free purification of plasmid constructs for to transfection was performed using an Endo-free maxi prep kit supplied by QIAGEN, Ltd, UK. From the glycerol stocks, plates were streaked for the −765G and −765C clones. After 16 hours, single colonies were picked and used to inoculate a 5ml starter culture. Cultures were incubated for 8 hours at 37°C in a shaking incubator (220rpm). 1ml of the starter culture was then used to inoculate a 250ml culture in a sterile 1 litre conical flask. Cultures were left at 37°C for 16 hours, shaking at 220 rpm. Bacterial cells were harvested by centrifugation at 4°C (6000g, 15 min). The supernatant was removed and bacterial pellets were resuspended in 10 ml buffer P1 containing RNase A. Pellets were thoroughly resuspended by pipetting. Following addition of 10 ml of buffer P2, samples were mixed by inversion (6 times) and incubated at RT for a maximum of 5 min. Chilled buffer P3 (10 ml) was added to the lysate and samples were mixed by inversion. The lysates were then poured into the barrel of a QIAfilter cartridge and left at ambient temperature for 10 min. The cap from the QIAfilter outlet nozzle was removed, the plunger was inserted gently into the QIAfilter Maxi Cartridge and the cell lysate was filtered into a 50 ml collecting tube. Buffer ER (2.5 ml) was then added to the filtered lysate and sample mixed by inversion (10 times) before incubating on wet ice for 30 min. A QIAGEN-tip 500 was equilibrated by applying 10 ml of buffer QBT and allowing the column to empty by gravity flow. The filtered lysate was then applied to the tip and allowed to enter the resin by gravity flow. The QIAGEN tip was washed twice with 30 ml buffer QC and DNA was eluted with 15ml buffer QN. DNA was precipitated by addition of 10.5 ml of isopropanol. Samples were mixed and centrifuged immediately at 4°C (15000g, 30 min). The DNA pellet was then washed with 5 ml of endotoxin-free 70% ethanol and centrifuged (15000g, 10 min). The supernatant was decanted carefully without disturbing the pellet. Pellets were left to air-dry for 15 min and DNA was redissolved in 50µl of endotoxin-free buffer TE.
2.9 REPORTER GENE ASSAYS

2.9.1 Transfections

Transfections were performed using a non-viral integrin targeted vector (peptide 6). The vector consists of a cationic lipid (L), an αβ1 integrin targeted peptide with a 16-cytosine tail (I) and a plasmid DNA (D) which combine electrostatically to form a lipopolyplex LID vector complex (detailed in Jenkins RG et al 2000). In brief, cells were set up in 12-well plates and incubated until dense, but subconfluent. On the day of transfection extra fresh serum was added to each well (100μl/1ml of medium). The transfection mixture for one sample was prepared as follows: 0.75μl of lipofectin [1mg/ml] (Invitrogen Ltd., UK) was combined with 100μl of OPTIMEM medium (Invitrogen Ltd., UK) in a 50ml polypropylene tube taking care to avoid the walls of the tube as lipofectin sticks to polypropylene. In a separate tube, 20μl of peptide 6 solution (0.2mg/ml) was combined with 80μl of OPTIMEM. In another tube, 1μg of -765G, -765C clone, or empty pGL3basic vector DNA with no insert was diluted in 100μl of OPTIMEM and 0.05μg of pRL-TK vector was added to each mix (1:20 ratio). Lipofectin and peptide 6 solutions were initially combined and mixed by pipetting up and down. The DNA solution was then added to the lipofectin-peptide 6 mix and contents mixed by pipetting. The mix was left for 1 hour at ambient temperature. Following incubation, 700μl of OPTIMEM was added to the mixture to make a total volume of 1 ml. The 12-well plates containing the subconfluent cells were aspirated and the cells rinsed once with DMEM that did not contain antibiotics or serum. Transfection mixture (1 ml) was then added to each well and plates were placed at 37°C (10% CO₂) for 6 hours. Following incubation, the transfection medium was removed and cells were rescued in DMEM containing 10% serum for 1 hour. The medium was then aspirated and cells were left for 16 hours in serum-free medium (2ml/well). The medium was changed and additions were made for treatments. Cells were treated for 8 hours prior to measuring luciferase activity.

2.9.2 Measurement of luciferase activity

Following treatments, the medium was removed from the cells and stored at -70°C. The cell layer was rinsed quickly (x2) with ice-cold saline, aspirated each time.
150μl of 1x Reporter Gene Assay Lysis buffer (Roche diagnostics, UK) was added to each well and plates were left on a rocking device for 10 min at RT. Lysates (15μl) were then transferred to a polystyrene 96-well plate (Optiplate, Perkin Elmer Life Sciences) and the plate placed in a plate-reading luminometer (TROPIX TR717 Microplate Luminometer, PE Applied Biosystems).

A dual reporter assay, Dual-Luciferase® Reporter Assay (Promega, UK), was used where two individual reporter enzymes (firefly and Renilla luciferases) are expressed and measured within a single system. The expression of the experimental reporter (firefly luciferase) is driven by the COX-2 promoter (1.9kb) and correlates with the effect of specific experimental conditions. The activity of the co-transfected Renilla luciferase serves as an internal control for baseline response. This normalises for the activity of the experimental reporter (firefly luciferase) and minimises experimental variability caused by differences in cell viability or transfection efficiency. The activities of the firefly and Renilla luciferases are measured sequentially from a single sample.

To measure promoter activity, the firefly luciferase reporter assay was initiated by the addition of Luciferase Assay Reagent II (LARII) to an aliquot of lysate, to generate a “glow-type” luminescent signal. Quenching of firefly luciferase luminescence and concomitant activation of Renilla luciferase were accomplished by the addition of Stop & Glo Reagent (supplied) to the sample, immediately after the quantitation of the firefly luciferase reaction. Luminometer injectors P and M were set to dispense 75μl of LARII and Stop & Glo® Reagent, respectively. For measurements, a 10 sec delay time was used.
CHAPTER III

CHARACTERISATION OF THE 5’ FLANKING REGION OF THE COX-2 GENE FOR SEQUENCE VARIANTS
3.1 INTRODUCTION

In normal tissue repair, COX-2 appears to have a protective role by driving the production of the anti-fibrotic mediator PGE\(_2\) and thereby limiting the fibroproliferative response. The anti-fibrotic properties of PGE\(_2\) are illustrated by its inhibitory effects on collagen gene expression (Saltzman LE et al 1982), fibroblast proliferation (Korn JH et al 1980) and fibroblast to myofibroblast transition (Kolodsick JE et al 2003).

Of interest is the observation that many of the mediators that stimulate collagen synthesis also induce fibroblasts to synthesise PGE\(_2\) (section 1.2.1.9). The evidence that has accumulated so far supports the presence of a tightly regulated feedback loop where the effects of stimulatory (pro-fibrotic) mediators such as PDGF, TGF-\(\beta\) and IL-1\(\beta\) are balanced by the actions of negative regulators such as PGE\(_2\) and IFN-\(\gamma\).

In pulmonary fibrosis this balance is altered. Part of this imbalance can be attributed to a ‘PGE\(_2\) deficiency’ occurring as a result of dysregulation of COX-2 expression. A diminished capacity to upregulate COX-2 has been shown in cells isolated from patients with IPF (Wilborn J et al 1995, Keerthisingam CB et al 2001, Vancheri C et al 2000). Failure to upregulate COX-2 in response to fibrogenic mediators (such as TGF-\(\beta\)) removes the inhibitory control of PGE\(_2\) leading to hyper-proliferation and excess collagen deposition by fibroblasts and myofibroblasts.

The failure to upregulate COX-2 expression in pulmonary fibrosis is central to the work presented in this thesis and leads to my hypothesis that the diminished capacity to upregulate COX-2 in pulmonary fibrosis is at least in part due to functional sequence variants in the promoter region of COX-2 that impact on gene expression.

At the start of this study there were no reported changes in the COX-2 promoter. Work in this centre by Dr Michael Hill investigated the promoter region of the COX-2 gene in healthy subjects and IPF patients. Consequently, this part of the study has the following specific aim:
• To investigate the 5’ flanking region of the COX-2 gene in sarcoidosis subjects for the presence of novel sequence changes that may prove functional and associate with fibrotic lung disease

The method of approach was PCR-Single Stranded Conformation Polymorphism (SSCP) analysis. In order to search for sequence alterations in the COX-2 promoter, oligonucleotide primers were designed, spanning the proximal promoter region of the gene. The primers were positioned to allow overlapping PCR products to be obtained (Chapter 2, Figure 2.1). The PCR products were denatured and run through a non-denaturing gel to screen for variants. SSCP was chosen as a safe (non-radioactive), high throughput and cost effective method with a detection rate exceeding 90% (Sheffield V.C et al 1993). As the aim of the study was to identify common variants this was considered acceptable.
3.2 METHODS

The approach used for identifying sequence variants was SSCP analysis (section 2.3.3). To maximise chances of detecting variants, the method was optimised by varying the running conditions of the gel and the constituents of the gel itself. One of the parameters that can affect the migration of a product, and therefore the sensitivity of SSCP detection, is the porosity of the gel. As the polyacrylamide gel matrix is formed by the polymerisation of acrylamide and a cross-linker (bis-acrylamide), the concentration of crosslinker can greatly affect the pore size of the matrix. For this reason acrylamide/bis acrylamide stock solutions were used with different concentrations of cross-linker (C). These included 2% C (19 parts acrylamide:1 part bis) and 5% C (49 parts acrylamide:1 part bis). The acrylamide concentration used for the SSCP was 10%. Glycerol can also enhance the detection rate of SSCP and so samples were analysed on gels with and without glycerol. Two concentrations of glycerol (5 and 10%) were tested. The role that glycerol plays is not exactly understood but it has been suggested that its effect is to reduce the pH of the buffer (TBE buffer) by chemical reaction with the boric acid. To determine the optimal concentration of glycerol SSCP gels were prepared with 0, 5 and 10% glycerol. Good quality of PCR product is also essential for SSCP analysis. Different conditions were used, however routinely the following conditions gave the best results: 10% acrylamide, 5% C (19:1), and 10% glycerol.
**Subject groups**

All subjects used for mutation detection were UK Caucasian subjects. At the start of this project, a UK Caucasian sarcoidosis population was available and has been used for mutation detection here. Sarcoid subjects were selected to represent a range of radiographic stages of sarcoidosis (see Chapter 1, Table 1.3). A total of 24 sarcoids were investigated for each primer set of whom 12 were CXR stage 4, 2 were CXR stage 3, 4 were CXR stage 1 and 6 were CXR stage 0. Later on a UK Afro-Caribbean sarcoidosis sample became available. Screening for variants in these samples has been initiated and work is currently ongoing in the centre, but for the purposes of this thesis only data on the Caucasian sarcoids will be presented.

**3.3 RESULTS**

PCR-SSCP analysis of the COX-2 5’flanking region led to the identification of the following sequence variants: -765G>C, -62C>G and -368T>C. Work initiated previously by Dr Michael Hill had identified the -490G>C variant that was only found in a single individual. Figures 3.1-3.8 are gel SSCP gels for primer sets 1, 2, 3, 5, 6, 8, 9, 10 (see Chapter 2, Table 2.1 for primer sequences and product sizes). A representative part of the gel is shown for each primer set in order to distribute SSCP band patterns and to highlight any differences. Subjects showing different band patterns on the SSCP gel were selected along with some subjects with the common band pattern for sequencing (see section 2.4.1). A representative electropherogram for the -765G>C variant is shown in Figure 3.9.
Figure 3.1 SSCP analysis of COX-2 promoter region -77 → +54 bp.

DNA was amplified from 24 Caucasian sarcoid patients using primer set 1 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 16.5 hours at 6W, 4°C. No sequence changes were identified in this region for these subjects.
Figure 3.2 SSCP analysis of COX-2 promoter region -193 → +8 bp.

DNA was amplified from 24 Caucasian sarcoid patients using primer set 2 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 22 hours at 7W, 4°C. A distinct band pattern was observed in subject SA4. The individual was sequenced along with subjects SA72 and SA87 showing the common band pattern. The different pattern seen in SA4 was shown to be the result of a C to a G substitution at position -62 (-62C>G).
Figure 3.3 SSCP analysis of COX-2 promoter region -323 → -60 bp.
DNA was amplified from 24 Caucasian sarcoid patients using primer set 3 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 28 hours at 7W, 4°C. There were no obvious band pattern differences in this region for these subjects.
Figure 3.4 SSCP analysis of COX-2 promoter region \(-601 \rightarrow -344\) bp.

DNA was amplified from 24 Caucasian sarcoid patients using primer set 5 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 24 hours at 7W, 4°C. A band pattern change was can be seen in subjects SA51 and SA120. Upon sequencing these subjects, a substitution of T to C was identified at position \(-368\) (-368 T>C).
Figure 3.5 SSCP analysis of COX-2 promoter region \(-677 \rightarrow -466\) bp.

DNA was amplified from 24 Caucasian sarcoid patients using primer set 6 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 23 hours at 7W, 4°C. No differences in band patterns were observed with these subjects.
Figure 3.6 SSCP analysis of COX-2 promoter region \( -883 \rightarrow -653 \) bp.

DNA from 24 Caucasian sarcoid patients was amplified by PCR using primer set 8 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single individual. Arrow point indicates double stranded DNA. The gel was run for 19 hrs at 10W, 4°C. An additional band can be seen in subjects SA25, SA35, SA21 and SA36. Sequencing analysis revealed that all the above subjects had a \(-G>C\) substitution at position \(-765\) \((-765G>C)\).
Figure 3.7 SSCP analysis of COX-2 promoter region -994 → -794 bp. DNA was amplified from 24 Caucasian sarcoid patients using primer set 9 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single individual. Arrow point indicates double stranded DNA. The gel was run for 22 hours at 7W, 4°C. There were no obvious band pattern changes with these subjects.
Figure 3.8 SSCP analysis of COX-2 promoter region −1122 → −935 bp.

DNA was amplified from 24 Caucasian sarcoid patients using primer set 10 (table 2.1). This image is a representative part of the gel showing results for 10 individuals. Each lane corresponds to a single subject. Arrow point indicates double stranded DNA. The gel was run for 20.5 hours at 7W, 4°C. No band pattern changes were identified in these subjects.
Figure 3.9. An ABI electropherogram for the -765G>C variant. Arrow head indicates the position of the variant. Panel A shows part of the sequence of an individual homozygous for the G allele (SA7). Panel B shows sequence from a subject heterozygous for the variant (SA25). Sequences were generated using primers CF8 and CR7 (Chapter 2, Table 2.1).
3.4 DISCUSSION

Investigation of the 5' flanking region of the COX-2 gene in sarcoidosis subjects led to the identification of 3 novel variants: a single nucleotide substitution G>C at position −765 (−765G>C), a C>G substitution at position −62 (−62C>G) and a T>C change at position −368 (−368T>C). Of those 3 variants, most noteworthy is the −765G>C variant located within a putative binding site for the transcription factor Sp1 between −766 to −761 bp upstream of the transcriptional start site. The location of the −765G>C variant suggests that it may have potential functional consequences. The −62C>G variant may also be of significance. Although not directly located within a transcription factor binding site the substitution occurs 2 nucleotides away from the cyclic AMP response element (CRE), an element that is essential for basal COX-2 gene expression (see Chapter 1, section 1.6.3.1) and may therefore also have functional implications.

Prior to starting this work, there were no polymorphisms reported in the COX-2 gene. The approach taken for screening was PCR-SSCP analysis. The choice of SSCP over automated sequencing also reflects the availability of equipment, as access to the ABI sequencer was limited at the time. Since starting this work there are now approximately 130 variants reported in the NCBI dbSNP database. Of those 130, approximately 40 locate in the 5' flanking region of the gene. It is however difficult to interpret and assess the relevance of these reported variants. Many of the SNPs submitted in the database are unconfirmed being identified through chip assays. Others are reported in very few subjects. Often, little information is given on the ethnic groups used to identify the SNPs or the geographical location of the subjects. In some cases, the population samples used to estimate the prevalence of variants has included a mixture of subjects of Caucasian, Hispanic, African American and Pacific Rim heritage.

The approach used in this study is focused on investigating subjects with disease, specifically sarcoidosis. All subjects used in the study were Caucasian in origin. The 5' flanking region of the COX-2 gene in healthy subjects was previously investigated in this centre as part of a study of IPF subjects.
The –765G>C appeared to be the most interesting of the SNPs given its location in a putative regulatory element of the COX-2 promoter. However, with the increased availability of published data several other polymorphisms would also be interesting to look at with respect to regulatory regions and therefore further study is warranted.

The dbSNP database does not allow to address which SNPs are likely to be functional and important within a disease context except perhaps for variants in the coding region resulting in amino acid changes. For promoter variants, inferences can be made based on location. However, the reported prevalences may be misleading as to what may exist and be relevant in disease and in different ethnic backgrounds. Investigation of the COX-2 5' flanking region in subjects of Afro-Caribbean origin was initiated during this project (data not shown) and preliminary results suggest that Afro-Caribbean subjects are considerably more polymorphic compared to Caucasians. However, dbSNP database does provide access to other SNPs and a future aspect of this work would be to genotype common variants and build a haplotype linked to disease. This haplotypes could then be used to identify subjects whose COX-2 gene would be of particular interest for further investigation.

In sarcoidosis for example, it would be highly relevant to explore haplotypes present in subjects with extreme phenotypes such as chest X ray (CXR) stage 4 with persistent disease and established fibrosis and compare them with haplotypes of subjects that present with a milder course of the disease and proceed to resolve spontaneously.

Of the 3 variants identified in this study, -765G>C was considered to be the most likely to have a functional outcome due to the nature of its location. The number of individuals with the -765G>C on the SSCP gel (8 out of the 24 subjects on the SSCP gel) suggests that the variant is common and therefore a good candidate for population-based studies. The remaining parts of this thesis concentrate on -765G>C. The chapter that follows (Chapter 4) investigates -765G>C in healthy subjects and subjects groups of sarcoidosis and IPF. Chapter 5 explores the functional implications of the variant while the final results chapter (Chapter 6) aims to demonstrate a phenotypic consequence for -765G>C in an inflammatory state.
CHAPTER IV

THE \(-765G>C\) VARIANT ASSOCIATES WITH INCREASED RISK OF SARCOIDOSIS AND INFLUENCES SEVERITY OF DISEASE
4.1 INTRODUCTION

Mutation detection studies (Chapter 3) examining the COX-2 promoter gave the first indication that the -765G>C variant was common, with 8 out of the 24 unrelated Caucasian individuals used in the SSCP analysis possessing at least one copy of the C allele. The substitution of a G for a C at position -765 disrupts a putative Sp1 site suggesting that this variant may also have functional consequences.

This section explores the investigation of the -765G>C variant in IPF and sarcoidosis. UK and Mexican IPF groups were available for the study along with ethnically matched controls. A UK sarcoidosis group was also investigated. Approximately, 5-10% of sarcoidosis subjects deteriorate and develop a persistent disease with an endstage of pulmonary fibrosis (see Chapter 1, Table 1.3). As the focus of this thesis is the role of COX-2 in the fibrotic process, the sarcoidosis sample was classified into subjects with non-persistent disease and subjects with fibrosis or persistent disease likely to lead to fibrosis. The variant was then tested for association with sarcoid disease overall and with the subset of patients that develop progressive disease with an endpoint of fibrosis.

A Mexican group of patients with pigeon breeder’s disease (PBD) was also available as a comparator group for these studies. PBD belongs to a group of granulomatous inflammatory diseases, termed hypersensitivity pneumonitis, occurring as a result of a hypersensitivity reaction in response to exposure to fine organic dust or low molecular weight chemicals (Selman M 1998). In the case of PBD, the cause is exposure to avian antigen. The clinical course of hypersensitivity pneumonitis is varied and can result in resolution, stabilisation, or progression to fibrosis. Approximately 30% of patients with hypersensitivity pneumonitis evolve into a diffuse fibrotic disorder that is usually lethal (Perez-Padilla R et al 1993).
4.2 METHODS

4.2.1 Subjects

A total of 83 UK Caucasian IPF and 200 Caucasian sarcoidosis subjects drawn from the London area were the primary focus of this study. Population controls (n=454) were obtained from the Northwick Park Hospital Study II, a large prospective study of heart disease in men (Humphries SE et al 2001). A smaller group of UK Afro-Caribbean sarcoidosis subjects (n=102) was also considered, however, the availability of data on their ethnically matched controls (n=110) was limited. Both patients and controls were drawn from the same geographical area, (London, UK). All Mexican subjects were Mexican mestizo with at least two generations born in Mexico. There were 107 subjects available with PBD and 92 subjects with IPF. The healthy subjects (n=313) were sequential unrelated blood donors from the Transfusion Department of the National Institute of Respiratory Diseases, Mexico.

The protocol was approved by the Joint University College London and University College London Hospitals Committees on the Ethics of Human Research in the UK and the Ethical Committee of the National Institute of Respiratory Diseases in Mexico. Written informed consent was obtained from each subject.

Diagnosis of IPF was supported by clinical, radiological, CT scans and functional findings. All patients had to fulfill the criteria of the ATS/ERS international consensus (American Thoracic Society 2000). In the Mexican group, diagnosis of IPF was corroborated by open lung biopsy in 43 out of 92 patients. None of the UK IPF subjects had biopsy-proven diagnosis.

Diagnosis of sarcoidosis was based on clinical, radiological and functional features of sarcoidosis. Approximately 5-10% of subjects with sarcoidosis deteriorate and develop pulmonary fibrosis. The sarcoidosis subjects were classified as having persistent disease if they had fibrosis or a history suggestive of progressive disease likely to lead to fibrosis (subjects with chest X-ray stage 4 or subjects having all of the following: a history of persistent pulmonary disease for ≥ 2 years, restrictive lung function with FVC and/or TLC and/or DLCO<80% predicted, receiving
corticosteroids for pulmonary disease). Those subjects not classified with persistent disease for ≥ 2 years from diagnosis were classified as having non-persistent disease, while the remaining subjects < 2 years from diagnosis wait to be classified.

The diagnosis criteria used for PBD included 1) pigeon exposure preceding disease and positive serum antibodies against avian antigens; 2) shortness of breath with partial improvement upon avoidance of the avian antigen exposure; 3) clinical and functional features of an interstitial lung disease; 4) HRCT opacities characterised by hazy increased parenchymal density, bilateral poorly defined centrilobular micronodules, and in more chronic cases, fine and coarse reticular opacities; 5) more than 40% lymphocytes in BAL fluid. PBD patients were followed up for at least 1 year, and after this time their condition was classified stable or persistent. Where possible, diagnosis was corroborated by histological evaluation of a surgical lung biopsy.

4.2.2 Genotyping
Restriction fragment length polymorphism (RFLP) analysis was carried out in order to determine the frequency of the −765G>C variant in healthy subjects. Restriction map analysis (Remap, EMBOSS suite of molecular biology programs) of the COX-2 promoter sequence revealed that the substitution of the G allele for a C, at position −765 results in the loss of a cut site for the restriction enzyme AcI I. A simple restriction enzyme digest was thus performed to distinguish between individuals that were homozygous for the G allele (GG), heterozygous (GC) or homozygous for the variant (CC).

Samples were amplified by PCR using primers CF8 and CR7 (Chapter 2, Table 2.1). This combination of primers amplifies a region of 305 bp. Digestion of this product with AcI I (Chapter 2, section 2.5.1) yields two fragments of 117 and 188 bp that are easily resolved on a 6% MADGE polyacrylamide gel run for 45 min (Chapter 2, section 2.5.2). The GG genotype is represented by the presence of a 188bp and a 117bp band, the GC heterozygote is represented by the presence of 3 bands (305, 188 and 117bp) and the CC homozygote is represented by single uncut 305bp band.
**4.2.3 Statistical analysis**

Allele frequencies were estimated by gene counting. Statistical analysis was performed using SPSSv11.5 (http://www.SPSS.com/). Chi-square tables were used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium and to compare genotype frequencies between the patient populations and the control groups. OR and 95% CI were derived from binary logistic regression analysis. For all tests, a $P \leq 0.05$ was taken as significant.

Power calculations using the StatCalc program of the EpiInfo (version 6) software predict that the lowest ratio we would expect to pick up with 80% power at the 5% significance level in the UK Caucasian sarcoid ($n=200$) and IPF ($n=83$) subjects is 1.72 and 2.12 respectively using 400 control subjects.
4.3 RESULTS

4.3.1 Genotyping
A representative MADGE gel image of an *Aci* I digest is shown in Figure 4.1. Samples were genotyped blind to phenotype data and checked independently.

4.3.2 Frequency and genotype distribution of \(-765G>C\) in Caucasians
The genotype distribution and rare allele frequency of the \(-765G>C\) variant in all Caucasian subject groups are shown in Table 4.1. All genotypes were checked independently for accuracy and the \(-765G>C\) variant is in Hardy-Weinberg equilibrium in all three subject groups (Table 4.1) There was no significant difference in the allele and genotypic frequencies between the IPF and healthy subjects \((P=0.84\) and \(P=0.35\), respectively). However the rare allele frequency in sarcoidosis subjects compared with the healthy subjects is significantly increased \((P=0.012)\) as is the genotype distribution overall between these two groups \((P=0.006)\).

The healthy subjects are not well-matched for age and gender with the IPF and sarcoidosis subjects and there is significant evidence of an interaction between age and genotype on risk when comparing the sarcoidosis and healthy subjects \((P=0.003)\). The age distribution does not however differ between the GG, GC and CC genotypes \((P=0.55)\). After adjusting for age, carriage of at least one rare allele of the \(-765G>C\) variant showed a significant risk for sarcoidosis subjects \((\text{OR}=1.88 \ [95\%\text{CI} \ 1.26-2.82], \ P=0.002)\) but not IPF subjects \((\text{OR}=1.06 \ [95\%\text{CI} \ 0.62-1.81], \ P=0.82)\) (Table 4.2). The risk in sarcoidosis is contributed mainly by subjects homozygous for the rare \(-765C\) allele \((\text{OR}=5.75 \ [95\%\text{CI} \ 1.80-18.42], \ P=0.003)\) (Table 4.2). Splitting the data by the median age of the data set (56 years) indicates a stronger effect of genotype on risk in older subjects (for carriage of at least one rare allele: subjects <56 years, \(\text{OR} =1.14 \ [95\%\text{CI} \ 0.70-1.87], \ P=0.60\); subjects \(\geq\)56 years, \(\text{OR}=2.79 \ [95\%\text{CI} \ 1.56-4.98], \ P=0.001)\). There is no difference in genotype frequencies between the genders in the sarcoidosis subjects (118 females, 82 males) to confound the relationship between genotype and disease \((P=0.456)\). However it cannot be excluded that gender may modify the relationship between genotype and disease.
When the sarcoidosis subjects are sub-grouped into patients with persistent and non-persistent disease, the allele and genotype frequencies of the non-persistent disease subjects are very similar to the frequencies found in the healthy subjects (Table 4.3). However both the allele and genotype frequencies of the persistent disease subjects are highly significantly different from the healthy subjects ($P=0.0027$ and $P=0.00037$, respectively). Comparing the persistent disease subjects with the healthy subjects, the OR for carriage of at least one rare allele of the $-765G>C$ variant in persistent sarcoidosis is 2.28 (95%CI 1.30-4.00), $P=0.004$) (Table 4.4). The effect of $-765G>C$ genotype on risk of sarcoidosis has been shown above to be contributed mainly by subjects homozygous for the rare $-765C$ allele. Subjects with persistent disease homozygous for the rare $-765C$ allele have an OR of 10.19 (95%CI 2.83-36.65), $P<0.005$) (Table 4.4). Within the sarcoidosis subjects the allele frequencies between persistent and non-persistent disease shows a trend consistent with the $-765C$ being increased in persistent disease ($P=0.09$), however sub-dividing the sarcoidosis group reduces the number of subjects and the ability to detect an effect of $-765G>C$ genotype on risk of poorer outcome within sarcoidosis (Table 4.4).
Figure 4.1 MADGE gel of an AcI I digest

Figure showing a representative MADGE gel of an AcI I digest. Molecular weight markers are marked M. Three wells are labelled to show mobility patterns of the GG, GC and CC genotypes resulting from AcI I digest of PCR product derived from primers CF8 and CR7.
TABLE 4.1. GENOTYPE DISTRIBUTION AND RARE ALLELE FREQUENCY (%) OF \(-765G>C\) IN CAUCASIAN HEALTHY SUBJECTS, SARCOIDOSIS AND IPF PATIENTS.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Rare allele frequency (95% CI)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>GC</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>454</td>
<td>0.13 (0.11-0.16)</td>
<td></td>
<td>339 (74.7%)</td>
<td>109 (24.0%)</td>
</tr>
<tr>
<td>IPF</td>
<td>83</td>
<td>0.15 (0.10-0.21)</td>
<td>0.84</td>
<td>61 (73.5%)</td>
<td>19 (22.9%)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>200</td>
<td>0.20 (0.16-0.24)</td>
<td>0.012</td>
<td>130 (65.0%)</td>
<td>61 (30.5%)</td>
</tr>
</tbody>
</table>

All genotypes were checked independently for accuracy. The \(-765G>C\) variant is in Hardy-Weinberg equilibrium in all three subject groups (χ² values were for healthy subjects 0.70 \((P=0.40)\), IPF 0.92 \((P=0.34)\) and sarcoidosis 0.29 \((P=0.59)\). *Allele and †genotype frequencies compared by χ² test with healthy subjects.
### TABLE 4.2. -765G>C AND RISK OF IPF AND SARCOIDOSIS IN CAUCASIANS.

<table>
<thead>
<tr>
<th>Category</th>
<th>Homozygote</th>
<th>Heterozygote</th>
<th>Carriage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P value*</td>
</tr>
<tr>
<td>IPF (n=83)</td>
<td>2.78</td>
<td>0.68-11.41</td>
<td>0.14</td>
</tr>
<tr>
<td>Sarcoidosis (n=200)</td>
<td>5.75</td>
<td>1.80-18.42</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* Adjusted for age

### TABLE 4.3. GENOTYPE DISTRIBUTION AND RARE ALLELE FREQUENCY (%) OF THE -765G>C VARIANT IN CAUCASIAN SARCOIDOSIS PATIENTS WITH PERSISTENT AND NON-PERSISTENT DISEASE.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Rare allele frequency (95% CI)</th>
<th>P value*</th>
<th>Genotype (%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>454</td>
<td>0.13 (0.11-0.16)</td>
<td>0.73</td>
<td>339 (74.7%)</td>
<td>0.52</td>
</tr>
<tr>
<td>Sarcoidosis (Non-persistent)</td>
<td>107</td>
<td>0.15 (0.11-0.20)</td>
<td>0.73</td>
<td>77 (72.0%)</td>
<td>0.52</td>
</tr>
<tr>
<td>Sarcoidosis (persistent)</td>
<td>62</td>
<td>0.25 (0.17-0.33)</td>
<td>0.0027</td>
<td>36 (58.1%)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

A total of 31 sarcoidosis patients remain unclassified. *Allele and †genotype frequencies compared by \(\chi^2\) test with healthy subjects.
TABLE 4.4. -765G>C AND RISK IN SARCOIDOSIS.

<table>
<thead>
<tr>
<th></th>
<th>Homozygote</th>
<th>Heterozygote</th>
<th>Carriage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR 95% CI</td>
<td>P value*</td>
<td>OR 95% CI</td>
</tr>
<tr>
<td>Sarcoidosis (persistent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs non Persistent</td>
<td>2.65 0.57-12.32 0.22</td>
<td>1.35 0.65-2.80 0.43</td>
<td>1.48 0.74-3.00 0.27</td>
</tr>
<tr>
<td>Sarcoidosis (persistent)</td>
<td>10.19 2.83-36.65 &lt;0.001</td>
<td>1.93 1.07-3.48 0.030</td>
<td>2.28 1.30-4.00 0.004</td>
</tr>
<tr>
<td>vs healthy subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Adjusted for age
4.3.3 Frequency and genotype distribution of -765G>C in Afro-Caribbean subjects

The genotype distribution and rare allele frequency of the -765G>C variant in the Afro-Caribbean subjects are shown in Table 4.5. All genotypes were checked independently for accuracy and the -765G>C variant is in Hardy-Weinberg equilibrium in both subject groups (Table 4.5). There was no significant difference in the allele and genotypic frequencies between sarcoidosis and healthy subjects (P=0.17 and P=0.129 respectively).

Unlike the Caucasian sarcoidosis subjects, carriage of at least one rare allele of the -765G>C variant appears protective in the Afro-Caribbean sarcoidosis subjects (OR=0.57 [95%CI 0.33-0.99], P=0.046) (Table 4.6). There is no difference in genotype frequencies between the genders in the sarcoidosis subjects (76 females, 26 males) to confound the relationship between genotype and disease (P=0.737). Data information on age and gender is not currently available for the control subjects and as such it cannot be excluded that gender may modify the relationship between genotype and disease. Furthermore, an interaction between age and genotype cannot be examined. The interpretation of results of the association study in Afro-Caribbeans is therefore treated cautiously.

When the sarcoidosis subjects are sub-grouped into patients with persistent and non-persistent disease, the allele and genotype frequencies of the non-persistent disease subjects are not significantly different from the frequencies found in the healthy subjects (Table 4.7). However both the allele and genotype frequencies of the persistent disease subjects are significantly different from the healthy subjects (P=0.044 and P=0.028, respectively). Comparing the persistent disease subjects with the healthy subjects, the OR for carriage of at least one rare allele of the -765G>C variant in persistent sarcoidosis is 0.40 (95%CI 0.18-0.92), P=0.028) (Table 4.8)
<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>(95% CI)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>-765G&gt;C</td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>110</td>
<td>0.39 (0.33-0.46)</td>
<td></td>
<td>38 (34.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td>58 (52.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>14 (12.7%)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>102</td>
<td>0.30 (0.24-0.37)</td>
<td>0.17</td>
<td>49 (48.0%)</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44 (43.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 (8.8%)</td>
<td></td>
</tr>
</tbody>
</table>

All genotypes were checked independently for accuracy. The -765G>C variant is in Hardy-Weinberg equilibrium in both subject groups ($\chi^2$ values were for healthy subjects 1.27 ($P=0.26$) and sarcoidosis 0.04 ($P=0.84$). *Allele and †genotype frequencies compared by $\chi^2$ test with healthy subjects.
### Table 4.6. -765G>C and Risk of Sarcoidosis in Afro-Caribbeans.

<table>
<thead>
<tr>
<th></th>
<th>Homozygote</th>
<th></th>
<th>Heterozygote</th>
<th></th>
<th>Carriage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P value</td>
<td>OR</td>
<td>95% CI</td>
<td>P value</td>
</tr>
<tr>
<td>Sarcoidosis (n=102)</td>
<td>0.50</td>
<td>0.20-18.42</td>
<td>1.27</td>
<td>0.59</td>
<td>0.33-1.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### Table 4.7. Genotype Distribution and Rare Allele Frequency (%) of the -765G>C Variant in Afro-Caribbean Sarcoidosis Patients with Persistent and Non-Persistent Disease.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Rare allele frequency (95% CI)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>GC</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>110</td>
<td>0.35 (0.33-0.46)</td>
<td></td>
<td>38 (34.5%)</td>
<td>58 (52.7%)</td>
</tr>
<tr>
<td>Sarcoidosis (Non-persistent)</td>
<td>49</td>
<td>0.35 (0.25-0.44)</td>
<td>0.76</td>
<td>22 (44.9%)</td>
<td>20 (40.8%)</td>
</tr>
<tr>
<td>Sarcoidosis (persistent)</td>
<td>30</td>
<td>0.22 (0.11-0.32)</td>
<td><strong>0.044</strong></td>
<td>17 (56.7%)</td>
<td>13 (43.3%)</td>
</tr>
</tbody>
</table>

* A total of 23 sarcoidosis patients remain unclassified. † Allele and genotype frequencies compared by χ² test with healthy subjects.
<table>
<thead>
<tr>
<th></th>
<th>Homozygote</th>
<th>Heterozygote</th>
<th>Carriage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR  95% CI</td>
<td>$P$ value</td>
<td>OR  95% CI</td>
</tr>
<tr>
<td>Sarcoidosis (persistent) vs healthy subjects</td>
<td>0.50 0.22-1.15 0.10</td>
<td></td>
<td>0.40 0.18-0.92 0.028</td>
</tr>
</tbody>
</table>
4.3.4 Frequency and genotype distribution of $-765G>C$ in Mexicans

The genotype distribution and rare allele frequency of the $-765G>C$ variant in all Mexican subject groups are shown in Table 4.9. All genotypes were checked independently for accuracy and the $-765G>C$ variant is in Hardy-Weinberg equilibrium in all three subject groups (Table 4.9). There was no significant difference in the allele and genotypic frequencies between the IPF group and the healthy subjects ($P=0.28$ and $P=0.25$, respectively). Similarly, there were no significant differences in allele or genotypic frequencies between the PBD group and the healthy subjects ($P=0.97$ and $P=0.97$, respectively).

In an attempt to draw a parallel between PBD and sarcoidosis, the PBD sample was sub-classified into subjects that were stable and subjects with a poorer outcome of the disease. No significant differences in allele frequency or genotype distribution overall were observed when each patient subgroup was compared with the healthy subjects (Table 4.10; $P=0.99$ and $P=0.96$ for stable PBD patients; $P=0.87$ and $P=0.63$ for patients with poorer outcome).
TABLE 4.9. GENOTYPE DISTRIBUTION AND RARE ALLELE FREQUENCY (%) OF -765G>C IN MEXICAN HEALTHY SUBJECTS, IPF AND PBD PATIENTS.

<table>
<thead>
<tr>
<th>Rare allele frequency</th>
<th>Category</th>
<th>n</th>
<th>(95% CI)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-765G&gt;C</td>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Healthy subjects</td>
<td>313</td>
<td>0.17 (0.14-0.20)</td>
<td>0.17</td>
<td>216 (69.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPF</td>
<td>92</td>
<td>0.22 (0.16-0.28)</td>
<td>0.28</td>
<td>55 (59.8%)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>PBD</td>
<td>107</td>
<td>0.16 (0.11-0.21)</td>
<td>0.97</td>
<td>75 (70.1%)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

All genotypes were checked independently for accuracy. The -765G>C variant is in Hardy-Weinberg equilibrium in all three subject groups ($\chi^2$ values were for healthy subjects 0.12 ($P=0.73$), IPF 0.12 ($P=0.73$) and PBD 0.01 ($P=0.92$). *Allele and †genotype frequencies compared by $\chi^2$ test with healthy subjects.
TABLE 4.10. GENOTYPE DISTRIBUTION AND RARE ALLELE FREQUENCY (%) OF THE -765G>C VARIANT IN MEXICAN PBD PATIENTS: STABLE vs POORER OUTCOME.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Rare allele frequency (95% Cl)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>GC</td>
<td>CC</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>313</td>
<td>0.17 (0.14-0.20)</td>
<td></td>
<td>216 (69.0%)</td>
<td>87 (27.8%)</td>
</tr>
<tr>
<td>PBD stable</td>
<td>42</td>
<td>0.17 (0.09-0.25)</td>
<td>0.99</td>
<td>29 (69.0%)</td>
<td>12 (28.6%)</td>
</tr>
<tr>
<td>PBD poorer outcome</td>
<td>28</td>
<td>0.14 (0.05-0.23)</td>
<td>0.87</td>
<td>20 (71.4%)</td>
<td>8 (28.6%)</td>
</tr>
</tbody>
</table>

*Outcome data available for 70 of the 107 genotyped subjects. *Allele and †genotype frequencies compared by χ² test with healthy subjects.
4.4 DISCUSSION

This part of the study investigates the \(-765G>C\) variant in IPF and sarcoidosis. The \(-765G>C\) variant associates with an increased risk of susceptibility to sarcoidosis in UK Caucasians and poorer outcome of the disease in this group. It does not however appear to contribute to susceptibility to IPF.

This is the first study implicating COX-2 variants in pulmonary fibrosis. The data lend support to hypothesis that functional COX-2 genotypes have a role to play in fibrotic lung disease. A major strength of this study was the availability of various sample groups through collaboration. Importantly, all collaborating centres used the same clinical criteria for phenotyping their samples. The UK samples of sarcoidosis and IPF formed the basis of the investigations. The non-UK samples were equally important to either replicate the UK results or use as a comparator group.

An issue surrounding the study however, is the small number of samples available for study. Although our numbers of subjects exceed many studies reported in the literature, they are still low. The frequency of IPF and sarcoidosis is rare compared to a respiratory disease such as asthma, and to recruit samples from the same geographical area and ensure they are separated by ethnicity resulted in the numbers that we have.

Sarcoidosis is a granulomatous disorder of unknown cause that can affect many organs but pulmonary involvement is seen in the vast majority of cases. Although the exact point when sarcoidosis starts is usually unclear, the initial event is thought to give rise to granuloma formation and the evolution of the response can either lead to resolution or chronic disease. Sarcoidosis is a complex disease and its genetic predisposition is likely to be determined by modifying effects of several genes. This study provides evidence that COX-2 is an important component of the sarcoid disease process. The \(-765G>C\) variant associates with sarcoidosis in our UK Caucasian population samples and this effect is attributable to a significant increase in homozygotes within the sarcoidosis sample. When the sarcoidosis subjects were classified into patients with persistent and non-persistent disease, carriage of the rare \(-765C\) allele associates with greater risk of poorer outcome in sarcoidosis suggesting
that variation in the COX-2 gene can influence the severity of the disease. The results are suggestive of a modifier rather than a causal effect of -765G>C in sarcoidosis. As there is currently no means of identifying sarcoid individuals that are pre-disposed to developing fibrosis, -765G>C may have important implications for the early detection of subjects at greater risk of poorer outcome. However, contrary to the result observed in the Caucasian sarcoidosis sample, the rare -765C allele appears protective in Afro-Caribbean sarcoids. The fact that the rare allele frequency of -765C in healthy Afro-Caribbean subjects (0.39) is considerably higher than that observed in the Caucasian healthy subjects (0.13) is the first indication that the two ethnic groups are very different with respect to -765G>C. However, the number of samples available in the UK Afro-Caribbean investigation is small. It is therefore possible that the apparent protective effect of the -765C in our Afro-Caribbean sample is misleading and due to chance. A more plausible explanation is that the -765G>C variant is in fact not the polymorphism that is causing the association with sarcoidosis, but that the -765G>C variant is in linkage disequilibrium with another as yet unidentified variant. The frequency of the unidentified variant may be disproportionate between the two ethnic groups, thus giving the apparent result that the -765C allele confers susceptibility in Caucasians and it protective in Afro-Caribbeans.

The effect of -765G>C may therefore be confounded by the presence of other variants in the COX-2 gene that are unique to Afro-Caribbean subjects. In view of that, it is worth noting that the impaired expression of COX-2 in pulmonary fibrosis has to date only been shown in Caucasians and therefore the possibility of a different situation in other ethnic groups cannot be excluded. In support of this notion is the well-documented variability in the prevalence and severity of sarcoidosis in individuals of different races. In spite of the limitations of such studies, the current available data point to an important role for COX-2 in sarcoidosis. Work conducted in parallel in this laboratory by Ms Giulia Chan showed an effect with another COX-2 promoter variant, -607T>C, that is only present in Afro-Caribbean subjects.
The genotype distribution of −607T>C in UK Afro-Caribbean subjects is shown below.

<table>
<thead>
<tr>
<th>Genotype distribution (%)</th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afro-Caribbean healthy subjects (n=168)</td>
<td>153 (91.1)</td>
<td>15 (8.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afro-Caribbean sarcoidosis (n=113)</td>
<td>91 (80.5)</td>
<td>21 (18.6)</td>
<td>1 (0.9)</td>
<td>0.026</td>
</tr>
</tbody>
</table>

The rare −607C allele is significantly increased in sarcoidosis and causes a significant difference in genotype distribution overall between sarcoidosis and healthy subjects. The results of the −765G>C and −607T>C variants in Caucasians and Afro-Caribbeans collectively support the notion that COX-2 is involved in genetic susceptibility to sarcoidosis. Together, these results provide the first evidence that genetic variation in the COX-2 gene contributes to susceptibility to sarcoidosis.

PBD shares similar features with sarcoidosis. Both are highly inflammatory granulomatous diseases with only a proportion of patients developing persistent fibrotic disease (Perez-Padilla R et al 1993). The aetiology of interstitial lung diseases is often unknown and it is therefore useful to use different disease groups in genetic studies as comparator groups. This study shows an association of −765G>C with sarcoidosis in UK Caucasians and furthermore, this association appears to be mostly explained by the patients with persistent disease (Table 4.3). It is therefore interesting to see in other interstitial lung diseases, in which a persistent form of a disease is found, whether a similar association of −765G>C may be observed. For this reason, the variant was also investigated in PBD in an attempt to draw comparisons between the two groups. Given the similarities in the varied presentation of the two diseases, it was postulated that there may be shared pathways between sarcoidosis and PBD that when defective, lead to fibrosis in a proportion of susceptible individuals.

Investigation of the −765G>C variant in the PBD sample showed no differences in allele or genotypic frequency between PBD and the healthy subjects. The results obtained for PBD did not mirror the sarcoidosis data as may have been expected. There may be a number of reasons for this discrepancy. One possibility is that the pathways leading to fibrosis in PBD patients are different from the ones contributing
to the fibrotic component in sarcoidosis. PBD has a known established cause whereas sarcoidosis is a disease of unknown aetiology and so in spite of the similarities in their clinical course, it is conceivable that distinct pathways, specific for each disease, are involved and this may confound the effect of \(-765G>C\) being seen in the PBD subjects. Moreover, the proportion of subjects with hypersensitivity pneumonitis that progress to the fibrotic stage is considerably greater than that of sarcoid subjects presenting with persistent disease (30% and 5-10% respectively), a fact that in itself may imply mechanistic differences in the passage to fibrosis in the two diseases.

Furthermore, the PBD group are made up of Mexican mestizo subjects that are a mix of Spanish and Indian blood. Although the Mexican controls are as well matched to the patients as possible, it remains possible that these subjects have more genetic heterogeneity than the UK Caucasians which may also confound an effect of \(-765G>C\) being seen.

As the original observation that COX-2 expression is impaired in pulmonary fibrosis was shown in fibroblasts taken from IPF patients (Wilborn J et al 1995, Keerthisingam CB et al 2001), the \(-765G>C\) variant was also investigated in an IPF sample. IPF is characterised by progressive fibrosis resulting in a 50% survival of patients within 3 years from diagnosis (Bjoraker JA et al 1998). There was no difference in allele and genotypic frequency for the \(-765G>C\) variant between the IPF group and the healthy subjects. There was also no risk associated with IPF for subjects homozygote or heterozygote for the rare 765G>C allele compared to the homozygote common \(-765G\) allele. Likewise, carriage of the rare allele \(-765G\) allele does not associate with risk of IPF. The results in the IPF group suggest that \(-765G>C\) does not contribute to susceptibility in IPF. If this is true, it raises questions as to why the variant does not appear to have an influence in IPF where fibrosis is a more prominent feature. One plausible explanation is that there is more heterogeneity in the factors leading to fibrosis in IPF compared to the factors promoting fibrosis in sarcoidosis and that these additional effects dilute the influence the COX-2 variant in IPF. Alternatively, the IPF sample may be too heterogeneous for an effect of the COX-2 genotype to be detected. There is currently a lot of debate between clinicians as to what should be considered IPF (section 1.1.1). As diagnosis of IPF in the UK cohort was not biopsy-proven, it is possible that our sample may include subjects that are not
Addendum. Clinical demographics for UK Caucasian sarcoidosis patients

<table>
<thead>
<tr>
<th></th>
<th>Persistent (n=67)</th>
<th>Non-Persistent (n=111)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>37/32</td>
<td>74/45</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>54.2 (14.7)</td>
<td>45.4 (12.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC % predicted</td>
<td>80.5 (27.8)</td>
<td>95.1 (19.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>FEV₁ % predicted</td>
<td>73.2 (26.5)</td>
<td>92.4 (21.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DLCO % predicted</td>
<td>68.0 (16.6)</td>
<td>88.1 (12.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KCO % predicted</td>
<td>80.6 (20.2)</td>
<td>92.4 (16.8)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

FVC indicates forced vital capacity, FEV₁ forced expiratory volume in 1 second, DLCO diffusing capacity of the lung for carbon monoxide, KCO carbon monoxide transfer coefficient.

*Remaining unclassified patients (n = 22) either do not have lung function data or are less than 2 years from diagnosis.
truly representative of IPF. For example, inclusion of subjects with NSIP may dilute any effect of the variant, as subjects with this histological pattern are known to have a better prognosis compared with the UIP pattern that is characteristic of IPF. However, the lack of association of the COX-2 variant in the UK IPF sample was also observed in the Mexican IPF group, where 43 out of 92 patients had biopsy-proven diagnosis, suggesting that −765G>C is unlikely to be of importance in the development IPF.

In summary, the −765G>C variant associates with sarcoidosis and increases risk of poorer outcome of the disease. The variant does not appear to influence susceptibility to IPF. The results with the sarcoidosis sample may have important implications for the early detection of subjects at greater risk of poorer outcome. Identification of the subset of sarcoid patients that are predisposed to developing fibrosis may in the future lead to more timely effective management of these subjects.
CHAPTER V

THE −765G>C VARIANT REPRESSES COX-2
GENE EXPRESSION AND ALTERS
TRANSCRIPTION FACTOR BINDING
5.1 INTRODUCTION

The association of the -765C allele of -765G>C with susceptibility and poorer outcome in sarcoidosis (Chapter 4) raises the question of what is the functional cause of this association. At this stage, it is not possible to know if the variant is functional. It is still possible that -765G>C may only act as a marker for functional polymorphisms located in other parts of the gene, or contribute to a larger functional haplotype. The work presented in this chapter addresses whether the -765G>C variant has direct functional consequences. At present, no functional variants have been reported in the promoter of the COX-2 gene.

Of interest is the location of the -765G>C variant, as it lies within a putative Sp1-binding motif. None of the sequence variants identified in the mutation detection screen in Chapter 3 located within putative response elements. To date, only one study has implicated Sp1 in the regulation of COX-2 gene expression. Xu Q et al (2000) demonstrated binding of Sp1 to the proximal Sp1 site in the human promoter and showed that this site is involved in the induction of COX-2 in hypoxic vascular endothelium. The site where the -765G>C variant locates has not been investigated to date.

The present chapter examines the functional effects of the -765G>C variant and has the following specific aims:

• to generate constructs of the COX-2 promoter region suitable for testing the effect of the -765G and -765C alleles on COX-2 promoter activity in in vitro assays in human lung fibroblasts.
• examine the mechanism by which the −765C allele may alter COX-2 promoter activity by investigating nuclear protein binding to the -765 region in the presence of the −765C allele.

• investigate the effect of −765G>C genotypes in the endogenous gene in normal human lung fibroblast cell lines previously characterised in terms of their PGE$_2$ response to TGF-β.
5.2 METHODS

*Preparation of 1.9kb COX-2 promoter constructs, -765G and -765C*

COX-2 promoter constructs for use in reporter gene assays were generated from the clone 973JM2 from the library RPC15 (kindly supplied by the Sanger Centre Clone Resource Group, Cambridge, UK). Previously, PCR amplification of genomic DNA samples failed to amplify the region and therefore the approach to generate the constructs from clone sequence was adopted (Chapter 2, section 2.8.3). In brief, clone 973JM2 was cultured and purified from the cultures (Chapter 2, section 2.8.1 and 2.8.2). The purified plasmid was checked by using primer set 1 to generate a PCR product. After confirming the presence of COX-2 sequence, primers CF11 and CR11 were used in a PCR reaction with the clone. Where they had previously failed on the genomic DNA, it was hoped that the clone would offer a ‘cleaner’ template. Figure 5.1 shows the initial approach taken to generate 1.9kb promoter constructs containing either a G base at position -765 upstream of the transcription start site (-765G clone) or a C base (-765C clone).

Problems were encountered at various stages, which resulted in modifications and adaptations of the initial strategy. This section describes in detail the various steps outlined in Figure 5.1 and explains how the approach was adapted to overcome the problem at each point of difficulty.

At step 1 (Figure 5.1), primers CF11 and CR11 with tagged ends containing *Nhe* I and *Hind* III sites respectively (see Table 2.8 for sequences) were used to PCR amplify the 1.9kb insert from the 973JM2 clone, but as for the genomic DNA the PCR failed. The problem was thought to lie at the 5’end as the 3’ end primer worked when used in combination with other primers. However, repositioning of primers eventually led to a PCR product (Chapter 2, section 2.8.3). The 5’ primer was moved out and designed without a tagged end in case this exasperated the problem. PCR was therefore performed using primers CF12 and CR11 (see Chapter 2, Table 2.9 for sequences) and a PCR product of extended size (2kb) was successfully generated (Figure 5.2, see Chapter 2, Table 2.9 for sequences).
1. PCR amplify 1.9kb fragment using primers with tagged ends.

2. Clip ends of PCR product and ligate into pGL3basic vector previously digested with Nhe I and Hind III.

3. Sequence insert, confirm with consensus database sequence (derived from sequences U44805, AF044206, U04636, D28235, U20548, L34209).

4. Use this plasmid in PCR-based site-directed mutagenesis reaction to introduce a C base at position –765 of the COX-2 promoter.

5. Dpn I digestion to remove methylated E.coli derived plasmid DNA.

6. Cut out insert.

7. Re-insert into original backbone of pGL3basic vector.

Figure 5.1. Schematic flow diagram of the initial strategy for the generation of the –765G and –765C constructs.
This PCR product was then diluted 1:100 and 2μl was used as template for a second round of PCR with the original primer CF11 that had failed to amplify from either genomic DNA samples and the 973JM2 clone (see section 2.8.3 for detail). This was important as the CF11 primer has a tagged end containing an Nhe I cut site which allows the product to be inserted into the pGL3 basic vector. This reaction successfully amplified the desired 1.9kb region (Figure 5.3a, lanes 2-10). Multiple PCR reactions were pooled and loaded onto 2 QIAquick columns for purification (Chapter 2, section 2.4.2). The purified 1.9kb fragment is shown in Figure 5.3b, lanes 2 and 3. After successfully generating a 1.9kb fragment of the COX-2 promoter it was possible to proceed to step 2 of the initial strategy (Figure 5.1).

The pGL3basic vector was digested with Nhe I and Hind III (Chapter 2, section 2.8.4) and the linearised product purified using the QIAGEN gel extraction kit as described in section 2.8.12 (Figure 5.4, lanes 3 and 4). Uncut vector was also loaded on the gel to control for the digests (Figure 5.4, lane 2). The purified 1.9kb COX-2 fragment was also digested with Nhe I and Hind III and purified as described in sections 2.8.4 and 2.8.12 (Figure 5.5). The purified digested vector was then dephosphorylated (Chapter 2, section 2.8.5) by treatment with Calf Intestinal Alkaline Phosphatase to prevent the ends from re-ligating and the product was purified using the QIAGEN gel extraction kit as described in chapter 2, section 2.8.12 (Figure 5.5).

The pGL3basic vector and the 1.9kb insert were then ligated (step 2, Figure 5.1) as described in section 2.8.6 and the product of the ligation reaction transformed into Epicurian Coli XL-1 Blue supercompetent cells (section 2.8.7). The transformation reaction was plated onto agar plates and 12 clones were isolated which were then used as templates in PCR (section 2.8.8) with primer set 9 [see Table 2.1 for primer sequences] (Figure 5.6, lanes 2-13). A clean product of the expected size was obtained from each clone, confirming the presence of the COX-2 promoter fragment.

Initially 10 clones were selected to be sequenced, to confirm that the sequence of the COX-2 promoter fragment agreed with the consensus sequence for COX-2. Sequencing (Chapter 2, section 2.8.9) revealed that none of the clones matched the published consensus sequence for COX-2. However, clones 2, 5 and 6 contained
large segments of consensus sequence that could be used to generate the desired constructs through a combination of site-directed mutagenesis, restriction enzyme digestion and re-ligation (Chapter 2, sections 2.8.10, 2.8.11, 2.8.12 and 2.8.13). Figure 5.7 shows the errors in sequence for clones 2, 5 and 6 the enzyme digestion strategy used to generate the final COX-2 promoter constructs used in the functional studies.

Site-directed mutagenesis (section 2.8.10) was performed on clone 6 using primer set 14 (see Table 2.10 for sequence) in order to introduce a C base at position -765 (Figure 5.8). Restriction enzyme digestion of clones 2, 5 and the -765G and -765C clone 6 were performed as described in chapter 2, section 2.8.11.

Clone 2 was digested with Nhe I and Ase I to generate a 654bp fragment. A restriction map of clone 2 illustrating the relative positions of the enzyme cut sites and the expected fragments is shown in Figure 5.9A. A gel image of clone 2 following digestion is shown in Figure 5.9B.

Afe I and Hind III were used to cut a 436bp fragment from clone 5. A restriction map for clone 5 is shown in Figure 5.10A and the results of the digest in Figure 5.10B.

The common allele (-765G) clone 6 and the rare allele (-765C) clone 6 were both digested with Ase I and Afe I. The restriction map for clone 6 is shown in Figure 5.11A. Gel images for the digests of the wild type and mutant clones are shown in Figures 5.11B and 5.11C respectively. The desired fragments from each clone were then gel extracted (Chapter 2, section 2.8.12) and the purified products were checked for purity on a 2% agarose gel (Figure 5.12).

Ligation reactions were set up as described in Chapter 2, section 2.8.13 and the products transformed into supercompetent cells as described in Chapter 2, section 2.8.7.

A PCR using the pGL3b forward and reverse primers (see Table 2.9 for sequences) was performed on 96 clones (48 containing -765G and 48 containing -765C). A gel image of the PCR result is shown in Figure 5.13. Only 4 clones generated a product of the expected size. One clone was contaminated and discarded (Chapter 2, section 2.8.17).
2.8.13), and the remaining sequenced. Of the 3 clones that were sequenced, 2 were consensus -765G and one was consensus with a C base at position -765 upstream of the transcription start site (-765C clone).

An endonuclease-free preparation (2.8.14) of the -765C clone and one -765G clone yielded the final purified constructs that were used in subsequent reporter gene assays.
Figure 5.2
PCR amplification of a 2kb COX-2 promoter fragment from clone 973JM2.
A 1% agarose gel (5V/cm, 1 hr in 1xTBE) showing the results of the CF12CR11 PCR (see Chapter 2, Table 2.8 for primer sequences). Only one reaction was successful (lane11). Four different products are visible (i-iv). The top band (i) was the expected fragment (2kb). A second unexpected band can be seen (ii) as well as two other less clear products (iii and iv). Lane 1 is the molecular marker (λ. Hind III). Sizes of the molecular marker fragments are shown in bp.
Figure 5.3 PCR product generated with primers CF11 and CR11.

A. A 1% agarose gel (5V/cm, 1 hr in 1xTBE) showing the CF11CR11 PCR (see Chapter 2, Table 2.8 for sequences). Molecular weight marker (λ Hind III) is loaded in the first lane. Multiple reactions were set up (lanes 2-10) in order to pool samples in a purification step. Products were pooled and then loaded onto two QIAquick columns for subsequent purification. B. A 1% agarose gel (5V/cm, 1 hr in 1xTBE) showing the purified 1.9kb insert in lanes 2 and 3. Lane 1 is the molecular weight marker (λ Hind III). Fragment sizes are shown in bp.
Figure 5.4 Purified pGL3 basic vector digested with Nhe I and Hind III. A 1% agarose gel (5V/cm, 1hr in 1xTBE) showing the purified linearised pGL3 basic vector (lanes 3 and 4) following digestion with Nhe I and Hind III. Molecular weight marker (λ Hind III) is loaded in lane 1. Uncut circular plasmid is shown in lane 2 as a control for the digestion. Fragment sizes are shown in bp.

Figure 5.5 Purified 1.9kb COX-2 promoter fragment and purified de-phosphorylated pGL3 basic vector. A 1% agarose gel (5V/cm, 1hr in 1xTBE) showing the digested COX-2 fragment after purification (lane 2) and the purified dephosphorylated vector (lane 3). These are the 2 components of the construct prior to their ligation. The gel image demonstrates that both products are free of contaminants. Lane 1 is the molecular weight marker (λ HindIII). Fragment sizes are shown in bp.
Figure 5.6 PCR of the construct to confirm presence of insert.

A 2% agarose gel (5V/cm, 30min in 1xTBE) of primer set 9 PCR (see Chapter 2, Table 2.1 for sequences) performed on the isolated construct. Molecular weight marker VIII is loaded in lane 1. Fragment sizes are shown in bp. Each lane (2-13) corresponds to product generated from construct isolated from a single clone following transfection of supercompetent cells with plasmid DNA containing the 1.9kb COX-2 promoter fragment.
Figure 5.7

A. Schematic representation of clones 2, 5 and 6 showing the relative position of the sequence errors revealed by sequencing (X). These clones contained large segments of sequence in consensus with published sequence, that could be isolated by restriction enzyme digestion and subsequently ligated, to generate the desired 1.9 kb insert. B. Restriction mapping analysis of the insert identified cut sites for Ase I and Afe I that were conveniently located to allow for clones 2, 5 and 6 to be digested so as to avoid the sequence errors. Nhe I and Hind III are the enzymes used initially to clone the insert into the pGL3basic vector.
Figure 5.8 Site-directed mutagenesis reaction to introduce a C base at position –765 upstream of the transcription start site.

A 1% agarose gel (5V/cm, 50min in 1xTBE) showing the mutagenesis product from the reaction performed on clone 6 in order to introduce a C base at position –765. Products were run in a 1% gel for 50min. Lane 2 is the undiluted stock sample. Lanes 3 and 4 contain 5 μl of a 50μl mutagenesis reaction set up with 25ng of DNA template. Reactions loaded in lanes 5 and 6 were set up with 50ng of template. Products were generated from primers CF14 and CR14 (see Chapter 2, Table 2.10 for sequences).
Figure 5.9
A. Plasmid map for clone 2. This is a plasmid restriction map for clone 2 showing the cut sites of the restriction enzymes Nhe I, Ase I, Afe I and Hind III. The fragment of interest is shown in orange. To isolate the desired 654 bp fragment, clone 2 was digested with Nhe I and Ase I. B. Gel image of clone 2 digests. A 1.2% low melting point agarose gel (5V/cm, 1hr in 1xTBE at 4°C). Lane 1 is the λ Hind III molecular weight marker. Uncut vector was loaded in lane 2. The two forms of plasmid DNA can be distinguished with supercoiled DNA migrating faster through the gel. Single enzyme digests with Ase I or Nhe I were performed as controls (lanes 3 and 4 respectively). Ase I cuts the vector at two sites and generates two fragments (lane 3). Nhe I cuts at a single site and linearises the plasmid resulting in a single band (lane 4). Lane 5 is the molecular weight marker VIII. Double digests with Nhe I and Ase I were performed in duplicate (lanes 6 and 7). The 3 expected fragments were obtained (4551, 1514, 654 bp). The 654 bp fragment was excised and gel purified.
Figure 5.10

A. **Plasmid map for clone 5.** This is a plasmid restriction map for clone 5 showing the cut sites of the restriction enzymes *Nhe* I, *Ase* I, *Afe* I and *Hind* III. The fragment of interest is shown in orange. To isolate the desired 436 bp fragment, clone 5 was digested with *Afe* I and *Hind* III. B. **Gel image of clone 5 digests.** A 1.2% low melting point agarose gel (5V/cm, 1hr in 1xTBE at 4°C). Lane 1 is the λ *Hind* III molecular weight marker. Uncut vector was loaded in lane 2. The two forms of plasmid DNA can be distinguished with supercoiled DNA migrating faster through the gel. Single enzyme digests with *Afe* I or *Hind* III were performed as controls (lanes 3 and 4 respectively). *Afe* I cuts the vector at two sites and generates two fragments (lane 3). *Hind* III cuts at a single site and linearises the plasmid resulting in a single band (lane 4). Molecular weight marker VIII was loaded in lane 5. Double digests with *Afe* I and *Hind* III were performed in duplicate (lanes 6 and 7). The 3 expected fragments were obtained (4200, 2083, 436 bp). The 436 bp fragment was excised and gel purified.
Figure 5.11. A. Plasmid map for clone 6. This is a plasmid restriction map for clone 6 (-765G and -765C) showing the cut sites of the restriction enzymes Nhe I, Ase I, Afe I and Hind III.. The fragment of interest is shown in orange. To isolate the desired 843 bp fragment, clone 6 was digested with Afe I and Ase I. B. Gel image of clone 6 ‘G’ digests. A 0.7% low melting point agarose gel (5V/cm, 2hrs in 1xTBE at 4°C). Lane 1 is the λ Hind III molecular weight marker. Uncut vector was loaded in lane 2. The two forms of plasmid DNA can be distinguished with supercoiled DNA migrating faster through the gel. Single enzyme digests with Afe I or Hind III were performed as controls (lanes 3 and 4 respectively). Ase I cuts the vector at two sites and generates two fragments (lane 3). Afe I also cuts at two sites and results in two bands (lane 4). The double digest with Afe I and Hind III is in lane 5. The 4 expected fragments were obtained (2519, 2168, 1189 and 843 bp). The 843 bp fragment was excised and gel purified. Molecular weight marker VIII was loaded in lane 6. C. Gel image of clone 6 ‘C’ digests. Same as for ‘G’ clone.
Figure 5.12: Gel extraction of COX-2 promoter fragments derived from clones 2, 5, 6G and 6C.

A 2% agarose gel (5V/cm, 30min in 1xTBE) showing the purified COX-2 promoter fragments. Lane 1 is the molecular weight marker. Lane 2 is the 436 bp fragments from clone 5 and lane 3 is the consensus 843 bp fragment from clone 6. The same fragment from clone 6 containing the C allele at -765 is shown in lane 4. Lane 5 is the 654 bp fragment from clone 2.
Figure 5.13 PCR to confirm ligation of fragments and vector
Figure showing the results of PCR using the pGL3b forward and reverse primers on the clones obtained from the ligation reaction. Each lane corresponds to a different clone. Only 4 clones generated a product of the expected size. The wells of the lanes containing products of interest are boxed for clarity. Molecular weight markers (VIII) are denoted with M.
5.3 RESULTS

5.3.1 Testing the activity of the −765G and −765C COX-2 promoter constructs in human lung fibroblasts.

Fibroblast activation and proliferation leading to excess collagen deposition is central to the development and progression of fibrosis. PGE$_2$ is implicated in the pathogenesis of pulmonary fibrosis through its effects on collagen production and fibroblast proliferation. It is the predominant prostanoid in lung fibroblasts and its synthesis is reduced in fibroblasts isolated from IPF patients (Wilborn J et al 1995, Keerthisingam CB et al 2001). For this reason, transfection experiments with the COX-2 constructs were carried out in human lung fibroblast cells.

For each sample, 1μg/ml of each vector was transfected into human foetal lung fibroblasts (HFL-1). The Dual-Luciferase® Reporter Assay (Promega, UK) was used (as described in Chapter 2, section 2.9). In this assay, two individual reporter enzymes (firefly and Renilla luciferases) are expressed and measured within a single system. The activity of the co-transfected Renilla luciferase serves as an internal control that allows for correction for cell number and transfection efficiency. The pGL3 basic vector (empty vector) was used in each experiment as a negative control.

Effect of serum

A serum dose-response for the -765G and the −765C construct is shown in Figure 5.14. Following quiescence for 16 hours in serum-free medium, the media was changed and fresh media containing either 0, 1, 2, 5 or 10 % serum was added to the cells for 8 hours. Following the addition of serum promoter activity was significantly increased in a dose-dependent manner for both the −765G and −765C constructs ($P<0.0000001$) for all concentrations of serum used compared to untreated). There is a trend for the −765C construct to have lower promoter activity than the −765G for all groups. Statistical significance is seen for serum concentrations of 1, 2 and 5% ($P=0.01$, $P=0.0002$ and $P=0.002$ respectively). In other experiments the trend for the −765C construct to have lower promoter activity than the −765G construct reached statistical significance basally (Figures 5.15 and 5.17). Subsequent repeat
experiments confirmed that there is a significant difference between the -765G and -765C constructs in unchallenged cells (data not shown).

**Timecourse of the COX-2 promoter**

A time-course for the two constructs in HFL-1 cells is shown in Figure 5.15. A time-dependent increase in promoter activity was observed with increasing time of treatment under both serum-free and serum-stimulated conditions. In serum-free media the difference in promoter activity between the -765G and -765C constructs was significant at 6 ($P=0.009$), 8 ($P=0.0008$) and 16 hrs ($P=0.0001$) but not at 3 ($P=0.09$) or 24 ($P=0.5$) hrs. In 2% serum the difference in promoter activity between -765G and -765C was significant at 3 ($P=0.001$), 6 ($P=0.04$), 8 ($P=0.006$) and 16 hrs ($P=0.006$) but not at 24 ($P=0.14$) hrs. At the 8 hr time-point (the time-point used in all other experiments) the -765C allele has significantly lower promoter activity compared with -765G, under basal conditions (28±3% lower). When serum was used as a stimulus both constructs responded but again the mutant construct displayed lower promoter activity (31±2% lower).

**Effect of cytokine treatments on COX-2 promoter activity**

The effect of IL-1β and TGF-β treatments on the COX-2 promoter constructs is shown in Figure 5.16. IL-1β and TGF-β were used at doses previously reported to stimulate the COX-2 promoter (10 ng/ml and 5 ng/ml respectively). A combination of IL-1β and TGF-β was also used to assess whether they could have a synergistic effect on the COX-2 promoter constructs. Following the addition of 2% serum promoter activity was significantly increased for both -765G and -765C constructs ($P<0.00001$ for both constructs). IL-1β and TGF-β alone or in combination had no effect on the -765G construct. Addition of IL-1β, TGF-β and their combination appeared to decrease the activity of the -765C construct ($P=0.002$, $P=0.02$ and $P=0.04$ respectively).

To investigate whether serum could act as a cofactor and stimulate a response, treatments with IL-1β, TNF-α (20 ng/ml), TGF-β and a combination of IL-1β and TNF-α, were conducted in the presence of a low percentage of serum (0.4%) (Figure 5.17). In the presence of 0.4% serum IL-1β had no effect on the -765G promoter but
reduced the activity of the -765C promoter ($P=0.003$). Treatment with TNF-α reduced the activity of both -765G and -765C constructs ($P=0.001$ and $P=0.0006$ respectively). The combination of IL-1β and TNF-α also lowered the activity of both constructs, $P=0.017$ and $P=0.0007$ for -765G and -765C respectively. Addition of TGF-β reduced the activity of both -765G and -765C constructs ($P=0.03$ and $P=0.01$ respectively).
Figure 5.14. Effect of serum on COX-2 promoter activity in HFL-1 cells.

Cells were treated with serum for 8 hours. Results represent Firefly luciferase activity normalised for Renilla luciferase activity, and are expressed as fold increase relative to untreated -765G clone. Data are expressed as the mean ± S.D. of four replicates. Data were analysed by Student’s t test. $P<0.05$ was considered significant.
Figure 5.15. Time-course of COX-2 promoter constructs in serum-free medium and 2% serum in HFL-1 cells. Cells were either left untreated or treated with 2% serum for 3, 6, 8, 16 and 24 hours. Results represent Firefly luciferase activity normalised for Renilla luciferase activity, and are expressed as fold increase relative to untreated −765G clone. Data are expressed as the mean ± S.D. of four replicates. Data were analysed by Student’s t test. P values reflect comparisons between −765G and −765C within the treatment groups. P<0.05 was considered significant.
Figure 5.16. Effect of IL-1β and TGF-β on COX-2 promoter activity in HFL-1 cells.

Cells were either left untreated or treated with 2% serum, IL-1β (10ng/ml) and TGF-β (5ng/ml) alone or in combination for 8 hours. Results represent Firefly luciferase activity normalised for Renilla luciferase activity, and are expressed as fold increase relative to untreated −765G clone. Data are expressed as the mean ± S.D. of four replicates. Data were analysed by Student’s t test. P<0.05 was considered significant.
Figure 5.17. Effect of inclusion of 0.4% on treatments with IL1-β, TNF-α and TGF-β

Cells were either left untreated or treated with 0.4% serum, 2% serum, and the following biological effectors in 0.4% serum: IL-1β (10ng/ml) or TNF-α (20ng/ml) alone or in combination, and TGF-β (5ng/ml) for 8 hours. Results represent Firefly luciferase activity normalised for Renilla luciferase activity, and are expressed as fold increase relative to untreated −765G clone. Data are expressed as the mean ± S.D. of four replicates. Data were analysed by Student’s t test. P<0.05 was considered significant.
The results with the cytokines were surprising. To exclude the possibility that the lack of response observed was due to the fact that the lung fibroblast used in these experiments were of foetal origin, the same experiments were carried out with adult lung fibroblasts (Figure 5.18 A and B). A minimal positive response was seen with IL-1β on the -765G construct \((P=0.05)\) but the activity of the -765C construct appeared reduced \((P=0.029)\). TNF-α had a clear inhibitory effect on both promoters \((P=0.02\) for -765G and \(P=0.00000005\) for -765C). The negative effect of TNF-α was dominant over IL-1β when these cytokines were used in combination \((P=0.01\) for -765G and \(P=0.0000007\) for -765C). TGF-β had no effect on the -765G construct but reduced the activity of the -765C promoter \((P=0.000018)\).

As in the experiments conducted in HFL-1 cells, cytokine treatments were repeated with the inclusion of 0.4% serum to investigate whether a small amount of serum could stimulate a positive response. A similar pattern of responses was observed. IL-1β had minimal positive effect on the -765G promoter \((P=0.01)\) but no effect on the -765C. TNF-α inhibited both constructs whether it was used alone \((P=0.0000028\) for -765G and \(P=0.000009\) for -765C) or in combination with IL-1β \((P=0.000001\) for -765G and \(P=0.000006\) for -765C). In the presence of serum, TGF-β inhibited both the -765G and -765C promoters, \(P=0.000047\) and \(P=0.0005\) respectively.
Figure 5.18. Effect of IL1-β, TNF-α and TGF-β on Adult Human Lung Fibroblasts in serum-free medium (A) and in the presence of 0.4% serum (B). Cells were either left untreated or treated with 0.4% serum, 2% serum, and the following biological effectors in 0.4% serum: IL-1β (10ng/ml) or TNF-α (20ng/ml) alone or in combination, and TGF-β (5ng/ml) for 8 hours. Results represent Firefly luciferase activity normalised for Renilla luciferase activity, and are expressed as fold increase relative to untreated −765G clone. Data are expressed as the mean ± S.D. of four replicates. Data were analysed by Student’s t test. *P<0.05 was considered significant.
5.3.2 Investigating nuclear protein binding to the –765 region.

The –765G>C variant locates within a putative Sp1 motif (Figure 5.19). Electrophoretic mobility shift assays (EMSAs) were performed in order to investigate whether the substitution of a G for a C at –765 altered transcription factor binding to that region. Oligonucleotides flanking the variant were designed and used in EMSAs together with nuclear extracts prepared from HFL-1 cells. Although simple in principle, there were several problems encountered with this technique. Initially a fluorescence-based method was used in an attempt to avoid the hazard of radioactivity. However, this method yielded irreproducible results. It also proved technically difficult to estimate comparable amounts of the –765G and –765C probes for use in experiments (see control of labeling efficiency, Chapter 2, section 2.7.1.4) and this was critical when comparing binding patterns and intensities formed with probes that only differed in a single base. The fluorescently-labelled probes also appeared to degrade very easily which also complicated matters when performing successive experiments. For this reason, a radioactive method was used. Another complication was secondary structure formation in the probe-alone reactions where in the absence of nuclear protein different species of probe were observed. This was particularly prominent with the oligonucleotide containing the C allele. In the presence of the C allele, the sequence in the middle of the probe becomes a run of six Cs and it is possible that this may lead to a loss of balance and subsequent loop formation. The problem of different species of probes in EMSAs is illustrated in Figure 5.20, panels A, B and C. This was resolved through gel extraction and purification of the probe of interest, Figure 5.20, panel D (see Chapter 2, section 2.7.2.4). After making sure that a single species of probe was obtained, an initial experiment with human recombinant Sp1 protein confirmed that Sp1 can bind to that region and that the site is disrupted when the G is changed for a C (Figure 5.21). Antibody supershift and blocking experiments (see Chapter 2, section 2.7.2.2) using nuclear protein derived from HFL-1 cells showed that Sp1, Sp3 and Egr-1 are involved in the regulation of this site (Figure 5.22). A supershifted complex was obtained with an anti-Sp1 antibody in the presence of the G but not the C probe (marked SS1, Figure 5.22, lane 5), whereas an anti-Sp3 antibody completely abolished formation of a complex that was only seen with the G probe (complex IV, Figure 5.22, lane 9). Antibodies to Sp2 and Sp4 had no apparent effect. An antibody
specific for the Egr-1 transcription factor lead to a supershift in the presence of the C probe (marked SS2, Figure 5.22, lane 14) and a reduction in the intensity of complex I of the G probe probe (complex I, Figure 5.22, lane 13).

Complex IV appears reduced with Sp1 and Sp3 antibodies (Figure 5.23, lanes 4 and 6) but this is likely to be non-specific as the same effect is observed with the negative IgG control (lane 8).

As Sp1 binding can be affected by phosphorylation, EMSAs were also performed with nuclear extracts prepared from HFL-1 cells in the presence of phosphatase inhibitors (1mM sodium orthovanadate, 2nM okadaic acid, 80pM cypermethrin, 15μM dephostatin and 100pM NIPP-1). Complexes I and II (Figure 5.23, lanes 1 and 2 respectively) are very similar to complexes I and II formed by the G and C probe respectively in the presence of non-treated extracts. A supershift is observed with the Sp1 antibody (marked SS, Figure 5.23, lane 3). Complex V formation is abolished in the presence of an Sp3 antibody (Figure 5.23, lane 5). Complex IV appears reduced with Sp1 and Sp3 antibodies (Figure 5.23, lanes 4 and 6 respectively) but this is likely to be non-specific as the same effect is observed with the negative IgG control (Figure 5.23, lane 8).

To investigate whether the -765 region could bind nuclear proteins from other cell types, EMSAs were performed with commercially available HeLa cell extracts. Several complexes are formed (I-VII) suggesting that the -765G>C variant may also be relevant in other cell types (Figure 5.24).
Figure 5.19: Schematic representation of the COX-2 5' flanking region showing the relative position of the -765G>C variant.
Figure 5.20. Annealed oligonucleotide probes across the –765 region containing either G or C at position –765.

Panels A, B and C illustrate the problems encountered with the oligonucleotide probes in EMSAs (see Chapter 2, Table 2.6 for probe sequences). In all cases shown, the probes are run in the absence of nuclear extracts where a single band is normally expected. Different species of probe (arrows) can clearly be observed indicating secondary structure formation. Panel D shows the probes following gel extraction and purification.
Figure 5.21: Binding of human recombinant Sp1 protein.

Commercially available human recombinant Sp1 protein was incubated with the G or the C oligonucleotide probe. The G probe can form six complexes (I-VI) in the presence of human recombinant Sp1. The probe containing the C allele is considerably less able to bind Sp1 protein. Probe alone reactions were loaded in the first two lanes. This is representative of 3 experiments.
Figure 5.22. Supershift and blocking antibody reactions. Several complexes are formed with both probes upon incubation with nuclear extracts derived from HFL-1 cells. Clear differences are observed between the binding profiles of the G and C probes. Pre-incubation with an anti-Sp1 antibody results in the formation of a supershifted complex (SS1, lane 5) in the presence of the G but not with the C probe. Anti-Sp3 antibody completely abolishes complex IV formation with the G probe (lane 9) but this is not seen with the C probe. Sp2 and Sp4 antibodies do not appear to have any obvious effects. Incubation with an Egr-1 antibody results in formation of a supershift with the C probe (SS2, lane 14) and reduction of the intensity of complex I with the G probe, lane 13. IgG antibody was used as a negative control (lanes 15 and 16).
Figure 5.23. EMSA with extracts treated with phosphatase inhibitors

This is an EMSA performed with HFL-1 nuclear extracts that were prepared in the presence of phosphatase inhibitors (1mM sodium orthovanadate, 2nM okadaic acid, 80pM cypermethrin, 15μM dephostatin and 100pM NIPP-1). Complexes I and II appear identical to the complexes I and II of the non-treated extracts (Figure 5.22, lanes 3 and 4). Pre-incubation with an anti-Sp1 antibody results in the formation of a supershifted complex (marked SS, lane 3) in the presence of the G but not with the C probe. Anti-Sp3 antibody blocks complex IV formation, lane 5. IgG antibody was used as a negative control (lanes 7 and 8).
Figure 5.24. EMSA with HeLa cell extracts

Figure showing results of an EMSA performed with commercially available nuclear extracts derived from HeLa cells. Several complexes are observed (I-VII). There are differences in the binding profiles obtained with the G and the C probes indicating that the functional consequences of the \(-765G>C\) variant may also be relevant in other cell types.
5.3.3 Investigating the effect of -765G>C genotypes in normal human lung fibroblast cell lines.

The final aim of the functional studies on -765G>C was to investigate whether there was a correlation between COX-2 genotype and PGE₂ levels in human lung fibroblast cell lines. Eleven normal human cell lines were available for genotyping the -765G>C variant. These cell lines had previously been characterised (McAnulty and colleagues) in terms of their PGE₂ responses to TGF-β. Intriguingly, some of the normal cell lines were found to be low PGE₂ producers and the reason for this is unknown. DNA from the cell lines was isolated by phenol-chloroform extraction and the -765G>C variant was genotyped. Cell line genotypes and corresponding PGE₂ levels are shown in Table 5.1. Of the 8 cell lines that were homozygous for the common G allele, 7 produced high levels of PGE₂ in response to TGFβ and only one was a low PGE₂ producer. However, 2 out of the 3 cell lines that were carriers of the rare allele C were found to produce low levels of PGE₂ upon challenge.

<table>
<thead>
<tr>
<th>Normal cell lines</th>
<th>genotype</th>
<th>High PGE₂</th>
<th>Low PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG (n=8)</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>GC (n=3)</td>
<td>1 (33.3%)</td>
<td>2 (66.7%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1. Cell line genotypes and PGE₂ levels.
5.4 DISCUSSION

In this chapter, 1.9kb COX-2 promoter constructs containing either the G or the C allele at position -765, were generated for use in reporter gene assays. *In vitro* studies in human lung fibroblasts revealed a significantly lower reporter expression from -765C compared with -765G (-30%). Both constructs are upregulated by serum but appear to be inhibited by treatment with IL-1β, TNF-α and TGF-β.

To explore the mechanism through which -765G>C reduces promoter activity, nuclear protein binding to the -765 region was investigated using EMSAs. EMSAs revealed that the probe containing the -765C allele was unable to bind human recombinant Sp1 protein. Studies with nuclear protein derived from human lung fibroblasts showed that Sp1, Sp3 bind to that region and that the presence of the C allele alters the interactions with these transcription factors. Binding of commercially available HeLa cell extracts indicates the possibility of a functional effect of the -765G>C variant in other cell types.

The effect of -765G>C genotypes was also investigated in normal human lung fibroblast cell lines. These cell lines had previously been characterised in terms of their PGE₂ response to TGF-β and classified into high and low PGE₂ producers. Of the 8 cell lines found to be homozygous for the common G allele only 1 (12.5%) was a low PGE₂ producer. However, 2 out of the 3 cell lines (66.7%) found to be carriers of the rare C allele were PGE₂ low producers.

The decision to generate 1.9kb constructs of the COX-2 promoter was based on the study by Yang X et al (1997) which showed that a 1.8kb construct of the human COX-2 promoter displayed maximal (2-fold) response to TGF-β (5ng/ml) in Bovine Pulmonary Artery Endothelial Cells (BPAC) after 24hrs. Significant difficulties were encountered in the process of generating the constructs. First, due to the high GC content of the COX-2 promoter, it proved very difficult to PCR amplify the region of interest and so to overcome this problem commercially available kits for difficult PCR had to be used. Second, the amplification reactions introduced sequence errors despite the claims that the polymerase enzymes provided with the
kits were of proof-reading quality. As many as 6 sequence errors were found in a single clone. This observation stresses the need to fully sequence any constructs intended for use in transfection experiments especially in the case of larger fragments where the chances of introducing an error are increased. As described in Chapter 2, Section 2.8, in order to generate the 1.9kb constructs used in this thesis, a ‘cutting and pasting’ approach was undertaken through appropriate use of restriction enzyme digests and ligation reactions. The final constructs were sequenced again to confirm that the only difference between the two plasmids was a single substitution of a G for a C at position –765.

Reporter gene assays in human lung fibroblasts with the 1.9kb construct generated in this study revealed that the –765C allele has significantly lower promoter activity compared with –765G, under basal conditions (28±3% lower, P<0.005). When serum was used as a stimulus both constructs responded but again the mutant construct displayed lower promoter activity (31±2% lower, P<0.005). In 3 out of 6 experiments presented in this chapter, the trend for the –765C construct to have lower promoter activity did not reach statistical significance under basal conditions. However, 4 subsequent repeat experiments (not shown) looking at untreated and serum-stimulated cells consistently showed a significant difference between the two constructs in untreated cells.

This is consistent with the hypothesis that the inability to upregulate COX-2 in pulmonary fibrosis may be due to sequence variants in the regulatory regions of the gene. In addition, the decreased promoter activity associated with the C allele is consistent with the increased frequency of –765C in sarcoid subjects with persistent disease, most of whom have a CXR stage 4 and have fibrosis (Chapter 3).

In an attempt to find a more specific stimulus than serum, TGF-β, IL-1β and TNF-α were used to treat cells transfected with the –765G and –765C constructs. TGF-β was of particular interest as it is known to induce COX-2 and its levels are significantly increased in pulmonary fibrosis (Khalil N et al 1991). IL-1β and TNF-α are also known stimulants of the COX-2 gene and synergy between the two cytokines in upregulating COX-2 has been reported (Diaz-Cazorla M et al 1999,
Wadleigh DJ and Herschman 1999). In view of that, the findings of the reporter gene assays were surprising. TGF-β failed to induce the COX-2 promoter constructs while IL-1β and TNF-α appeared to inhibit the reporter gene expression and this effect was more pronounced when these cytokines were used in combination.

There have been many studies with varying lengths of the COX-2 promoter in a plethora of cell types and as may be expected each study yielded different results. Surprisingly, relatively few studies of the COX-2 promoter have been done in fibroblasts. A study by Saunders MA et al (2001) in quiescent human fibroblasts showed an upregulation of -891/+9 bp and -459/+9 bp COX-2 promoter constructs in response to IL1-β (1ng/ml) and TNF-α (1ng/ml) after 6 hrs of treatment. A 1.5-fold induction with IL1-β (200pg/ml) was observed with the -1432/+59 bp promoter in human gingival fibroblasts (Nakao S et al 2000). In human foreskin fibroblasts, the -891/+9 bp promoter was induced by 2-fold in response to IL1-β (1ng/ml) and 3-fold in response to TNF-α (1ng/ml) after 6 hrs (Schroer K et al 2002). A larger promoter construct -1840/+123 bp, similar to the one used in this study, was induced 2.5-fold by a 2-hour treatment with IL1-β (10ng/ml) in human orbital fibroblasts (Han R et al 2002). In human synovial fibroblasts, a -2390/+34 bp construct was stimulated by 2-fold after treatment with IL1-β (100pg/ml) for 6 hrs (Faour WH et al 2001). However, the authors concluded that the induction of COX-2 mRNA, protein and PGE₂ release by IL1-β, was primarily the result stabilization of the COX-2 mRNA and stimulation of translation through activation of p38 MAPK signalling (Faour WH et al 2001).

Diaz A et al (1998) performed nuclear-run-off assays to study the regulation of COX-2 gene transcription in human lung fibroblast. Their study showed that incubation of fibroblasts with IL-1β and TNF-α caused an increase of 14.5- and 2.1-fold respectively on the transcription of the COX-2 gene while TGF-β lacked any detectable effect. Furthermore, the addition of TGF-β to IL-1β-treated cells did not increase, but rather reduced COX-2 gene transcription. However, the overall effect of TGF-β on COX-2 expression was positive as addition of TGF-β potentiated the effects of IL-1β or TNF-α on COX-2 mRNA and PGE₂ production. The authors concluded that in human lung fibroblasts, IL-1β and TNF-α stimulate the
transcription of the COX-2 gene, whereas TGF-β acts post-transcriptionally by increasing the stability of the COX-2 transcripts (Diaz A et al 1998).

By contrast, TGF-β was shown to act transcriptionally to induce COX-2 expression in pulmonary artery smooth muscle cells. A short 2-hour treatment with TGF-β (10ng/ml) resulted in a 4-fold induction of a 1kb promoter in these cells (Bradbury DA et al 2002). In the same study, addition of IL-1β (10ng/ml) caused a 3-fold induction of the COX-2 promoter (Bradbury DA et al 2002).

Newton et al showed that in type II A549 epithelial cells, IL-1β (1ng/ml) produced a rapid and prolonged induction of COX-2 mRNA which was primarily due to increased transcription rather than mRNA stabilization (Newton R et al 1997). Using nuclear run on assays they showed a 8-fold increase in transcription. Interestingly, transient transfection assays with a -917/+49 bp promoter failed to show IL-1β-dependent activation after a 24-hour treatment. Lack of IL-1β-inducibility was observed under various transfection conditions and stimulation times and even in different cell types including human airway epithelial BEAS2B and mouse LA-4 cells. They also comment that a -2307/+49 construct as well as shorter constructs down to -85/+49 bp were tested in A549 cells but also failed to show IL-1β-inducibility. The authors concluded that the system did not faithfully mimic the in vivo situation (Newton R et al 1997).

An inability of IL-1β to activate the COX-2 promoter was also observed in human endometrial stromal cells (Tamura M et al 2002). Tamura et al used a -825/+56 bp promoter construct and a shorter -360/+56 bp promoter but failed to obtain a response after 24 hrs of treatment with IL-1β (1ng/ml). They did however observe an increase in COX-2 mRNA stability, a process that appeared to involve PKA, NF-κB, and/or the ERK1/2 signalling pathways (Tamura M et al 2002).

In human airway smooth muscle cells, addition of IL-1β (0.2ng/ml) or TNF-α (1ng/ml) had no effect on a -471/-4 promoter construct after 20 hrs, while the combination of the two cytokines resulted in 1.5-fold induction (Moore PE et al 2001). An increased dose of IL-1β (20ng/ml) alone stimulated COX-2 promoter by 1.5-fold (Moore PE et al 2001).
In human messangial cells, combination of IL-1β (2ng/ml) and TNF-α (100ng/ml) resulted in a 3-fold induction of a -1772/+106 bp construct after 18 hrs (Diaz-Cazorla M et al 1999). A similar effect was obtained in mouse osteoblast MC3T3-E1 cells where a 4-hour treatment with IL-1β (10ng/ml) and TNF-α (20ng/ml) stimulated the murine -724/+7 construct by approximately 3-fold (Wadleigh DJ and Herschman 1999). In human alveolar epithelial NCI-H292 cells, addition of TNF-α (30ng/ml) for 6 hrs caused a 3.2-fold induction of a -459/+9 construct (Chen CC et al 2000).

Taken together, these studies highlight the variability of the mode of action of biological effectors between cell types. For IL-1β in particular, it is clear that while in some cells IL-1β-dependent COX-2 induction is primarily the result of increase transcription, in others mRNA stabilization appears to be the predominant mechanism. Furthermore, the effect on COX-2 mRNA stability may stem from the actions of distinct signaling pathways in different cell types. It becomes increasingly difficult to compare the results of these studies as most were done under different conditions with varying lengths of the COX-2 promoter, different doses of cytokine and variable treatment times.

The transfection experiments discussed thus far show that -765G>C is functional but provide no information on the molecular mechanism that leads to reduced promoter activity. As the variant locates within a putative Sp1 binding site it was reasonable to speculate that the substitution of a G for a C would alter transcription factor binding to that region. For this reason the next aim of this section of the thesis was to investigate DNA-protein interactions using electrophoretic mobility shift assays (EMSAs). Incubation of -765G and -765C probes with purified human recombinant Sp1 protein showed that the -765C probe was virtually unable to form any complexes thereby providing proof of principle for the removal of an Sp1 site in the presence of the C allele. EMSAs using nuclear extracts from human foetal lung fibroblasts revealed differences in the abilities of the two alleles to bind nuclear proteins. Several complexes were observed and supershift and blocking experiments confirmed the presence of Sp1 and Sp3 in complexes formed with the -765G probe. Loss of Sp1 binding was observed in the presence of -765C as shown by the absence of a supershift in the presence of an anti-Sp1 antibody. Interestingly, a supershift was
observed with an anti-Egr-1 antibody in reactions containing the −765C probe. In order to discuss the possible effects of the differential binding profiles observed and their relevance to regulation of COX-2 expression, it is necessary to consider first Sp1 and Egr-1. A brief background on these factors is presented followed by a discussion of the results and their relevance to the observations of the reporter gene assays.

**The transcription factor Sp1**

Sp1 belongs to a family of related factors binding to GC-rich sequences known as GC boxes. The Sp family includes Sp1, Sp2, Sp3 and Sp4 which contain highly conserved DNA-binding zinc finger domains and glutamine-rich transactivation domains adjacent to serine/threonine stretches in their N-terminal region. Sp1, Sp3 and Sp4 are more closely related to each other than Sp2 (Suske G 1999). Sp1 was the first mammalian transcription factor to be cloned (Kadonaga JT et al 1987). Sp1 and Sp3 are ubiquitously expressed but can fulfill different functions. Sp1 is a typically a transcriptional activator that is required for the expression of a variety of genes (Kadonaga JT et al 1987). The transactivation potential of Sp3 is influenced by an inhibitory domain (Dennig J et al 1996) which is not found in the other Sp proteins. In most promoters, Sp1 and Sp3 recognise the classical Sp1 consensus element 5'GGGGCGGGG3' with comparable affinity and specificity (Suske G 1999). Sp3 has been reported to act as a bi-functional regulator (repressor/activator) whose activity depends on the context of DNA binding sites in a promoter (Majello B et al 1997, Suske G 1999). It has been suggested that promoters containing a single binding site are activated, whereas promoters with multiple binding sites often do not or only weakly respond to Sp3 (Dennig J et al 1996). Whether Sp3 acts as an activator or a repressor might also depend on the cellular context. For instance, in the Sp-deficient Drosophila SL2 cells, Sp3 is a weak activator of the COX-2 promoter whereas in HUVEC cells Sp3 acts as a repressor of the COX-2 promoter (Xu Q et al 2000).

The abundance of factors may vary among different cell types and under certain conditions. The Sp1/Sp3 ratio is often critical for gene activation as both Sp1 and
Sp3 compete for the same site. In HUVECs, hypoxia enhances the amount of Sp1 protein while Sp3 levels remain unaltered (Xu Q et al 2000). By contrast, in myoblasts, the increase in Sp1/Sp3 ratio in response to hypoxia is achieved through post-transcriptional down-regulation of Sp3 protein levels (Discher DJ et al 1998). Given the dual nature of Sp3, variation in the expression of Sp1 and Sp3 can be expected to have important consequences for those genes whose transcription is regulated through the co-operative action of both transcription factors (Bouwman P & Philipsen S 2002).

The induction of transcription by Sp1 reflects an interaction with the basal transcriptional machinery complex, including the TATA-box binding protein (TBP) (Emili A et al 1994) and the TBP-associated factors (TAFs) within the TFIID complex (Hoey T et al 1993, Chiang CM and Roeder RG et al 1995, Tanese N et al 1996). Furthermore, a transcriptional cofactor complex CRSP (cofactor required for Sp1 activation) was shown to be required for transcriptional activation by Sp1 (Ryu S et al 1999). Sp1 can also interact with cell cycle regulators such as the retinoblastoma-related protein p107 (Datta PK et al 1995) and transcription factors such as E2F (Karlseder J et al 1996, Lin SY et al 1996).

Sp1 multimers have been reported to induce synergistic superinduction of gene promoters. (Mastrangelo IA et al 1991, Su W et al 1991). When two binding sites are present and both sites are bound by Sp1, the Sp1 molecules at both sites can physically associate with each other and bridge over large distances by looping out the intervening DNA.

Although the amount of Sp1 protein is regulated to a large extent at the mRNA level (Saffer JD et al 1991) there are also other post-translational control mechanisms. Protein modifications can be expected to impact on interactions with target sequences or other cofactors. Indeed, Sp1 is subject to extensive post-translational modification by phosphorylation (Jackson SP et al 1990) and glycosylation (Jackson SP and Tjian R 1988), which can influence its activity.

Several cellular kinases have been reported to phosphorylated Sp1. The first Sp1-kinase to be characterised was DNA-dependent protein kinase (Jackson SP et al
Phosphorylation of Sp1 by this kinase occurs upon binding to DNA. An unknown growth-regulated kinase is responsible for the increased phosphorylation of Sp1 in response to serum in serum-starved fibroblasts (Black AR et al 1999). Whereas phosphorylation by these kinases does not appear to affect DNA binding, phosphorylation of Sp1 by casein kinase II, a serine/threonine kinase, inhibits its DNA binding activity (Armstrong SA et al 1997). However, Sp1 phosphorylation can also result in increased binding. For instance, cyclin A binds directly to Sp1 and phosphorylation of Sp1 by a cyclin A-associated kinase increases its DNA binding activity (Haidweger E et al 2001). Phosphorylation of Sp1 by the cAMP-dependent protein kinase (PKA) also increases its transactivating activity, at least in part through enhancing its binding to DNA (Rohlff C et al 1997). The activity of Sp1 has also been reported to be regulated by protein phosphatases (Armstrong SA et al 1997, Garcia A et al 2000) indicating that steady state levels of Sp1 phosphorylation are established through both active phosphorylation and dephosphorylation processes.

Another mode of regulation of Sp1 activity is glycosylation. O-glycosylation of Sp1 with N-acetylglucosamine has been reported to block Sp1 protein interactions (Roos MD et al 1997). Glycosylation of the carboxy-terminal part of Sp1 activation domain B inhibits its transactivation potential by decreasing the interaction between Sp1 and components of the basal transcriptional machinery complex (Roos MD et al 1997). However, the same mechanism can activate transcription by blocking Sp1 interactions with repressor proteins such as the retinoblastoma-related protein p107 (Datta PK et al 1995). Furthermore, a low glycosylation state of Sp1 has been associated with increased susceptibility to proteasome-dependent degradation (Han I and Kudlow JE, 1997).

Interestingly, treatments that increase glycosylation of Sp1 (e.g. with acetylglucosaminidase inhibitors) lead to a decrease in its phosphorylation and it has been proposed that the regulation of Sp1 activity by glycosylation and phosphorylation may be coordinated (Haltiwanger RS et al 1998).
**The transcription factor Egr-1**

The early growth response (Egr)-1 transcription factor, also known as nerve growth factor induced-A (NGFI-A), Krox-24, ZIF268 and TIS8, is the prototype of a family of zinc-finger transcription factors that includes Egr-2, Egr-3, Egr-4 and NGFI-B. Egr-1 binds to GC-rich DNA sequences (5'-GCGGGGGCG-3') and can alter gene transcription through mechanisms dependent on both coactivators and corepressors (Silverman ES and Collins T 1999). Egr-1 contains both transactivation and repression domains and can act as a negative or a positive regulator of gene transcription depending on the arrangement of other DNA binding motifs near the Egr-1 site (Gashler AL et al 1993). The phosphorylation status of Egr-1 is another significant determinant of Egr-1’s ability to activate gene transcription as it can affect both protein-protein and protein-DNA interactions (Jain N et al 1996).

Egr-1 is an immediate-early response gene that is rapidly and transiently induced by serum, growth factors, cytokines and injurious stimuli (Cao XM et al 1990). Interestingly, induction of Egr-1 mRNA by PGE\(_2\) has been demonstrated in fibroblasts (Danesch U et al 1994). Egr-1 acts primarily as transcriptional inducer of gene expression. Several studies have shown that Egr-1 can activate gene transcription by displacing prebound Sp1 in promoters where Sp1 serves as a weak activator (Khachigian LM et al 1995, Kachigian LM et al 1996, Silverman ES et al 1997). Conversely, for Sp1 inducible promoters, the result of Egr-1 displacement of Sp1 could be transcription repression. It appears that Egr-1 and Sp1 can displace one another from many promoters, and that binding site occupancy is dependent on a balance determined by their concentration and affinity for the binding site (Silverman ES and Collins T 1999).

Egr-1 can repress transcription either by direct repression of promoter activity via DNA binding (Gashler AL et al 1993, Du B et al 2000) or by ‘squelching’ transcription through interactions with Sp1 that can be independent of Sp1 binding to DNA (Srivastava S et al 1998). For instance, interaction of Egr-1 with an Ets-like site was shown to have an inhibitory effect on the TGF-\(\beta\) type II receptor (Du B et al 2000) and more recently Egr-1 was reported to act as a repressor of the type II collagen gene by preventing interactions between Sp1 and the general transcription machinery (Tan L et al 2003). Sp1 sequestration by Egr-1 has also been reported.
Egr-1 is able to bind directly to free (unbound) Spl and prevent it from binding to DNA (Srivastava S et al 1998). This association can be modulated by phosphorylation as phosphorylated Egr-1 binds less avidly to Spl (Srivastava S et al 1998).

It is possible that Egr-1 and Sp-1 bind in an adjacent, non-competitive manner to the -765 region. Egr-1 may bind to the -765G probe but in a different conformation that does not allow detection of a supershift. The substitution of a G for C at -765 may however result in a site that resembles more closely the consensus recognition site for Egr-1 rather than Sp1. This would result in a higher affinity for Egr-1 to bind, possibly by competing with Sp1. It is of course difficult to extrapolate this to a possible scenario in vivo when chromatin structure adds a further level of complexity but despite this limitation, the information obtained from the EMSA studies may explain the difference in promoter activities observed in the reporter gene assays with the -765G and -765C COX-2 promoter constructs. Sp1 binding to the COX-2 promoter at another Sp1 site close to the transcription start site has been shown to stimulate COX-2 gene transcription in HUVEC cells. There is currently no information available on Sp1 binding to the site examined in this study in any cell type. Although the regulation of COX-2 gene expression varies considerably between cell types, Sp1 is more often than not an activator and it is therefore very likely that this is also the case in fibroblasts. In this context, loss of Sp1 binding at -765C due to increased binding of Egr-1 may explain at least in part the reduced promoter activity of the mutant construct. In view of that, Egr-1 may act as a repressor or as a less potent activator of the COX-2 promoter.

Of great interest is also the observation that Egr-1 is one of the most highly upregulated transcription factors in sarcoidosis (Personal communication, Martin Brutche). Increased levels of Egr-1 combined with a higher affinity for Egr-1 in the presence of the -765C allele may be very relevant in the role of COX-2 in sarcoidosis. This scenario would be consistent with the results of the association studies (Chapter 4), which showed that sarcoid patients carrying the -765C allele are more likely to deteriorate and develop persistent disease.
Finally, preliminary data were presented on a small number of cell lines. These had been previously characterised in our laboratory by McAnulty and colleagues. Assessment of the PGE\(_2\) levels generated in response to TGF-\(\beta\) lead to the intriguing observation that some normal cell lines responded less efficiently to TGF-\(\beta\). This prompted the classification of these cells into high and low PGE\(_2\) responders. Cell line DNA was extracted and genotyped in an attempt to see whether there was a correlation between COX-2 genotype and PGE\(_2\) levels. Although the numbers of cell lines genotyped are clearly very low, the first preliminary indication is that PGE\(_2\) levels are influenced by \(-765\mathrm{G}>\) genotype. In contrast to the reporter gene assays where the effect of the variant is investigated in isolation, this type of study involves the endogenous COX-2 gene promoter where the presence of other functional variants cannot be excluded. Therefore the effect of the variant may be masked or confounded by other variants and this limitation can only be overcome by substantially increasing the numbers of cell lines that are being investigated. It would be particularly interesting to look at cell lines isolated from patients with sarcoidosis and IPF.
CHAPTER VI

A PHENOTYPIC CONSEQUENCE FOR –765G>C

IN VIVO
6.1 INTRODUCTION

**COX-2 in the cardiovascular system and inflammation**

The importance of COX-2 in the cardiovascular system is highlighted by results emerging from studies on the use of COX-2 selective inhibitors. The topic is surrounded by considerable controversy with inconclusive results as to whether COX-2 inhibition exerts beneficial or detrimental effects on cardiovascular events in atherosclerosis. Awareness of a possible risk of cardiovascular events associated with the use of COX-2 selective inhibitors was first raised by the outcome of a study comparing rofecoxib (a COX-2 inhibitor) and naproxen (a COX-1 and COX-2 inhibitor) (Bombardier C *et al* 2000).

The aim of the study by Bombardier and colleagues was to assess whether selective inhibition of COX-2 by rofecoxib (Vioxx) would be associated with a lower incidence of clinically important upper gastrointestinal events than the nonselective non steroidal anti-inflammatory drug (NSAID) naproxen in patients with rheumatoid arthritis. This study is referred to as the VIGOR trial (Vioxx Gastrointestinal Outcomes Research). As NSAIDs are amongst the most commonly used medications in the world, the gastrointestinal toxicity that can be associated with their use is of major concern. The study showed that selective inhibition of COX-2 is associated with significantly fewer clinically important upper gastrointestinal events than nonselective inhibition. However, the results of this study drew attention to a different issue. The investigators reported that rofecoxib treatment increased the incidence of myocardial infarction by a factor of 5 (Bombardier C *et al* 2000).

Conceptually this was perhaps not surprising, but the need for a new generation of NSAIDs that lacked gastrointestinal toxicity was such, that the potential side effects associated with selective COX-2 inhibition were overlooked.

Nonselective NSAIDs may be protective through their inhibitory effects on thromboxane A2 (TXA$_2$), a vasoconstrictor that causes platelet aggregation. Conversely, COX-2 inhibitors may have adverse cardiovascular effects because they do not block the COX-1-dependent production of platelet TXA$_2$, and appear to
selectively inhibit the production of prostacyclin (PGI$_2$), a vasodilator and potent inhibitor of platelet activation.

A substantial body of work indicates that the balance between TXA$_2$ and PGI$_2$ is critical in maintaining cardiovascular homeostasis. Using knockout mice that lack receptors for either TXA$_2$ or PGI$_2$ or both, Cheng and colleagues (2002) showed that PGI$_2$ modulates the cardiovascular actions of TXA$_2$ in vivo. Tight regulation of the interaction between TXA$_2$ and PGI$_2$ appears to be crucial for homeostatic balance. In vitro, agonists of the TXA$_2$ receptor evoke the release of PGI$_2$ from endothelial cells (Nicholson NS et al 1984) and in vivo, inhibition of the TX synthase enhances rediversion of endoperoxides to platelet inhibitory prostanoids such as PGI$_2$ (Nowak J et al 1989). In addition, platelet-derived TXA$_2$ evokes COX-2 dependent PGI$_2$ formation by endothelial cells (Caughey GE et al 2001). As the formation and activity of the two prostanoids may interact via several mechanisms, the disruption of this homeostatic balance between TXA$_2$ and PGI$_2$ by selective inhibition of COX-2 may be relevant to the deleterious cardiovascular effects of COX-2 inhibitors. In this regard, PGI$_2$ inhibition with relatively unopposed platelet TXA$_2$ generation may lead to increased pro-thrombotic risk.

The results of the VIGOR study may also reflect a cardioprotective effect of naproxen rather than an increased incidence with rofecoxib (or perhaps both). Not all NSAIDs inhibit platelet aggregation. Naproxen inhibits the production of TXA$_2$ by 95% and platelet aggregation by 88% suggesting that the effects of regular use of naproxen may be similar to those of aspirin (also a COX-1 and COX-2 inhibitor) (Bombardier C et al 2000). A recent study by Mukherjee and colleagues, analysed the results of the VIGOR study and also a further study on the COX-2 inhibitor celecoxib, the Celecoxib Long-term Arthritis Safety Study (CLASS), and compared their myocardial infarction (MI) rates with the annualized MI rates in the placebo group of a meta-analysis of patients in 4 aspirin primary prevention trials (Mukherjee D et al 2001). The study reports significantly higher annualized MI rates for the COX-2 inhibitors in both VIGOR and CLASS compared with those in the placebo group of the meta-analysis (Mukherjee D et al 2001). The findings of Mukherjee and colleagues further substantiated reasons for concern regarding the prothrombotic effects of COX-2 inhibitors. However, there are inherent limitations about this kind
of analysis as comparisons across multiple studies can be problematic. Subsequent to these reports, a retrospective cohort study revealed that users of high-dose rofecoxib were 1.7 times more likely than non-users to have coronary heart disease (Ray WA et al 2002). Further observational studies of naproxen failed to show a protective effect sufficient to explain the difference (Ray WA et al 2002, Solomon DH et al 2002). Together, these results suggest that the increased rates of cardiovascular events are possibly due to a pro-thrombotic effect of COX-2 inhibitors and not simply a failure to offer the protection of aspirin-like NSAIDs.

Furthermore, the use of COX-2 inhibitors has been associated with elevations in blood pressure (Muscara MN et al 2000). The Heart Outcomes Prevention Evaluation (HOPE) study reported a significant reduction in cardiovascular events with reduction in blood pressure (Yusuf S et al 2000), suggesting that elevation of blood pressure may be another way in which COX-2 inhibitors can contribute to adverse cardiovascular outcomes. In addition, up-regulation of COX-2 was shown to play an essential role in the cardioprotection against myocardial stunning and MI afforded by the late phase of ischemic preconditioning (Shinmura K et al 2000).

Because of concerns about the cardiovascular safety of COX-2 inhibitors, current guidelines recommend that patients at high risk of cardiovascular disease should also receive aspirin when prescribed a COX-2 inhibitor (Pitt B et al 2002). Indeed, a recent study demonstrated that selective inhibition of COX-2, on top of standard therapy with aspirin and statins improved endothelial function and reduced markers of inflammation and oxidative stress in patients with coronary artery disease (Chenevard R et al 2003).

Critically, COX-2 is known to be induced at sites of inflammation and to promote inflammatory responses. As atherogenesis is an inflammatory process (Koenig W 2001, Ross R 1999), inhibition of COX-2 may potentially have anti-atherogenic effects by inhibiting inflammation and thus in this context, lower levels of COX-2 would be beneficial. In support of this is the observation that COX-2 expression is increased in human atherosclerotic lesions (Schonbeck U et al 1999).
In view of these reports, a functional COX-2 variant that reduces COX-2 promoter activity, may act as natural endogenous ‘brake’ on COX-2. The variant would have a protective anti-atherogenic role in the cardiovascular setting by virtue of decreasing inflammation, as a reduced induction of COX-2 would result in lower levels of inflammatory prostaglandins being generated. Furthermore in the presence of such a variant, while reduced, a proportion of PGI₂ will presumably still be produced and allowed to exert its anti-thrombotic effects. The variant could conceivably define a new prostanoid balance that would prove advantageous in individuals at risk of cardiovascular events. The -765G>C variant therefore presents intriguing questions as to its role in the cardiovascular setting.

Chapter 5 presented *in vitro* evidence that the -765G>C variant represses COX-2 transcription by altering transcription factor binding to that region. In this investigation, a cohort of patients undergoing coronary artery bypass surgery was used as an *in vivo* model of inflammation with the aim to see if a biological correlate for this functional variant could be identified. Bypass surgery is a well-characterised inflammatory stimulus (Brix-Christensen V *et al* 1998, Aouifi A *et al* 1999, Kilger E *et al* 1998) that causes a significant acute-phase reaction with induction of inflammatory stimuli associated with COX-2, for example Interleukin-6 (IL-6), C-reactive protein (CRP) and fibrinogen. Given the role of COX-2 in the cardiovascular system and inflammation, it was postulated that -765G>C may have implications for cardiovascular disease as well as other chronic inflammatory disorders.

- The aim of this section is to determine whether -765G>C impacts on an acute inflammatory response and demonstrate a phenotypic consequence of the variant in vivo.
6.2 METHODS

6.2.1 Subjects
Patients undergoing elective first-time coronary artery bypass graft surgery (n=173) were recruited from the Middlesex Hospital (London, UK) as part of the Coronary Artery Surgery Inflammation Study (CASIS) (Brull DJ et al 2001). The study had approval of the hospital ethics committee and written informed consent was obtained from all participants. The 454 Caucasian subjects used to estimate genotype frequencies in healthy UK subjects were a random subset of males, taken from the Northwick Park Hospital Study II (Humphries SE et al 2001).

6.2.2 Genotyping
Bypass subjects were genotyped by Aci I digest as described previously in Chapter 2, section 2.5.1

6.2.3 Measurement of CRP, IL-6 and fibrinogen
Undertaken by Dr. D. J. Brull, Centre for Cardiovascular Genetics, UCL.

Citrated blood samples (4.5mL) were initially drawn before surgery and then again on the first 5 postoperative days. These were immediately centrifuged (3500g, 10 min) and plasma was separated and frozen at ~20°C until analysis. IL-6 and fibrinogen were measured as previously described (Brull DJ et al 2001 and 2002). CRP was measured on a BN Prospec (from Dade Behring). Inter-assay and intra-assay coefficients of variation were <4% and <2% respectively, and assay sensitivity was 0.20mg/L.

6.2.4 Statistical analysis
Allele frequencies were estimated by gene counting. A χ² test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. IL-6 and CRP values were not normally distributed; thus data were logarithmically transformed before analysis and geometric mean ± SE values are quoted. Fibrinogen values at all time points were normally distributed. Differences between genotypes were assessed by ANOVA and by the Student t test
for unpaired data. One-way ANCOVA was performed as previously described (Brull DJ et al 2002).
Baseline characteristics of the 173 bypass patients are shown in Table 6.1. A total of 123 subjects (71%) were receiving statin therapy and 82 (47%) were receiving β-blockers. Baseline characteristic for the healthy subjects were as follows (mean ± SE): age 56.0 ± 3.4 years, cholesterol 5.71 ± 1.0 nmol/L, triglycerides 1.78 ± 0.94 mmol/L, body mass index (BMI) 26.2 ± 3.4 kg/m² and systolic blood pressure 136.7 ± 18.6 mm Hg (Humphries SB et al 2001). There was no significant difference in the genotype distribution overall comparing the bypass patients with the 454 white healthy subjects (Table 6.2; P=0.10). The rare allele frequency appeared higher in the bypass in the bypass group than the healthy subjects but this was not significant (Table 6.2; P=0.19). However, the frequency of carriage of the rare allele (GC+CC combined) was higher in the bypass group compared with the healthy control group (33.5% versus 25.3% respectively; odds ratio 1.49 [95% CI 1.02 to 2.17]; P=0.04).

Carriers of 1 or more −765C allele were combined to assess CRP, IL-6 and fibrinogen levels by genotype, because there were only 2 individuals who had the genotype −765CC. At baseline, mean CRP levels were lower in carriers of 1 or more −765C allele (2.1±0.2 mg/L for GG versus 1.8±0.3mg/L for GC+CC), although this difference was not statistically significant (P=0.37). As expected, BMI was positively correlated with basal CRP levels (r=0.21, P=0.02), but after adjustment for this confounder, levels were not significantly associated with the −765G>C genotype (P=0.18).

After bypass surgery, CRP levels were significantly higher at all subsequent points compared with baseline (P<0.005 for all comparisons), with peak CRP levels recorded on the third postoperative day (mean 166±5mg/L). The magnitude of this rise was strongly genotype dependent (Figure 6.1). Mean CRP values were lower for carriers of ≥ 1 rare −765C allele at all times after surgery. This difference remained significant after multivariate analysis for all CRP values recorded for days 2, 3, 4 (by ANCOVA, P=0.024, P=0.013 and P=0.026). Data were adjusted for age, sex, smoking, cholesterol levels, diabetes, BMI, statin therapy, duration of surgery, and bypass and aortic cross-clamp time. No single parameter was associated with a
significant CRP-lowering effect after surgery (BMI and operation duration showed
significant positive correlations with peak CRP values). At the peak of CRP levels on
day 3, mean CRP levels in carriers of −765C (149.57±8.58 mg/L) were 14±0.05% (P<0.05) lower than patients homozygous for −765G (173.64±6.30mg/L).

IL-6 levels were not significantly different by −765G>C genotype at baseline or at
anytime after bypass surgery (Figure 6.2). Fibrinogen levels were not significantly
different by −765G>C genotype at baseline or at anytime after bypass surgery with
the exception of day 3, P=0.015 (Figure 6.3).

| Table 6.1 Patient Baseline Characteristics and Operative Details |
|---------------------------------|---------------- |
| Age, years                      | 63.2 ± 9.8     |
| Sex, M/F                        | 137/36         |
| Current smokers                 | 31 (18%)       |
| Ex/non smokers                  | 142 (82%)      |
| Treated hypercholesterolaemia   | 123 (71%)      |
| Treated Hypertension            | 68 (40%)       |
| Diabetes                        | 34 (20%)       |
| Family history of CAD           | 90 (52%)       |
| Mean number of grafts           | 2.8 ± 0.7      |
| Operation duration              | 193 ± 39       |
| CPB time, min                   | 66 ± 19        |
| AoXC time, min                  | 33 ± 13        |
| Length of Ventilation, hours    | 10.2 ± 4.6     |
| Stay in Intensive care, days    | 2.3 ±1.8       |
| Post operative stay, days       | 6.8 ± 3.9      |

CAD indicates coronary artery disease; CBP, cardiopulmonary bypass;
AoXC, aortic cross-clamp
<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Rare allele frequency (95% CI)</th>
<th>P value*</th>
<th>Genotype(%)</th>
<th>P value(\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>GC</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>454</td>
<td>0.13 (0.11-0.16)</td>
<td></td>
<td>339 (74.7)</td>
<td>109 (24.0)</td>
</tr>
<tr>
<td>Bypass subjects</td>
<td>173</td>
<td>0.17 (0.13-0.21)</td>
<td>0.19</td>
<td>115 (66.5)</td>
<td>56 (32.4)</td>
</tr>
</tbody>
</table>

All genotypes were checked independently for accuracy. The \(-765G>C\) variant is in Hardy-Weinberg equilibrium in both subject groups \(\chi^2\) values were for healthy subjects 0.70 \((P = 0.40)\), bypass subjects \((P = 0.09)\). *Allele and \(\dagger\)genotype frequencies compared by \(\chi^2\) test.
Figure 6.1. Circulating plasma levels of CRP by -765G>C genotype.
Following bypass surgery, CRP levels were significantly higher at all time points compared to baseline ($P<0.05$, for all comparisons). The magnitude of this rise was strongly genotype dependent. Mean CRP values were lower for carriers of one or more –765C alleles at all times post-surgery (ANOVA $P=0.032$, $P=0.023$, $P=0.034$, $P=0.048$ and $P=0.13$ at 1, 2, 3, 4 and 5 days respectively).
Figure 6.2. Circulating plasma levels of IL-6 by -765G>C genotype.
IL-6 levels were not significantly influenced by -765G>C genotype at baseline or at any time after bypass surgery. (ANOVA $P=0.25$, $P=0.47$, $P=0.25$, $P=0.95$ and $P=0.87$ at 0, 1, 2, 3, and 4 days respectively).
Figure 6.3. Circulating plasma levels of fibrinogen by -765G>C genotype.
Fibrinogen levels were not significantly influenced by -765G>C genotype at baseline or at any time after bypass surgery with the exception of day 3, $P=0.015$. 
6.4 DISCUSSION

In patients undergoing bypass surgery, individuals with \( \geq 1 \) copy of the \(-765C\) allele were found to have significantly lower plasma CRP levels after surgery compared with patients homozygous for \(-765G\). This observation is completely consistent with the \textit{in vitro} data which show \(-765C\) with lower promoter activity than \(-765G\), and demonstrate a phenotypic consequence of \(-765G>C\) in an inflammatory state. It is possible that these results are an underestimation of the true effect of the COX-2 genotype on CRP levels \textit{in vivo}, as 71% of patients were on statin therapy which is known to lower CRP levels in addition to lowering LDL cholesterol (Ridker PM \textit{et al} 1999, Albert MA \textit{et al} 2001).

In order to understand the possible implications of this result, it is necessary to consider the functions of CRP. The role of CRP as a predictor of cardiovascular events will be discussed in detail before exploring the relationship between CRP and COX-2.

\textbf{CRP is an acute phase protein}

Proteins that show dramatic alterations in response to inflammation and tissue injury are collectively termed acute phase reactants. These acute phase proteins amplify, sustain, attenuate and resolve inflammation either directly or through stimulating the production of other molecules from a variety of cell types (Gabay C and Kushner I 1999). In general, the magnitude of the acute-phase response is related to the severity of the inflammatory state or the extent of tissue injury (Ballou SP and Kushner I 1992).

Most notable in terms of the magnitude of the increase, is C-reactive protein (CRP). Within 2-3 days after the onset of inflammation serum levels of CRP can increase to as much as 1000-fold. CRP has been known since the 1930s (Tillett WS and Francis T 1930) as a serum protein that binds to the C-polysaccharide antigen of the pneumococcal cell wall. The primary function of CRP remains unclear but the high degree of conservation throughout evolution suggests an important biological role. Since its discovery, many biological properties have been attributed to CRP (Table 6.3).
Table 6.3: Known biological properties of CRP

CRP can...

- recognise pathogens and damaged cells of the host and mediate their elimination by recruiting the complement system and phagocytic cells (Volanakis JE 2001).
- suppress the activation of neutrophils (Buchta R et al 1988, Dobrinich R and Spagnuolo PJ 1991)
- restrain neutrophil movement through inhibition of their chemotactic responses (Kew RR et al 1990).
- prevent neutrophil adhesion to endothelial cells and thus decrease migration to tissues (Zouki C et al 1997).

CRP is a predictor of cardiovascular events

Recent reports indicate that basal and slightly elevated plasma levels of CRP associate with risk of developing cardiovascular disease (Haverkate F et al 1997, Koenig W et al 1999, Rohde LE et al 1999, Ridker PM et al 2000). This is perhaps not surprising as CRP is an inflammatory marker and from a pathological viewpoint, all stages of atherosclerosis can be regarded as an inflammatory response to injury (Ross R 1999).

Previously, basal levels of 2mg/L were at the lower limits of detection of CRP assays. Newer, high sensitivity assays can measure circulating CRP levels with acceptable precision down to or below 0.3 mg/L, and it is within these lower, previously ‘normal’ ranges that CRP levels seem to have predictive abilities for cardiovascular events (Ridker PM 2003). The current recommendations of the AHA/CDC scientific statement are that CRP should be measured in metabolically stable persons without obvious infection or inflammatory condition (Pearson TA et al 2003 AHA/CDC scientific statement). Two separate measurements are adequate to classify a persons risk level with the cutpoints being <1.0 mg/L for low risk of cardiovascular disease, 1.0-3.0 mg/L moderate risk and >3mg/L high risk. If a level of >10 mg/L is identified there should be a search for an obvious source of infection or inflammation. The result should be discarded and the test repeated again in 2 weeks to allow acute inflammation to subside before retesting. The writing group of
the AHA/CDC scientific statement assigned a level of evidence B to the measurement of CRP as a means of assessing risk in patients without known cardiovascular disease, and recommended it as an adjunct to the major risk factors. The measurement of CRP at present is considered optional and at the discretion of the physician (Pearson TA et al 2003 AHA/CDC scientific statement).

Several studies have also reported a strong link between circulating plasma levels of CRP on admission for myocardial infarction and long-term clinical outcome (Tommasi S et al 1999) including risk of dying (Lindhal B et al 2000, Nikfardjam M et al 2000). Measuring CRP several months after a coronary event appears to predict long-term outcomes (Ridker PM et al 1998). CRP’s strong predictive value may be explained by its long-term stability during storage, its long half-life, its lack of diurnal variation and its lack of age and sex-dependance (Meier-Ewert HK et al 2001). CRP retains an independent association with incident coronary events after adjusting for age, total cholesterol, HDL cholesterol, smoking, BMI, diabetes, history of hypertension, exercise level and family history of coronary disease (Ridker PM et al 1998, 2001, 2002).

Intriguingly, increased CRP concentrations also appear to predict coronary events in apparently healthy men with no prior history of cardiovascular disease. (Ridker PM et al 1997, Koenig W et al 1999, Danesh J et al 2000). Studies assessing risk in healthy women also reported similar findings. The Women’s Health Study (WHS) showed that CRP was the single strongest predictor of future coronary event amongst the examined biochemical markers and that the relationship remained significant after adjustment for cardiovascular disease risk factors (Ridker PM et al 2000). An extension of this study to confirm this also suggests that CRP is a better predictor of the risk of cardiovascular events than low-density lipoprotein (LDL) cholesterol (Ridker PM et al 2002). Furthermore, CRP levels appear to be of predictive value even in a young healthy population (Ridker PM et al 2000 Circ), suggesting that elevated CRP levels in young adults may provide an early warning for a later risk.

A study of patients undergoing coronary pulmonary bypass surgery showed that increased pre-operative levels of CRP, in the absence of any sign of infection, were associated with significantly poorer outcome, higher systemic inflammatory response
complications and a prolonged stay in the intensive care unit (Boeken U et al 1998). Removal of inflammatory cytokines reduces coronary pulmonary bypass surgery morbidity and indicates that limiting the inflammatory response would remove the largest threat to the survival of critically ill patients or even elective surgical cases (Johnson D and Mayers I 2001, Marshall JC 2001).

The exact role of CRP in cardiovascular disease is unclear and the relative contributions of CRP as a marker, a mediator or a consequence of coronary heart disease, are debatable. The simplest interpretation of the above observations is that CRP is just a sensitive marker of inflammation, a process known to underlie the cardiovascular pathology. Slightly elevated concentrations of CRP may reflect the presence of low-grade inflammation in the coronary arteries or elsewhere. Alternatively, they may reflect pro-inflammatory effects of CRP itself (Ballou SP and Lozanski G 1992). CRP can promote vascular inflammation by inducing an increase in the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), selectins, and the chemokine monocyte chemotactic protein-1 (MCP-1), and by increasing local infiltration by monocytes and lymphocytes (Pasceri V et al 2000, 2001). CRP may also contribute to cardiovascular disease by exerting pro-thrombotic effects and thereby promoting coagulation. Aggregated CRP binds to low and very low-density lipoprotein, which in turn activates complement, stimulates tissue factor production by macrophages, and thus starts coagulation (de Ferranti S and Rifai N 2002, Arici M and Walls J 2001).

Complement activation by CRP appears to play a major role in atherosclerotic pathogenesis. Contrary to previous beliefs that CRP is mainly produced in the liver (Gabay C and Kushner I 1999, Lagrand WK et al 1999) and delivered to plaques by serum, several observations support the concept that CRP may be an endogenous activator of complement within atherosclerotic lesions. In atherosclerotic plaque tissues CRP mRNA levels were 10 fold higher than in normal artery tissue, and protein levels of CRP and complement proteins were also found to be up-regulated (Yasojima K et al 2001). This suggests that inflamed arteries could be the source of the mildly elevated CRP levels reported to predict cardiovascular events. CRP
mRNA was found to reflect the vulnerability of the atheromatous lesion and the likelihood of a plaque to rupture (Libby P 1995). CRP appears to co-localise with the membrane attack complex of complement in early atherosclerotic regions of human coronary arteries (Torzewski J et al 1998). Co-localisation of CRP with activated complement in human hearts during acute myocardial infarction has also been reported (Lagrand WK et al 1997). Activated complement attacks cells in plaque tissue resulting in a self-sustaining autotoxic mechanism operating within the plaques as a precursor to thrombotic events (Yasojima K et al 2001). In view of that, anti-inflammatory agents such as NSAIDs would be anticipated to be helpful in interrupting such a cycle.

There is currently no definitive evidence that lowering circulating plasma CRP levels will necessarily reduce cardiovascular event rates, however, many interventions known to reduce cardiovascular risk also lower CRP levels. Lipid-lowering therapy with statins to reduce vascular risk leads to a decline by 25-50% in plasma levels of CRP (Ridker PM et al 1999, Plenge JK et al 2002, Albert MA et al 2001). Importantly, primary and secondary-prevention trials have shown that treatment with statins reduces levels of CRP and rates of cardiovascular disease, with the beneficial effects of treatment being greatest amongst those with elevated base-line levels of CRP (Ridker PM et al 1998, 2001).

More recently, statin therapy was shown to result in inhibition of COX-2 expression in plaque macrophages (Cipollone F et al 2003). The data raise the intriguing possibility that inhibition of the COX pathway may partly account for the clinical benefits of statins. This may also explain why patients with low LDL levels can also benefit from statin therapy (Ridker PM et al 2001). The beneficial anti-inflammatory effects of aspirin are also well established in the secondary prevention of myocardial infarction in patients with known cardiovascular disease (He J et al 1998). Interestingly, the magnitude of relative risk reduction attributable to aspirin in primary prevention appears to be greatest among those with elevated CRP and declines proportionately in direct relation to CRP levels (Ridker PM et al 1997). In a recent study of patients with severe coronary artery disease receiving standard background therapy with aspirin and statins, selective inhibition of COX-2 resulted in significantly lower levels of CRP (Chenevard R et al 2003).
**A common pathway for COX-2 and CRP?**

The finding that a polymorphism in the promoter of the COX-2 gene associates with levels of CRP raises the intriguing possibility that COX-2 and CRP expression may be coordinately regulated. COX-2 genotype determines the levels of CRP, suggesting a common pathway in which COX-2 up-regulation is either upstream of CRP synthesis or part of a feedback loop. Although a relationship between COX-2 and CRP has not been described to date, there is indirect evidence in the literature to support such a link.

The concept that a COX-mediated pathway might be connected with the acute phase response is not completely unprecedented. In fact, earlier studies raised the possibility that prostaglandins may play a role in the initiation of the acute phase response. Administration of the prostaglandins PGE₁, PGE₂, PGF₂α and PGA₂ has been shown to increase the synthesis of the acute phase protein haptoglobin by the liver in rabbits, to an extent sufficient to raise plasma levels (Shim BS 1976). Furthermore, PGE₁ was reported to cause an acute phase response in CRP and serum amyloid A (SAA) protein in man (Whicher JT *et al* 1980). Infusion of PGE₁ was also shown to cause changes in serum concentration of a wide range of acute phase proteins including α₁-antitrypsin and α₁-antichymotrypsin and haptoglobin (Whicher JT *et al* 1984).

Ikuta T *et al* (1986) reported that T-cell preparations co-cultured with monocytes, synthesised CRP following stimulation by a monocyte-derived factor that was part of the arachidonate cascade. In this study, CRP synthesis was reduced when monocytes were treated with agents inhibiting phospholipid metabolism including inhibitors of PLA₂, lipoxygenase and the COX inhibitor indomethacin (Ikuta T *et al* 1986). However, in a study by Schultz D *et al* (1982) in cultured mouse hepatocytes prostaglandins did not induce CRP synthesis, reflecting perhaps a need for interaction with factors released by distinct cell populations or differences in the regulation of CRP between cell types.

Further support for the notion that COX-2 and CRP may be co-ordinately regulated comes from the observation that CRP is also significantly increased during the onset...
of labour when prostaglandin levels are known to be elevated (De Meeus et al 1998). A significant correlation between PGE$_2$ and CRP has been observed in patients with temporomandibular joint inflammatory disorders (Alstergren P and Kopp S 2000). Finally, NSAIDs have been shown to modify acute phase response levels thereby supporting a role for prostaglandins in the onset of an acute phase reaction (Bienvenu et al 1985). However, as these early studies have not been pursued further, the interaction between prostaglandins and CRP remains an open question.

In contrast to CRP, IL-6 levels were not significantly influenced by $-765G>C$ genotype at baseline or at any time after bypass surgery. This was surprising as the induction of COX-2 is known to precede IL-6 gene expression and PGE$_2$ has been shown to regulate IL-6 synthesis in macrophages (Williams JA and Shacter E 1997). There is also evidence that IL-6 regulates CRP levels (Humphries SE et al 2001). IL-6 is the principal inducer of the CRP gene (Toniatti C et al 1990). The CRP promoter contains several IL-6 response elements, and the functional IL-6 promoter variant $-174G>C$ is reported to affect levels of CRP (Humphries SE et al 2001, Akira S 1995). It is unclear whether the influence of the COX-2 $-765G>C$ variant on CRP levels is through IL-6. The interaction between IL-6 and CRP is complex and not entirely clear-cut. CRP was shown to mediate shedding of membrane-bound IL-6 receptor from neutrophils, suggesting that CRP can also modulate IL-6 activity (Jones SA et al 1999). The ability of CRP to increase the secretion of IL-6 has also been reported (Verma S et al 2002).

It is possible that IL-6 levels need to be measured at an even earlier time-point in order to detect an effect of the COX-2 genotype as the peak of IL-6 may be earlier than day 1. IL-6 plasma levels start to decline as early as day 2 following bypass surgery, whereas CRP levels peak at day 3 and at day 5 are still considerably above baseline. It is also possible that COX-2 may have a more direct effect on CRP than on IL-6. In macrophages, the effect of PGE$_2$ on IL-6 in response to inflammatory agents appears to be dependent on the presence of ancillary signals (Williams JA and Shacter E 1997). This may explain the lack of significance despite the presence of a trend for carriers of the C allele to have lower plasma IL-6 levels at days 1 and 2 post bypass surgery.
Like CRP, fibrinogen is an acute phase protein. The transcription of fibrinogen is stimulated by IL-6; its synthesis is suppressed by IL-1β and TNF-α (Green F and Humphries S 1989, Woods A et al 2000). Fibrinogen is a circulating glycoprotein that acts at the final step in the coagulation response to vascular and tissue injury (Herrick S et al 1999). Fibrinogen has a central role in thrombosis as cleavage of fibrinogen by thrombin generates soluble fibrin fragments, which are the most abundant components of blood clots.

Several studies have demonstrated an association between increased plasma levels of fibrinogen and risk of cardiovascular disease. Meade TW et al (1986) performed a prospective study, the Northwick Park Heart Study (NPHS) and showed that increased plasma fibrinogen levels indicated the risk of an MI event. The results have been confirmed by numerous prospective studies (reviewed by Koenig W 1999). Two meta-analyses of 18 and 22 prospective long-term studies, demonstrated statistically significant risk ratios for individuals in the upper tertile of baseline fibrinogen concentration compared with subjects in the lower tertile (risk ratio 1.8; 95% CI = 1.6-2.0 and OR 1.99; 95% CI = 1.85-2.13 respectively) (Danesh J et al 1998, Maresca G et al 1999). Although the data have shown consistency between studies it remains to be determined whether fibrinogen has a causal role in atherothrombosis or is merely a marker of the degree of vascular damage.

In the study presented here, fibrinogen levels were not significantly different by −765G>C genotype at baseline or at anytime after bypass surgery with the exception of day 3 (Figure 6.3). It is probable that the difference observed at day 3 is due to chance as an effect of the −765G>C on fibrinogen levels over the 5 day period is not seen. However, this needs to be confirmed in a separate study.

In summary, this study demonstrates a phenotypic consequence of the −765G>C variant in an inflammatory state. The −765C allele is associated with lower CRP levels compared with the levels observed with −765G. This raises the possibility that this variant may have implications for a number of inflammatory and chronic disorders. Finally, genotyping −765G>C in subjects using NSAIDs may prove to be useful in predicting an individual’s response to treatment.
CHAPTER VII

FINAL DISCUSSION
An overview

Pulmonary fibrosis is the end-stage of a heterogeneous group of lung disorders that includes IPF and sarcoidosis. It is characterised by fibroblast proliferation and excessive accumulation of extracellular matrix proteins, notably collagen. This aberrant response results in loss of the normal lung architecture and ultimately leads to death from respiratory failure. At present, the molecular mechanisms underlying the pathogenesis of pulmonary fibrosis are poorly understood. However, it is becoming increasingly clear that fibrosis is the result of an imbalance between stimulatory and inhibitory mediators, and there is now convincing evidence that at least a part of this imbalance can be attributed to a defect in the cyclooxygenase COX pathway. In normal lung fibroblasts, up-regulation of COX-2, the inducible COX isoform, leads to production of prostaglandins including PGE$_2$. PGE$_2$ is the predominant prostanoid in the lung, and in addition to having a bronchoprotective role, it exerts potent anti-fibrotic effects by negatively regulating collagen production and fibroblast proliferation. By contrast, fibroblasts isolated from patients with IPF have a diminished capacity to upregulate COX-2 and synthesise PGE$_2$. This may in part be due to sequence variants in the regulatory regions of COX-2 that modify gene expression. Functional variants that suppress COX-2 may thus contribute to the fibroproliferative response by removing an important inhibitory control.

In this thesis, the promoter of the COX-2 gene was investigated for novel sequence changes. At the start of this thesis, there were no known variants in the COX-2 gene. There are now over 130 variants reported in the NCBI dbSNP database, 40 of which are promoter variants. Despite the availability of SNPs in the database, it proves very difficult to select functionally relevant polymorphisms. Contrary to sequence changes in the coding region, functional polymorphisms in regulatory regions of genes cannot be readily distinguished simply by considering their sequence context. Recent data suggest that up to a third of promoter variants may alter gene expression to a functionally relevant extent (Hoogendoorn B et al 2003). These data highlight the need to investigate the promoter regions in genes suspected of involvement in a specific disease.
The promoter region of the COX-2 gene was screened for sequence variants in sarcoidosis subjects using PCR and single strand conformation polymorphism (SSCP)-based methods. The data demonstrate that a number of sequence variants exist in the promoter of the COX-2 gene. Three sequence changes were identified: -765G>C, -62C>G and -368T>C. The -62C>G, was found in a single subject. -765G>C, is carried by ~25% of a healthy UK Caucasian population. The -765G>C variant was genotyped in UK IPF and sarcoidosis samples and tested for association with disease. There was no difference in the genotypic frequencies between the IPF sample and the healthy subjects (P=0.66) and similar results were obtained with a sample of Mexican IPF subjects. By contrast, the genotype distribution overall in the UK Caucasian sarcoidosis sample was significantly different from that in the healthy subjects (P=0.006). As 5-10% of sarcoidosis subjects develop persistent disease with an endpoint of fibrosis, the -765G>C variant was further tested for association with this particular subgroup, to support a link between COX-2 genotypes and fibrosis. When the sarcoidosis sample was classified into patients with persistent and non-persistent disease, a significant genotypic distribution overall was observed between the subjects with persistent disease and the healthy subjects (P=0.00037). The genotype distribution in the subjects with non-persistent disease was not significantly different from the healthy subjects (P=0.52). In the persistent disease subjects, carriage of the rare -765C allele (GC+CC combined) was associated with greater risk of poorer outcome (OR=2.28; 95%CI 1.30-4.00, P=0.004). The effect of -765G>C on risk of sarcoidosis is mainly contributed by subjects homozygous for the rare -765C allele. Subjects with persistent disease that are homozygous for the -765C allele have an OR of 10.19 (95%CI 2.83-36.65, P<0.005).

The biggest implication of this result is the intriguing possibility of identifying patients with sarcoidosis who are at risk of going on to develop persistent disease. At present this is not possible. Identification of such subjects may allow a more aggressive approach to the management of these subjects at risk of poorer outcome. The lack of effect observed in IPF is likely to reflect the complexity of the genetic factors that result in IPF. Furthermore, the IPF group may be more heterogeneous as no subjects in the UK Caucasian group had biopsy proven diagnosis, and may include patients with NSIP (Chapter 1) which are known to have better prognosis than true IPF.
Importantly, the findings of the association studies suggest that COX-2 has a modifying rather than a causal effect in pulmonary fibrosis. This reflects the complexity of fibrotic disease and its polygenic nature. An additional level of complexity is added in fibrosis resulting from exposure to a known environmental agent, such as PBD, where gene-environmental interactions can be expected to be crucial.

Functional studies were undertaken to determine the effect of the -765G>C polymorphism on gene expression. Constructs were generated containing 1.9kb of the COX-2 promoter with either a G or a C at position -765. It proved particularly difficult to amplify the region of interest directly from genomic DNA and so a clone containing the entire COX-2 gene (clone 973JM2, kindly supplied by the Sanger Centre Clone Resource Group, Cambridge, UK) was used as a template. Despite the use of commercially available kits for difficult PCR containing proof-reading Taq polymerase, sequencing revealed that a significant number of sequence errors had been introduced in the amplification process. As a consequence, a 'cutting and pasting' approach was adopted (Chapter 5, section 5.2) and the final constructs sequenced again cautiously to ensure that the only difference between the two constructs was a G to a C substitution at position -765.

The functionality of the variant was then tested by transfecting the -765G and -765C 1.9Kb COX-2 promoter constructs into human lung fibroblasts, and measuring reporter gene activity. In transfection experiments, the mutant -765C allele is significantly less efficient that the wild type -765G in directing transcription of the luciferase reporter gene, under both basal (28±3% lower, \( P<0.005 \)) and serum stimulated (31±2% lower, \( P<0.005 \)) conditions. These data lend support to the original hypothesis that failure to upregulate COX-2 in pulmonary fibrosis may be due to naturally occurring promoter variants that affect COX-2 expression. The -765G and -765C constructs were also challenged with cytokines such as IL-1β, TNF-\( \alpha \) and TGF-\( \beta \) that are known to stimulate the endogenous COX-2 gene (Chapter 5). The results with the cytokines were surprising as rather than enhancing promoter activity they appeared to have an inhibitory effect on the COX-2 promoter. It is
possible that in HFL-1 cells the upregulation of the endogenous COX-2 gene by these cytokines is due to an increase in COX-2 mRNA stability rather than an effect on the transcription rate of the gene.

The functional studies are also consistent with the population studies in this thesis investigating fibrotic lung disease. The finding that -765G>C associates with susceptibility and poorer outcome in sarcoidosis is in keeping with a role for -765C in lowering COX-2 expression. Furthermore, preliminary results in a small number of normal human lung fibroblast cell lines indicate that PGE$_2$ levels are influenced by -765G>C genotypes.

The results of the reporter gene assays warranted studies aimed at providing a molecular basis for the effect of -765G>C on the regulation of COX-2 gene expression. The substitution of a G for a C at position -765 removes a putative Sp1-like binding site. To address this, EMSAs were performed using 30bp probes of the -765 region containing either the G or the C allele. Significant difficulties were encountered in the annealing process of the probes as the introduction of the C allele resulted in formation of secondary structures and therefore several species of probes. This occurred presumably due to a loss of balance of the oligonucleotide probe as the substitution of a G for a C results in a run of six Cs in the middle of the probe. The problem was overcome by extracting the radio-labelled probe species of interest from polyacrylamide gels.

EMSA analysis demonstrated an altered capacity between the -765G allele and the -765C allele to bind nuclear proteins derived from human lung fibroblasts. The complexity of the binding patterns observed is likely to reflect the active participation of this promoter region in the regulation of COX-2 expression. In the presence of an anti-Sp1 antibody, a supershifted complex was observed with the -765G allele but not with -765C. Furthermore, DNA fragments containing the -765C allele were unable to bind purified human recombinant Sp1 protein. Taken together, the data suggest that the decreased promoter activity observed in the presence of the -765C allele can at least in part be explained by loss of Sp1 binding. Incubation with an Sp3 antibody abolished the formation of a complex that is only seen with the -765G allele. Intriguingly, an Egr-1 antibody supershifted a complex formed in the
presence of the -765C allele, suggesting that Egr-1 may also be involved in the regulation of this region. This is particularly interesting as Egr-1 is one of the most highly upregulated transcription factors in sarcoidosis (Martin Brutche, personal communication). Furthermore, results from EMSAs performed with commercially available HeLa cell extracts indicate that the -765G>C variant may also be relevant in other cell types.

As COX-2 is a key player in inflammation, it was reasonable to suppose that -765G>C could also impact on inflammatory processes. To test this possibility, COX-2 genotypes were also investigated in an in vivo model of inflammation of patients undergoing coronary artery bypass graft surgery. In the coronary bypass model of inflammation, the magnitude of rise in levels of the CRP, was strongly -765G>C genotype dependent. Subjects carrying the -765C allele had significantly lower plasma CRP levels at 1 to 4 days following surgery (14% lower at the peak of CRP levels on day 3) compared with patients homozygous for -765G (P<0.05 for all time-points). This finding raises the possibility that COX-2 and CRP may be co-ordinately regulated.

In order to examine the possible implications of the -765G>C variant in disease with respect to CRP, it is necessary to consider that both pro-inflammatory and anti-inflammatory functions have been attributed to CRP (reviewed by Heurtz and Webster, 1997). Consequently, the effect conferred by the COX-2 genotype may have very different functional consequences depending on the setting. In the context of the lung CRP appears protective. CRP is shown to inhibit acute lung injury in animal models by reducing neutrophil influx and protein leakage into the lung (Heuertz et al 1993 and 1994, Ahmed et al 1996). From this perspective, the COX-2 -765C allele could be argued to exacerbate and maintain inflammation. Furthermore, CRP plays a major role in the innate immune system through its ability to recognise foreign pathogens and activate complement. It is therefore conceivable that the variant could facilitate infection by lowering CRP levels. By contrast, the -765C allele may be protective in the context of cardiovascular disease where raised CRP levels have been shown to predict cardiovascular events. (Haverkate et al 1997, Toss et al 1997, Gaspardone et al 1998, Buffon et al 1999).
It seems unlikely that the association of $-765G>C$ with sarcoidosis is through CRP. Active pulmonary sarcoidosis without pulmonary fibrosis has been associated with minor or no increases in serum CRP concentrations despite evidence of ongoing tissue inflammation (Hind CRK et al 1987) and there have been no reports linking CRP with fibrosis in sarcoidosis. In IPF, elevation in CRP levels has been reported in patients presenting with acute exacerbation (Kondoh Y et al 1993). The disease is normally chronic in nature but abrupt worsening occurs in some patients. As the cause of the exacerbation is unknown, the significance of elevations in CRP levels is at present unclear. However, a relationship between COX-2 and CRP appears more evident in systemic sclerosis, a chronic inflammatory disease leading to fibrosis. Prostaglandins can induce an increase in CRP and this response in is greatly diminished in patients with systemic sclerosis (Whicher JT et al 1980).

The studies in this thesis point towards a potential clinical role for the COX-2 $-765G>C$ polymorphism in cardiovascular disease. A growing body of evidence indicates that traditional risk factors do not fully account for the occurrence of cardiovascular disease and it is estimated that only about half of patients with coronary heart disease have hypercholesterolemia (EUROASPIRE study 1997). This highlights the need to identify at risk individuals that would otherwise be missed if only lipid screening was used. For example, patients with elevated CRP but low LDL are at high vascular risk (Ridker PM et al 2002). COX-2 genotypes, including the COX-2 variant described here may prove to be important in identifying those with higher risk of cardiovascular disease. In this regard, knowledge of the COX-2 genotype would enhance the current prognostic and therapeutic capabilities. It remains possible however that $-765G>C$ is just a marker for other more important variants and therefore these studies need to be extended in order to define functional haplotypes across the COX-2 gene.

Risk stratification for cardiovascular disease is important because the information about the probability of a cardiovascular event in the future can help target therapy and resources to those most likely to benefit. It is now becoming increasingly clear that pharmacogenetic factors need to be taken into account. Patients receiving the same treatment may have very different responses that can be explained by the presence of genetic variability. For example, the therapeutic response to a 5-
lipoxygenase (5-LO) inhibitor can vary with 5-LO promoter polymorphisms containing variable numbers of GC-boxes capable of binding Sp1 and Egr-1 transcription factors (Funk CD et al. 1989, Drazen JR et al. 2001). In view of this, COX-2 genotype information might help in stratifying patients into risk groups to allow the most appropriate use of aspirin or to refine indications for COX-2 inhibitors.

As the GG genotype is associated with higher COX-2 expression and CRP levels, individuals that are ‘GG’ would be expected to be better responders to aspirin and COX-2 inhibitors and therefore have the largest benefit from treatment. At present this remains vastly speculative and would need to be substantiated by large-scale clinical trials. It is conceivable however, that the presence of the C allele might act as a natural endogenous ‘inhibitor’ by dampening down COX-2 expression and thus conferring a protective effect, while a GG genotype might highlight the need for more aggressive therapeutic intervention with NSAIDs.

If the clinical usefulness of the variant was to be confirmed by clinical trials, for example in patients taking NSAIDs or COX-2 inhibitors, knowledge of the COX-2 genotype may also have public health implications. If the variant adds prognostic information this could be important in the primary prevention of cardiovascular disease, as it would help to identify high-risk individuals who can be targeted for smoking cessation, diet, exercise and blood pressure control. Individuals with the GG genotype and a previous family history of cardiovascular disease should be advised to adhere carefully with lifestyle interventions and additional information of COX-2 genotype may lead to better compliance and adherence with treatment guidelines. It is well recognised that compliance with lifestyle recommendations is directly related to the absolute risk perceived by individual patients. Knowing about the greater risk incurred by the presence of the GG allele might provide the extra incentive needed for a high risk patient e.g. a smoker or an overweight patient, to modify their behaviour and reduce their risk of cardiovascular disease.

Similarly, genotyping -765G>C may prove useful in identifying individuals at risk of poorer outcome in sarcoidosis where there is currently no means of predicting which
patients are more likely to deteriorate and develop persistent disease with fibrosis. As sarcoidosis can either resolve spontaneously or progress to fibrosis (Chapter 1, section 1.1.2), subjects must be followed up for 2 years after presentation before they can be classified as having persistent or non-persistent disease. It would be therefore particularly useful to identify susceptible individuals at an early stage in this 2-year period before irreversible fibrotic scarring has occurred in the lung. In sarcoidosis where the rare -765C allele associates with severity of disease, individuals homozygous for the C allele could be selected for more aggressive treatment in the hope of preventing the deterioration of their symptoms.

**Future directions**

It is highly probable that other variants in the COX-2 gene may augment or attenuate the influence of the -765G>C polymorphism. It will therefore be important to explore in the future the relationship between -765G>C and other COX-2 variants in larger functional haplotypes. The -62C>G variant identified in Chapter 3 may also be of interest. Although rare (with 1 subject showing the change on the SSCP gel), -62C>G may have functional implications due to its location. -62C>G occurs just 2 nucleotides away from the CRE, an element essential for basal COX-2 gene expression. The -62C>G variant is unlikely to have a broad impact but it may be very important for a small number of individuals that carry it. The effect of a combination of COX-2 variants could be investigated in reporter gene assays with COX-2 promoter construct bearing more than one variant. Furthermore, cell types other than fibroblasts (e.g. epithelial cells) could also be investigated. In the context of sarcoidosis, it would be particularly relevant to examine COX-2 haplotypes in individuals with extreme phenotypes such as CXR stage 4. Furthermore, the functional cause of the decreased COX-2 expression in IPF and sarcoidosis still needs to be elucidated and may include variants in other genes influencing COX-2 expression as well as factors affecting COX-2 mRNA stability.

In chapter 5, EMSAs showed that the regulation at the -765 region is likely to involve interactions with Sp1, Sp3 and Egr-1 and that the -765C allele may alter these interactions. Although informative, this assay remains an artificial system where only a 30bp oligonucleotide probe is considered, and chromatin structure, and
therefore availability of sites for binding, is not taken into account. A further aspect of this work could therefore be to investigate the effect of the -765G>C variant on transcription factor binding in the endogenous gene in relevant cell types using a chromatin immunoprecipitation assay.

Further investigation of -765G>C in human cell lines is also warranted. Preliminary data were generated in chapter 5 using normal human lung fibroblast cell lines. It would be of interest to investigate cell lines isolated from IPF and sarcoidosis patients with respect to -765G>C and their ability to produce PGE₂ in response to pro-fibrotic mediators such as TGF-β.

The effect of -765G>C on endogenous COX-2 gene expression in normal cell lines and cell lines isolated from patients with IPF and sarcoidosis could also be investigated by Quantitative Real Time PCR. Although the contribution of other biological variables cannot be excluded, this approach could provide valuable information if a substantial number of cell lines is investigated. Quantitative Real Time PCR could also be performed to assess induction of COX-2 mRNA in cells with different -765 genotypes in response to IL-1β, TNF-α and TGF-β. Furthermore, looking at nascent and mature transcripts with Quantitative Real Time PCR should elucidate whether the effect of these mediators in humal lung fibroblasts is through a transcriptional or a post-transcriptional mechanism. Alternatively, nuclear run-on assays could be used to assess whether the effect of IL-1β, TNF-α and TGF-β is transcriptional. The effect of these cytokines on COX-2 mRNA stability could also be investigated by challenging cells in the presence of a transcriptional inhibitor such as Actinomycin D.
Conclusions

The results of this thesis indicate that the -765C allele of the -765G>C variant represses COX-2 expression by altering transcription factor binding to that region. Furthermore, -765G>C associates with susceptibility and poorer outcome in sarcoidosis. Although further studies are clearly warranted to elucidate the contribution of genetic variation in hereditary susceptibility to fibrosis, the present study provides the first evidence that naturally occurring functional variants in the COX-2 gene increase risk of susceptibility to pulmonary fibrosis and influence the course and severity of disease. This observation may help to identify individuals at increased risk of developing fibrosis. The association of the variant with CRP levels suggests that -765G>C may also influence the course of other chronic disorders and inflammatory states. The -765G>C variant may impact on human disease through various distinct pathways, a possibility which would be in keeping with the many diverse roles of the COX-2 enzyme in biology and disease. In cancer, where COX-2 is usually overexpressed, the -765G>C variant may confer a protective effect. Furthermore, it is conceivable that the -765G>C polymorphism may contribute to the variability in responses to COX-2 inhibitors and may in the future prove to be useful in predicting those individuals that are more likely to benefit from treatment.
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APPENDIX

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS THESIS:

ABSTRACTS


  presented in the form of an oral presentation at the American Thoracic Society Conference, San Francisco, USA, May 2001


  presented in the form of a poster presentation at the British Association for Lung Research Summer Meeting, Leuven, Belgium. September 2001


  presented in the form of a poster presentation at the American Thoracic Society Conference, Atlanta, USA, May 2002


  presented in the form of an oral presentation 12th International Fibrosis Colloquium, Glion-Montreux, Switzerland, October 2002


  presented in the form of a poster discussion at the American Thoracic Society Conference, Seattle, USA, May 2003

Editorial on this manuscript:
Cyclooxygenase (COX) is a key regulatory enzyme in eicosanoid metabolism, converting free arachidonic acid to prostaglandin (PGH₂), from which a number of prostanoids, including PGE₂, PGI₂, PGD₂, and thromboxane, are produced.† The prostanoids are important mediators in the control of normal tissue homeostasis and regulate inflammation in response to trauma or infection. Two isoforms of COX have been identified, COX-1 and COX-2, which have common and specific roles. COX-1 is expressed constitutively in most cell types; however, COX-2 is inducible on cell activation and is mainly expressed at sites of inflammation. COX-2 expression is raised in several pathophysiological states, and the use of COX inhibitors to reduce COX-2 activity has proven beneficial in attenuating chronic inflammatory conditions, such as arthritis and inflammatory bowel disease.‡ Several million people worldwide regularly use COX inhibitors. Regular use has been shown to decrease the relative risk of developing cardiovascular disease, stroke, and colorectal cancer.§

Objective—Cyclooxygenase (COX)-2 is a key regulatory enzyme in the synthesis of prostanoids associated with trauma and inflammation. We investigated the COX-2 gene for functional variants that may influence susceptibility to disease.

Methods and Results—The promoter of COX-2 was screened for variants in healthy subjects by use of polymerase chain reaction–based methods. Promoter activity was investigated by using reporter expression experiments in human lung fibroblasts. Patients undergoing coronary artery bypass graft surgery, with measurements of plasma markers linked to COX-2 activity, were genotyped for association studies. A common COX-2 promoter variant, -765G>C, was found and shown to be carried by >25% of a group of healthy UK subjects. The -765C allele had significantly lower promoter activity compared with -765G, basally (28±3% lower, P<0.005) and in serum-stimulated cells (31±2% lower, P<0.005). In patients subjected to coronary artery bypass graft surgery, the magnitude of rise in levels of C-reactive protein (CRP) was strongly genotype dependent. Compared with -765G homozygotes, patients carrying the -765C allele had significantly lower plasma CRP levels at 1 to 4 days after surgery (14% lower at the peak of CRP levels on day 3, P<0.05 for all time points).

Conclusions—For several acute and chronic inflammatory diseases, -765G>C may influence the variability of response observed. (Arterioscler Thromb Vase Biol. 2002;22:1631-1636.)

Key Words: cyclooxygenase-2 ■ promoter variant ■ coronary artery bypass graft surgery ■ C-reactive protein ■ inflammation
also reported to be associated with disease.\textsuperscript{11,12} The 5' flanking region of the human COX-2 gene, principally involved in regulating gene transcription, contains a canonical TATA box and several putative transcription-factor binding sites, including cAMP-responsive element, nuclear factor-{kappa}B, nuclear factor-IL-6, glucocorticoid response element, poliovirus enhancer activator 3, activator protein-2, CAAT box/enhancer binding protein, stimulatory protein-1 (Sp1), and a transforming growth factor-{beta} response element, suggesting that a complex array of factors is involved in its regulation.\textsuperscript{13-16} Deletion and forced mutation experiments altering this sequence have identified critical regions and elements involved in inducing COX-2 gene transcription.\textsuperscript{15-17}

Naturally occurring gene polymorphisms have been reported in COX-2; however, the functional significance of those found in the 5' flanking region is unclear.\textsuperscript{18,19}

We hypothesized that dysregulation of COX-2 expression has a role in COX-2-mediated pathology and that this, in part, may be due to functional changes in the 5' transcriptional regulatory promoter region of the gene. We report in the present study the presence of novel promoter variants in COX-2 and show that 1 of them, lying in a putative Sp1 binding site, affects the transcription rate of a reporter gene in a transient transfection studies. To find a biological correlate for this functional promoter variant, we have examined an in vivo model of inflammation of patients undergoing coronary artery bypass graft surgery. Bypass surgery causes a significant acute-phase reaction, with a rise in the plasma levels of markers such as IL-6 and C-reactive protein (CRP),\textsuperscript{20-22} which are associated with COX-2 activity. IL-6 synthesis has been shown to be regulated by COX-2 via the production of prostaglandin (PG)E\textsubscript{2}.\textsuperscript{23} Using this model, we demonstrate a phenotypic consequence of the functional promoter variant in an acute inflammatory response.

**Methods**

**Subjects**

Patients undergoing elective first-time coronary artery bypass graft surgery (n = 173) were recruited from the Middlesex Hospital (London, UK) as part of the Coronary Artery Surgery Inflammation Study (CASIS), whose design has been previously described.\textsuperscript{24} The study had approval of the hospital ethics committee, and written informed consent was obtained from all participants. The 454 white subjects used to estimate genotype frequencies in healthy UK subjects were a random subset of males who were taken from the Northwick Park Hospital Study II and who had baseline characteristics as follows (mean±SE): age 56.0±3.4 years, cholesterol 5.71±1.0 mmol/L, triglycerides 1.78±0.94 mmol/L, body mass index (BMI) 26.2±3.4 kg/m\textsuperscript{2}, and systolic blood pressure 136.7±18.6 mm Hg (see Humphries et al\textsuperscript{25} for full details).

**Measurement of IL-6, CRP, and Fibrinogen**

Citrated blood samples (4.5 mL) were initially drawn before surgery and then again on the first 5 postoperative days. These were immediately centrifuged (3500g, 10 minutes), and plasma was separated and frozen at −20°C until analysis. IL-6 and fibrinogen concentrations were measured as previously described.\textsuperscript{26,27} CRP was measured on a BN Prospect (from Dade Behring). Interassay and intra-assay coefficients of variation were <4% and <2%, respectively, and assay sensitivity was 0.20 mg/L.

**SSCP and Genotyping**

The proximal promoter region of the COX-2 gene was polymerase chain reaction (PCR)-amplified by using overlapping primer sets from normal healthy control subjects, and the products were examined by single-strand conformational polymorphism (SSCP); the methods used have previously been described.\textsuperscript{28,29} For PCR primers and their positions relative to the first transcribed nucleotide of the COX-2 gene, please see the expanded Methods section (available online at http://atvb.ahajournals.org). After electrophoresis, the product was visualized by SyBr Gold stain (Molecular Probes) on a Fuji FLA3000 imager. Within each primer set, PCR products with altered mobility patterns were sequenced with AmpliTag DNA polymerase FS (Perkin-Elmer) fluorescently labeled dye-terminator chemistry and with the use of an Applied Biosystems 377 PRISM automated sequencer.

DNA was extracted from peripheral blood leukocytes by standard phenol/chloroform extraction techniques or a salting-out method.\textsuperscript{30,31} The −765G>C variant was genotyped by AccI (NEB) restriction endonuclease digest of the PCR product generated by the use of primers CF8 and CR7 (Table I, available online at http://atvb.ahajournals.org). Digested products were separated in an 8% MAGE gel,\textsuperscript{32} and genotype was determined by 2 independent observers blinded to clinical details. The primers CF8 to CR7 gave a 306-bp band that in the presence of −765G was digested by AccI into 2 fragments of 188 and 118 bp. Positive and negative controls were included in all gels.

**Reporter Gene Constructs**

PCR primers CF11 and CR11 (Table I) containing recognition sites for Nhrl and HindIII, respectively, were used to generate a 1933-bp product of the COX-2 gene (−1811 +108) from clone 973 M2 from the library RPCIS (kindly supplied by the Sanger Center Clone Resource Group, Cambridge, UK). A commercially available kit (Advantage Genomic PCR, Clontech) was used for PCR, and the product was purified by using a QiAquick PCR purification kit (Qiagen). After restriction enzyme digestion, the product was directly ligated into pGL3 Basic Luciferase vector (Promega), linearized by digestion with Nhrl and HindIII, and transfected into XL1-Blue supercompetent cells (Stratagene). The sequence of the plasmid was confirmed by automated sequencing. Clone 973 M2 has a G residue at position −765 upstream from the COX-2 transcription start site. To generate a plasmid with a C residue at position −765, a QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used, along with the −765G plasmid DNA as a template and the following oligonucleotide and its reverse complement sequence, GAATTAC-
Culture Collection) cells were seeded into a 12-well plate and grown containing the obtained from Life Technologies. The HFLI cells were cotransfected of each plasmid DNA were generated for transfection by using the

purification of the novel sequence variants, -490 C > G and -765 G > C, are shown. The arrow and +1 denote the transcriptional start site.

CTTTCCCCCTCTTTTTCC. The sequence of the -765C plasmid was confirmed by automated sequencing. Two separate preparations of each plasmid DNA were generated for transfection by using the EndoFree Plasmid Maxi Kit (Qiagen).

Transfections
Human fetal lung fibroblast (HFL1, purchased from American Type Culture Collection) cells were seeded into a 12-well plate and grown to confluence in DMEM, supplemented with 10% heat-inactivated FCS and penicillin (100 U/mL)/streptomycin (100 μg/mL), all obtained from Life Technologies. The HFL1 cells were cotransfected with 1 μg reporter plasmid and 0.05 μg control plasmid pRL-TK containing the Renilla luciferase gene by a synthetic integrin-targeted nonviral vector.32 Negative controls included transfections with an empty pGL3 Basic vector. After transfection, cells were resuspended in 10% serum for 1 hour, made quiescent for 16 hours in serum-free medium, and then stimulated with 2% FCS or left untreated and harvested 8 hours later. Firefly and Renilla luciferases were measured sequentially by using the Dual-Luciferase Reporter Assay System (Promega) in a Tropix TR717 microplate luminescence reader (PE Applied Biosystems). Each transfection experiment was carried out in quadruplicate, and experiments were performed twice, each with separate preparations of plasmid DNA.

Statistical Analysis
Allele frequencies were estimated by gene counting. A χ2 test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Statistical significance was set at P<0.05. IL-6 and CRP values were not normally distributed; thus, data were logtransformed before analysis. Fibrinogen values at all time points were normally distributed. Geometric mean±SE values have been quoted where data are shown. Differences between genotypes were assessed by ANOVA and by the Student t test for unpaired data. One-way ANCOVA was performed as previously described.26

Results
Screening of 5' Flanking Region of COX-2 Gene
Screening of the COX-2 gene by PCR-SSCP, covering the region from -1122 to 54 bp, revealed variation at positions -490 and -765 from the transcription start site. Sequencing identified that in each case this involved a single nucleotide substitution. A C residue was replaced by G at position -490 (-490C>G), and a G residue was replaced by C at position -765 (-765G>C); see Figure 1. The -490C>G and -765G>C variants are identified by BsiEI and Acel restriction endonucleases, respectively. These enzymes were used to genotype the variants in 454 white healthy UK men to assess allele frequencies. The -490C>G variant was rare; it was present as a heterozygote genotype only in the single individual for whom PCR-SSCP identified a change. For

-765G>C, the genotype distribution was GG=339, GC=109, CC=6, and the rare allele frequency was 0.13 (95% CI 0.11 to 0.16).

Functional Studies
The -490C>G variant is not located within any known transcription factor binding site; however, the -765G>C is located within a putative Sp1 binding site (Figure 1). Transfection of -765G>C—1811-108 constructs into HFL1 cells revealed the -765C allele to have significantly lower expression than the -765G allele in untreated (28±3%) compared with the healthy control group (33.5% versus 28% lower, P<0.005) and serum-stimulated (31±2% lower, P<0.005) cells (Figure 2). Treatment of HFL1 cells with serum resulted in a 1.64±0.08-fold induction (P<0.001) of luciferase expression in the presence of the -765G allele and a 1.57±0.05-fold induction (P<0.001) in the presence of the -765C allele, relative to their corrected untreated alleles (Figure 2).

Coronary Artery Bypass Graft Study
Baseline characteristics of the 173 bypass patients are shown in the Table. A total of 125 (71%) subjects were receiving statin therapy, and 82 (47%) were receiving β-blockers. There was no difference in the genotype distribution of the bypass patients (GG=115, GC=56, and CC=2) compared with the 454 white healthy subjects (P=0.10). The rare allele frequency (0.17, 95% CI 0.13 to 0.21) was slightly but not significantly higher than that found in the healthy subjects (P=0.19). However, the frequency of carriage of the rare allele (GC+CC combined) was higher in the bypass group compared with the healthy control group (33.5% versus 25.3%, respectively; odds ratio 1.49 [95% CI 1.02 to 2.17]; P=0.04). Carriers of 1 or more -765C allele were combined to assess IL-6, fibrinogen, and CRP levels by genotype, because there were only 2 individuals who had the genotype -765 CC. IL-6 and fibrinogen levels were not significantly different by -765G>C genotype at baseline or at any time after bypass surgery (Figure 3A, data not shown for fibrino-
At baseline, mean CRP levels were lower in carriers of 1 or more −765C allele (2.1±0.2 for GG versus 1.8±0.3 for GC+CC), although this difference was not statistically significant (P=0.37). As expected, BMI was positively correlated with basal CRP levels (r=0.21, P=0.02), but after adjustment for this confounder, levels were not significantly associated with the −765G>C genotype (P=0.18). After bypass surgery, CRP levels were significantly higher at all subsequent postoperative time points compared with baseline (P<0.005 for all comparisons), with peak CRP levels recorded on the third postoperative day (mean 166±5 mg/L). The magnitude of this rise was strongly genotype dependent (Figure 3B). Mean CRP values were lower for carriers of ≥1 rare −765C allele at all times after surgery. This difference remained significant after multivariate analysis for all CRP values recorded for days 2 to 4 (by ANCOVA, P=0.024, P=0.013, and P=0.026 at 2, 3, and 4 days after surgery, respectively). Data were adjusted for age, sex, smoking, cholesterol levels, diabetes, BMI, statin therapy, duration of surgery, and bypass and aortic cross-clamp time. No single parameter was associated with a significant CRP-lowering effect after surgery (BMI and operation duration showed significant positive correlations with peak CRP values). At the peak of CRP levels on day 3, mean CRP levels in carriers of −765C (149.57±8.58 mg/L) were 14±0.05% (P<0.05) lower than patients homozygous for −765G (173.64±6.30 mg/L).

Discussion

COX-2 has a major regulatory role in the production of prostanoids associated with trauma and inflammation. In the present study, we describe a functional COX-2 promoter polymorphism, −765G>C, which is carried by >25% of a healthy UK white population. In vitro studies in untreated human lung fibroblast cells revealed a significantly lower reporter expression from −765C compared with −765G of ~30%, and this difference was maintained, although not enhanced, after stimulation with serum. We also report on in vivo observations of patients undergoing bypass surgery. The mechanism through which −765G>C reduces promoter activity has yet to be shown. However, it is located within a putative Sp1 site in the promoter of COX-2 between −766 to −761 bp upstream from the transcriptional start site and may thus alter Sp1 binding to this region. Sp1 is considered to be a positive activator of transcription, and it can bind and act through G-rich elements, such as the GC box. However, it is also possible that −765G>C may alter the binding ability of other DNA binding elements to this region, including Sp3. Sp3 will compete for the same binding site as Sp1 and has been shown to be a repressor of Sp1-mediated transcription in promoters containing multiple binding sites.

Our in vivo observations of patients undergoing bypass surgery, in which patients with ≥1 copy of the −765C allele were found to have significantly lower plasma CRP levels after surgery compared with patients homozygous for −765G, are completely consistent with the in vitro data, which show −765C with lower promoter activity than −765G and demonstrate a phenotypic consequence of −765G>C in an inflammatory state. The mean CRP levels at baseline (2.0±0.2 mg/L) were also lower in carriers of 1 or more −765C allele, although this difference was not statistically significant. In this case, the influence of −765G>C on CRP measurements is likely to be confounded by other factors when CRP levels are so low. Bypass surgery is a well-characterized inflammatory stimulus that causes a significant acute-phase reaction with induction of inflammatory stimuli associated with COX-2. The effect of −765G>C on in vivo proinflammatory and prothrombotic arachidonic acid derivatives such as PGE2 and thromboxane is not known; however, the association of −765G>C with plasma CRP levels suggests that COX-2 and CRP may be coordinately or sequentially regulated. Indirect evidence to support this includes several studies reporting a concomitant rise in plasma levels of acute-phase proteins linked to prostaglandin activity. NSAIDs lowering COX activity have also been shown to modify acute-phase responses. There is evidence suggesting that IL-6 regulates CRP levels; however, whether the influence of −765G>C on CRP levels is through IL-6 is unclear. The CRP promoter contains several IL-6 response elements, and the functional IL-6 promoter variant, −174G>C, is reported to affect levels of CRP. Induction of COX-2 precedes IL-6 gene expression, and, via the production of PGE2, has been shown to regulate IL-6 synthesis in macrophages. We did not find a significant association
between -765G>C and plasma IL-6 levels after surgery. Possibly, after such an acute severe injury, other mechanisms and stimulatory responses may compensate for any effect of -765G>C on the production of IL-6. Similarly, no association was observed between -765G>C and fibrinogen levels. However, it is likely that -765G>C has a direct effect on CRP expression and that CRP is more sensitive to small changes in COX-2 levels than IL-6 or fibrinogen.

The functional differences between the -765G and -765C alleles, although statistically significant, are relatively small, and it remains to be seen whether the variant will have clinical relevance. It is possible that the influence of -765G>C on CRP levels may have implications for various disease and inflammatory conditions. CRP is one of the earliest of the acute-phase proteins to be elevated and is increased in plasma in response to a wide range of disorders, including infection, trauma, surgery, and cancer. It is also associated with chronic inflammatory diseases, such as rheumatoid arthritis and cardiovascular disease. CRP has a proinflammatory and anti-inflammatory functions. The -765C variant may be protective in cardiovascular disease, inasmuch as raised CRP levels predict cardiovascular events. In acute lung injury, however, the -765C variant may increase risk, inasmuch as CRP acts as a major anti-inflammatory agent, inhibiting neutrophil function.

The -765C variant may also facilitate infection, inasmuch as a major function of CRP is to activate complement and bind to foreign pathogens in innate immunity.

The presence of other naturally occurring polymorphisms in the promoter of COX-2 has been reported; however, none have so far been shown to be functional or to be associated with inflammatory disease. None of these changes are located within recognized transcription factor binding sites. We did not identify these changes in the present study; however, this may be due to low allele frequencies and the ethnic group studied.

The effect of -765G>C and other COX-2 gene variants may have implications in the use of NSAIDs. Regular use of NSAIDs has proven effective in reducing the risk of developing diseases, in particular, cardiovascular disease and bowel cancer. The role of -765G>C may be potentially important, inasmuch as prospective studies have shown that subjects with elevated CRP levels are most likely to benefit from NSAIDs as a preventative measure for myocardial infarction and stroke.

In summary, we have described a common promoter variant in the COX-2 gene, -765G>C, and we have shown in reporter expression studies that the -765C allele, compared with -765G allele, reduces promoter activity. We demonstrate a phenotypic consequence of -765C in an inflammatory state by showing that it is associated with lower CRP levels compared with the levels associated with -765G. Thus, the presence of -765G>C may have implications for a number of chronic and acute inflammatory states associated with disease. Furthermore, genotyping -765G>C in patients using NSAIDs might be of predictive value in terms of their response.

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References