The a_1-*Antichymotrypsin* -51bp Promoter Polymorphism; Functional Activity and its Role in Alzheimer's Disease

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ABSTRACT

There is biochemical evidence that the acute phase protein, a_1-antichymotrypsin (ACT), is involved in Alzheimer's disease (AD), the most common form of dementia. Inflammation increases the level of ACT in the plasma, and it has been suggested that AD provokes chronic neuroinflammation. One of the key proteins in AD is ß-amyloid which associates with ACT in the senile plaques characteristic of AD. Studies to demonstrate a genetic association between polymorphisms in the *ACT* gene and AD have, to date, been inconclusive and contradictory. The discovery, in our laboratory, of a single nucleotide polymorphism in the promoter region of ACT suggested a new marker to test for a genetic association between ACT and AD.

To determine if the *ACT* promoter polymorphism had a functional effect which could modify the risk of AD, the promoter region was cloned into reporter constructs and transfected into mammalian cells in culture. These included hepatocytes, astrocytes, neuronal cells and mixed population neural cells, and these were stimulated with oncostatin M. In astrocytes, the T allele of the promoter allele demonstrates 208% and 146% higher activity than the G allele under basal and stimulated conditions, respectively. In neuronal cells these values are 37% and 46%. Electrophoretic mobility shift assays demonstrated differences in binding affinity of a DNA-binding protein, probably TFIIB, and the two alleles of the *ACT* promoter polymorphism. However, an analysis of *ACT* promoter genotypes in AD cases (n= 389) and controls (n= 335) revealed no significant difference in the distribution of genotypes between cases and controls (p= 0.250), nor did any *ACT* promoter genotype appear to modify the age of onset of AD (p= 0.997). The association between the *ACT* –51bp polymorphism and early onset AD was not investigated, due to the small number of EOAD cases in the population studied.

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1 INTRODUCTION

1.1 General Overview

A vast body of information has been accumulated about Alzheimer's disease in the past century. A number of factors have been discovered that contribute to the aetiology and pathology of this disease, and advances in several branches of biomedical sciences have led to a greater understanding of this condition. As an example the progress made in molecular biology has led to a number of genes being implicated in the aetiology and pathology of Alzheimer's disease, and this understanding improves hope of producing treatments, and possibly cures and preventative measures to combat the disease.

This introduction is intended to give a summary of some of the advances in the molecular pathology related to Alzheimer's disease. The focus of this thesis will be on a_1-antichymotrypsin also known as SERPINA3, and it's role in AD.

1.2 Alzheimer's Disease

1.2.1 Features and Scale of Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population accounting for 50 – 70% of all cases (Borchelt, 1998; Kelly, 2000). It afflicts an estimated 10% of people over the age of 65, with the incidence and prevalence doubling every 5 years, past the age of 60, with the proportion rising to 50% of those older than 85. AD reduces life expectancy, and has a marked effect on the quality of life of sufferers and family (Gracon and Emmerling, 2001). According to the most recent data available, AD is the third most expensive disease after heart disease and cancer in the USA. When the direct and indirect costs of caring for the estimated 4 million Americans suffering from this disease are calculated $100 billion a year is spent on the

disease (Leifer, 2003). Studies in the US that counted cause of death on death certificates showed that there are 50,000 deaths per year caused by AD, making it the eighth leading cause of death in the US, although these figures are likely to be an underestimate (Fu et al., 2004). There is no reliable way, at present of predicting who is at risk, or when symptoms will develop. In addition the age group at risk is the fastest growing sector of the world population, and at the present rate of growth, by the year 2050 there will be a worldwide incidence of 30 million patients with AD (Gracon and Emmerling, 2001). The progress of the disease can be divided into three phases; early, middle and late (Kelly, 2000) (See table 1.2.1).

Phase	Symptom
Early	Absent mindedness, difficulty recalling names / words
	Increasing forgetfulness
	Difficulty learning new information
	Disorientation in unfamiliar surroundings
	Minor, but uncharacteristic, lapses in judgement and behaviour
	Reduction in social activities
Middle	Obvious loss of cognitive skills and marked memory loss
	Deterioration in verbal skills: range and content of speech diminishes
	Increased behavioural disturbance, characterised by; frustration, impatience, restlessness, and verbal or physical aggression
	Obvious decline in social skills
	Emergence of psychotic phenomena; paranoid delusions and hallucinations
Late	Speech becomes monosyllabic and later disappears
	Fleeting psychotic symptoms, because of superimposed delirium
	Behavioural and emotional disturbance
	Loss of bladder and bowel control
	Mobility deteriorates with a shuffling gait or involuntary movements

Table 1.2.1: The progression of symptoms in AD, from (Gracon and Emmerling, 2001; Kelly, 2000).

The symptoms of AD are not uniform, and may be present in a greater or lesser degree in individuals. Depression and anxiety are common in the early clinical stages in mildly impaired patients, while severely impaired patients may have delusions, hallucinations, paranoia, agitation, hostility and aggression (Gracon and Emmerling, 2001).

The chief histopathological characteristics of AD are extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs). The pathology is most profound in the limbic system, the temporal, frontal and association neurocortices, as well as the basal forebrain areas (Friedland et al., 2001). These areas are involved in learning, memory, emotion, judgment, and language (Selkoe, 1999).

Diffuse plaques are present in normal ageing brain, but can develop into senile plaques (Burnett, 2000). The number of senile plaques in AD patients is greater than in ordinary ageing brains (Howlett et al., 2001). Senile plaques form around degenerating nerve endings, which become surrounded by a ß-amyloid (Aß) central core (Howlett et al., 1995). In AD the amyloid is mainly $A\beta_{1-42}$ and the core also contains heparan sulphate proteoglycan, $a_1$antichymotrypsin (ACT, also called SERPINA3), $a_2$macroglobulin (a2M), apolipoprotein E, complement factors C1q, C3d, C4d and components of dying cells (Gracon and Emmerling, 2001). Around this are activated microglia, astrocytes, and dystrophic neurites (Burnett, 2000). Senile plaque count does not correlate with severity of dementia, but plaque count in specific brain areas correlates with degree and type of mental impairment (Cummings and Mega, 1996). Plaques may be present for 10 years before AD is clinically detectable (Carr et al., 1997; Zubenko, 1997). The Aß in senile plaques has been the focus of much study into AD, although recent research has focussed on soluble, intracellular Aß oligomers between 4 and 12kDa (Dahlgren et al., 2002; Delacourte et al., 2002; Walsh et al., 2002b; Walsh et al., 2000; Yang et al., 1999). The processing of the amyloid precursor protein (APP), which also results in APP-C-terminal fragments (APP-CTFs), generates these oligomers. This is thought to trigger the tau pathology, as a decrease in APP-CTFs correlates with an increase in tau pathology, however, the mechanism for this is unknown (Sergeant et al., 2002).

Neurofibrillary tangles (NFT) are an interneuronal structure. They consist of paired helical fragments, containing microtubule associated tau protein and glycolipids (Gracon and Emmerling, 2001). Tau participation is dependent on the degree to which that protein has been phosphorylated, which is controlled by at least eight protein kinases and various phosphatases (Burnett, 2000; Imahori et al., 1998). An increase in intracellular calcium concentration leads to the formation of NFTs and the loss of calcium homeostasis may be a common factor in senile plaque and NFT formation (Cummings and Mega, 1996).

In it's abnormally hyperphosphorylated state tau forms paired helices that disrupt microtubule formation and cause cell death. NFTs accumulate in response to a variety of degenerative, toxic and traumatic insults to the brain. The density of NFTs in the neocortex correlates with the severity of dementia. Age related deposits are present in the hippocampus and inferior temporal cortex of non-demented elderly people (Burnett, 2000). NFTs only develop in a few types of cortical pyramidal cells, starting in the entorhinal cortex. The destructive processes then spread in a predictable fashion to the isocortex (Braak et al., 1993).

1.2.2 Pathology of Alzheimer's Disease

Although definitive diagnosis of AD can only be made on autopsy, often decades after the onset of disease (Gracon and Emmerling, 2001; Kelly, 2000; Walker, 2000), there are gross changes to the brain during the course of the disease. These include a decrease in brain weight, brain atrophy, specifically in the cortex and hippocampus, and the selective loss of the medial temporal lobe (Gracon and Emmerling, 2001). It can be seen that these changes take place in the brain and so, are difficult to study, as extraction of live tissue is almost impossible. In addition, the brain decreases in weight during normal ageing, from about the 5^{th} decade in females, and the 6^{th} in males (Burnett, 2000). AD affects the rate and pattern of weight loss in the brain. There is progressive neuronal shrinkage and death, that affects the hippocampus, brain stem, basal forebrain and temporoparietal areas of the neocortex and this leads to diffuse

cortical atrophy and, later, dilated ventricles, which are characteristic of AD (Burnett, 2000) (See figure 1.2.1).

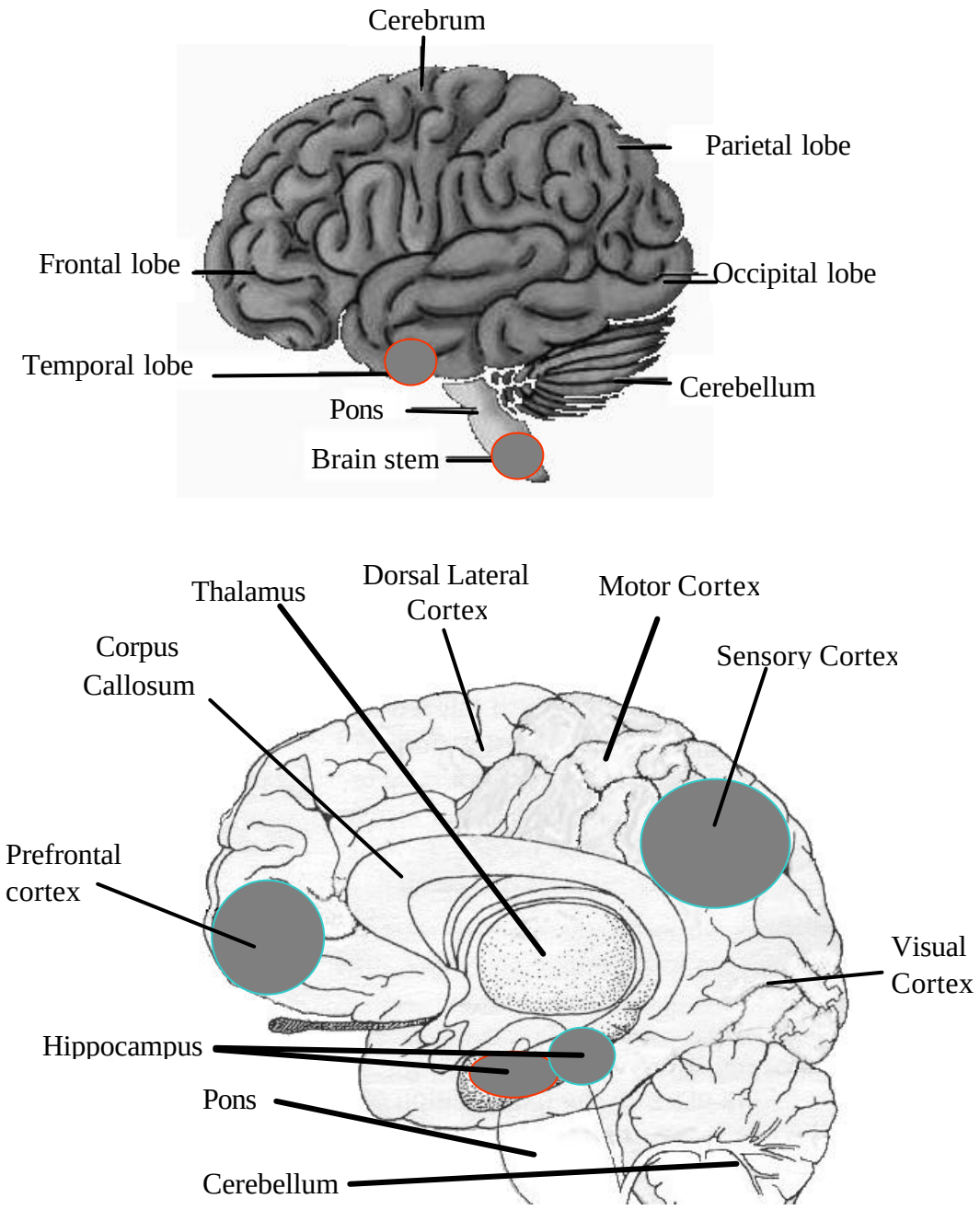

Figure 1.2.1: Diagrams of the human brain, showing some of the areas affected by Alzheimer's disease. Sections highlighted in red are some of those that show neuronal shrinkage, while the blue areas are amongst those that decrease in mass.

There are histopathological changes to the brain during AD as well. These include synaptic loss, and granulovacuolar degeneration, as well as neuronal cell death, amyloid plaque formation and amyloid angiopathy, and NFT formation, consisting of paired helical fragments of hyperphosphorylated tau protein and glycolipids. There are also changes that indicate an inflammatory response, such as the activation of microglia and astrocytes. The most consistent change in AD brains is cholinergic loss in CA1 region of hippocampus, but the timing and its connection in relation to the onset of symptoms is unknown (Gracon and Emmerling, 2001). Cell death in the forebrain and stem cell nuclei leads to decreased synthesis of neurotransmitters. Cholinergic cells project from the basal forebrain into the cortex and hippocampus, areas that are involved in AD (Gracon and Emmerling, 2001). The cholinergic system is involved in memory and learning, and neuronal loss in the nucleus basilis of Meynert (nbM), and loss of choline acetyltransferase (CAT) activity correlates with plaque formation, possibly by promoting the amyloidogenic metabolic pathway of APP (see 1.2.2) (Burnett, 2000). The cholinergic deficits associated with AD include the atrophy of cells in nbM, a loss of cholinergic cortical neurones, decreased CAT synthesis, which leads to decreased acetyl choline synthesis, a decrease in nicotinic receptor density, and possibly a functional change of the muscarinic receptors (Burnett, 2000). The loss of cholinergic neurones, and the disruption of choline activity may contribute to memory and cognitive changes, and these neurones are believed to be especially sensitive to $Aß_{1-42}$ compared with other cell types (Gracon and Emmerling, 2001).

The direct causes of neuronal damage are thought to come about from a number of sources. These include direct toxic effects of Aß, in particular oligomers of $Aß_{1-42}$ (Sergeant et al., 2003), free radicals that promote Aß, disruption to cells caused by NFT formation, altered cell membrane function, leading to calcium influx. These factors can promote Aß and NFT formation (Cummings and Mega, 1996), as well as immune mechanisms resulting in the cell lysis due to complement activation (Burnett, 2000).

It is believed that there are a number of amyloid isoforms found in AD, including insoluble $Aß_{40}$ and soluble $Aß_{42}$ oligomers (Delacourte et al., 2002). In this spectrum of Aß isoforms, it has been found that oligomers are 10 times more neurotoxic than insoluble fibrils and 40 times more neurotoxic than the unaggregated peptide. Oligomers of $Aß_{42}$ are more toxic than $Aß_{40}$, and $Aß_{42}$ oligomers correlate with the severity of neurodegeneration in AD (Dahlgren et al., 2002). The oligomeric form of Aß is produced intracellularly, especially in neural cells (Walsh et al., 2000) and can be induced by exogenous Aß fibrils (Yang et al., 1999). Aß oligomers are able to inhibit hippocampal long-term potentiation in rat brains, and in mouse models, dysfunctional amyloid processing appears to precede tau pathology in pre-clinical AD (Sergeant et al., 2003). Dysfunction of amyloid precursor protein processing has an effect on neuron to neuron propagation of tau pathology (Delacourte et al., 2002), indicating that Aß production may be one of the earlier events in AD aetiology. The balance between production and clearance of Aß is altered in AD (Evin and Weidemann, 2002).

Diagnosis of AD can only be made *post mortem*, for a number of reasons. It is rare that patients present a classical form of AD, but instead they show mixed pathologies, and there are unresolved issues about the pathological classification of all the main dementias (Walker, 2000). There are two conditions in particular which overlap with AD; vascular dementia, and dementia with Lewy bodies (Gorelick et al., 1996). In addition, there are a number of conditions that can be confused with AD on clinical examination. These include; pseudodementia, delirium, fronto-temporal dementia, alcohol related dementia, toxic and drug encephalopathies, normal pressure hydrocephalus, chronic subdural haematoma, certain infectious diseases, Cruetzfeldt-Jakob disease and various endocrine and metabolic disorders (Walker, 2000) all in addition to vascular dementia and dementia with Lewy bodies.

1.2.3 Causes and Risk Factors of Alzheimer's Disease

Although there is no known specific cause of AD in most patients (Burnett, 2000), there are a number of risk factors associated with AD, and these can be

placed into various categories. Demographic factors, psychological factors, medical factors, environmental factors and genetic factors are all thought to play a role in the development of AD. Demographic factors are probably the most important category of risk factors for AD. The most important risk factor for AD is increased age. An increase in age results in more time for cells to be exposed to toxins, and greater neuronal cell death (Burnett, 2000). Ischaemic events, hypoxic events, oxidative stress, free radical generation, inflammation and repair occur all through life, and this leads to loss of neurones and synapses, and decreased cortical acetylcholine levels. The situation is exacerbated by increased concentrations of $A\beta_{1-42}$ (Gracon and Emmerling, 2001; Licastro, 2001; Licastro et al., 2001). Even accounting for the effects of longevity, women have a greater risk for AD, although there is a decreased incidence of AD in women who undergo hormone replacement therapy, and oestrogen has been linked to improved cognitive performance (Richard and Amouyel, 2001).

It has been reported that low educational levels and poor linguistic skills increases susceptibility to AD as an increase in educational levels indicates greater cerebral reserve and stronger synaptic connections (Snowdon et al., 1997). A history of depression leads to an increased risk of late onset AD, and the depressive episodes can occur up to 10 years before the onset of AD (Burnett, 2000). Depression and AD are both associated with over activity of the hypothalamus – pituitary – adrenal axis, with an increase in circulating levels of cortisol (Burnett, 2000). In rats and primates increases in corticosteroid levels can cause hippocampal damage, which worsens with age.

There are medical conditions that can alter the risk of developing AD (see table 1.2.2). The excessive microvascular pathology and blood-brain barrier abnormalities seen in AD have challenged the traditional division between AD and vascular dementia, and has led to the implication of blood vessel abnormalities in the pathogenesis of AD (Burnett, 2000). While blood pressure decreases immediately before the onset of dementia, hypertension early in life is a risk factor for the disease (Kilander et al., 1998).

Medical condition	Reference
Hyper- and hypotension	Gracon & Emmerling, 2001
Hyperthyroidism	van Duijn, et al, 1992
Epilepsy	van Duijn, et al, 1992
Hypercholesterolaemia	Gracon & Emmerling, 2001
Coronary artery disease	Gracon & Emmerling, 2001
Atherosclerosis	Gracon & Emmerling, 2001
Cerebrovascular disease	Gracon & Emmerling, 2001
Atrial fibrillation	Gracon & Emmerling, 2001
Down's syndrome	Richard & Amouyel, 2001
Parkinson's disease	Richard & Amouyel, 2001
Damage to endothelial cells	Gracon & Emmerling, 2001
Damage to blood-brain barrier	Gracon & Emmerling, 2001

Table 1.2.2: Medical factors that increase the risk of AD.

Head injury involving loss of consciousness is also a risk factor for AD (van Duijn et al., 1992). The risk may be greatest during the pre-symptomatic phase of AD when the capacity for recovery is reduced. On *post mortem* an over-expression of APP has been observed, and NFT and senile plaques have been found in the brains of boxers with dementia pugilistica. In addition, Aß deposition has been found in 33% of people who die after severe brain injury, and the combination of head injury, and an *apolipoprotein E* ε4 allele (see below) leads to a 10 fold greater risk of AD (Mayeux and Ottman, 1998).

Another medical condition that can modify the risk of AD is diabetes. It has been shown that experimentally induced diabetes results in memory deficits, and hyperglycaemia and hyperinsulinaemia could increase the vulnerability of neurones to damage by pathological events, for instance; hypoxia, hypoglycaemia or Aß deposition (Messier and Gagnon, 1996). In addition, adults with mature onset diabetes mellitus have a 1.7 fold increase in their risk of dementia, rising to 2 fold greater in men (Leibson et al., 1997).

Nutrition and toxins can also alter the chances of developing AD, although a significant effect of toxins as risk factors has yet to be conclusively

demonstrated (Burnett, 2000). Heavy metal poisoning has long been considered to play a role in the development of AD. It has been suggested that redox metals such as copper, iron and zinc may play a role in both Aß and tau pathologies (Cuajungco and Faget, 2003; Shin et al., 2003; Sparks and Schreurs, 2003). It is thought that aluminium may enter cells and interfere with production of Aß, induce aggregation of Aß and contribute to the formation of NFTs, by a mechanism yet to be fully demonstrated. Conversely, occupational exposure to solvents and lead may result in a small drop in the risk of developing AD (Burnett, 2000). Other toxins thought to modify the risk of developing AD are heavy alcohol consumption, which lowers the risk of AD, at a cost of increasing the likelihood of developing other forms of dementia, and controversially, an increased number of cigarettes smoked per day may lead to a lower risk of developing AD (Burnett, 2000). Differences in nutrition have been shown to modulate the risk of AD. A deficiency of thiamine leads to vascular endothelial cell changes, which lead to local leakage and local Aß expression. High fat diets, and high blood cholesterol increases the risk of AD, while lowering these decreases the risk of AD. High cholesterol levels are believed to interfere with a-secretase production, and therefore favour the production of $Aß_{1-42}$ (Burnett, 2000). Providing support to the theory that AD can result due to a build up of damage by free-radicals and reactive oxygen species over life, a diet high in anti-oxidants might provide some benefit in lowering the risk of developing AD (Behl and Moosmann, 2002; Burnett, 2000; Carr et al., 1997; Goodman et al., 1994; Lauderback et al., 2002).

The theory of neuronal reserve is used to explain the finding that having a small head circumference leads to a more rapid progression of AD (Schofield et al., 1995). This theory states that a small head circumference, a small brain size, a low IQ or a lack of education are indicative of a lower number of synapses, decreased neuronal plasticity, and therefore a diminished ability to establish alternative circuitry to bypass damaged pathways, and therefore a more rapid onset of symptoms (Gracon and Emmerling, 2001).

The long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, indomethacin or dapsone, reduces risk by delaying the onset of

symptoms, probably mediated by decreasing the levels of microglial cell activation by Aß, and providing support for the belief that AD is an inflammatory disease (Gracon and Emmerling, 2001; Richard and Amouyel, 2001).

Many non-demented elderly patients come to autopsy with pathologies that significantly resemble AD, and it has been hypothesised that there is a final common pathway between genetically determined AD, familial AD, sporadic AD, Down's syndrome and normal ageing. It is also believed that ones genetic background may determine the cause of dementia, although mutation accounts directly for less than 5% of AD cases, there are a large number of putative genetic risk modulators for AD. The genes known to cause familial Alzheimer's disease are listed in table 1.2.3, while those thought to promote sporadic Alzheimer's disease are listed in table 1.2.4.

Chromosome	Gene	Number of pathogenic mutations	Effect of mutation
21	*Amyloid Precursor Protein*	12	Promotes amyloidogenic metabolism and Aß deposition
1	*Presenilin 2*	10	Promotes Aß deposition
14	*Presenilin 1*	135	Promotes Aß deposition

Table 1.2.3: Amyloid-processing genes directly implicated in Alzheimer's disease. A list of the pathogenic mutations implicated in familial Alzheimer's disease can be found on the Alzheimer disease & Frontotemporal Dementia Mutations Database (www.molgen.ua.ac.be/ADMutations).

Mutations in the genes listed in table 1.2.3 are known to cause familial early onset AD. These genes are all involved in the processing of amyloid precursor protein to ß-amyloid, which is central to the pathogenesis of AD. Only one gene has been proven to alter the risk of AD, the lipid transporter *apolipoprotein E* (*APOE*) gene (Rocchi et al., 2003; Saunders et al., 1993). Table 1.2.4 lists some of the genes that have been suggested as AD

susceptibility genes, that modify the risk of late onset sporadic and familial AD. This is not a complete list and as more knowledge is gained to determine the mechanisms of AD aetiology and pathology other genes will be added to the list of genes linked to AD. Indeed, as high throughput genotyping becomes more readily available the number of potential candidate genes are being discovered with increasing frequency (Bertram et al., 2000; Blacker et al., 2003; Tanzi and Bertram, 2001), although care is required to validate such studies, to avoid false-positive associations (Emahazion et al., 2001).

Gene	Chromosome	Role
Interleukin-6	1q21	Regulation of inflammatory response
Nicastrin	1q23	Component of ?-secretase
Transferrin C2	3q21	Inhibits lipid peroxidation
Neprilysin	3q21-q27	Degrades Aß
Butyrylcholinesterase	3q26.1-26.2	Maturation of plaques
Nitric Oxide Synthase	7q35	Regulates production of nitric oxide
N-Acetyltransferase	8p23.1-21.3	De-toxification of exogenous toxins
Very Low Density Lipoprotein Receptor	9p23	Alters lipoprotein metabolism
Insulin Degrading Enzyme	10q23-q25	Degrades Aß
Cathepsin D	11p15.5	Cleaves APP, degrades tau protein
LBP-1c/CP2/LSF Transcriptional Factor	12q13	Protective effect
a2-Macroglobulin	12q13.3-p12.3	Clearance and degradation of Aß
Low Density Lipoprotein Receptor-Related Protein	12q13-q14	Production and clearance of Aß
Presenilin 1 Promoter	14q24	Regulates PS1 activity
a_1-Antichymotrypsin	14q32.1	Promotes Aß fibrilisation
5-HT (Serotonin) Transporter	17q11.1-q12	Involved in serotonin transmission
Bleomycin Hydrolase	17q11.1q12	Secretion of Aß
Angiotensin Converting Enzyme	17q23	Inhibits Aß aggregation
Myeloperoxidase	17q23.1	Oxidation induced damage
Transforming Growth Factor – ß1	19q13.1-q13.3	Modulates cellular response to injury
Apolipoprotein E (Apo E)	19q32.2	Aß deposition
Apo E Promoter	19q32.2	Regulates Apo E
Cystatin C	20p11.2	Co-localises with Aß

Table 1.2.4 : Susceptibility genes in Alzheimer's disease (Combarros et al., 2002; Ling et al., 2003; Nicholl et al., 1999; Rocchi et al., 2003).

1.2.3.1 Apolipoprotein E

The *Apolipoprotein E* (*APOE*) gene is found on chromosome 19, and codes for the major serum lipoprotein involved in cholesterol metabolism (Lendon et al., 1997). The apolipoprotein consists of 299 amino acids (Berr et al., 1994), making it far too big to cross the blood brain barrier, but it is synthesised in the brain, mainly by astrocytes (Lambert et al., 1997; Lendon et al., 1997).

Apolipoprotein E level has been identified as being a risk factor for hypercholesteraemia (Berr et al., 1994) and the e4 allelic variant of Apolipoprotein E gene is the single most common genetic determinant of AD susceptibility (Lendon et al., 1997). The gene has three alleles, ε2, ε3, and ε4 resulting from polymorphisms at positions 112 and 158, with ε4 associated with a greater risk of developing late onset AD (Lendon et al., 1997). For ε4 heterozygotes the risk of developing AD is 2 to 4 fold greater, and ε4 homozygotes the risk is 5 to 18 fold higher for developing AD compared to those with other *APOE* alleles (Rojas-Fernandez et al., 2002). While the mechanism by which *APOE* alleles lead to AD is unknown, the pathology could result from the absence of ε3 allele, or the presence of the ε4 allele, although the risk of AD appears to rise in a dose dependent manner with the number of ε4 alleles present. The more ε4 alleles present, the lower the age of onset of AD and the greater the rate of Aß deposition (Artiga et al., 1998; Burnett, 2000; Lendon et al., 1997). In addition to this, the same studies have demonstrated a protective effect of ε2 alleles (Artiga et al., 1998; Burnett, 2000; Lendon et al., 1997). It is known that lipoproteins that contain the e4 isoform of the protein are cleared more effectively from the blood (Lendon et al., 1997). There have been suggestions that e4 facilitates the deposition of Aß (Burnett, 2000), or that since ApoE binds to tau protein (Burnett, 2000), the isoform may influence formation of NFTs, for example e3 decreases the level of tau protein phosphorylation, so that e4 promotes the assembly of paired helices, and NFT formation (Berr et al., 1994). Another suggestion is that *APOE* alleles interact with other genetic or environmental factors, such as brain injury (Lendon et al., 1997; Snowdon et al., 1997). The expression of ApoE increases with neuronal damage, and it is possible that it may be part of a compensatory mechanism to replace synapses (Lambert et al., 1997). The risk of AD could be related to ApoE expression rate, and it is known that levels of ApoE expression rise after insult. It is also the case that different alleles are expressed at different rates (Lambert et al., 1997). It is possible that the additional risk of AD associated with e4 may not be entirely due to intrinsic biochemical properties of isoforms of ApoE, but instead results from the expression rate (Artiga et al., 1998). The

level of ApoE expression rises with the ε3 allele over ε4, in both patients and controls (Lambert et al., 1997). In addition there are polymorphisms in the regulatory regions of the *APOE* gene, such as that at position –491, that is in linkage disequilibrium with *APOE* polymorphisms, and can alter *APOE* expression (Thome et al., 1995). The *APOE* –491 promoter polymorphism is not thought to be in direct association with AD, but this polymorphism could act as a genetic modifier for AD risk, although other studies suggest otherwise (Licastro et al., 1999b). This promoter polymorphism is not the only modifying factor associated with *APOE*, while genetic factors are not the sole type of risk modifiers that act in tandem with the *APOE* gene. Aside from the synergy between *APOE* e4 and brain injury, other biological factors can act with this gene to influence risk of developing AD. There is an inverse relationship between number of ε4 alleles and residual brain choline acetyltransferase activity and nicotinic receptor density (Poirier et al., 1995), which may have an implication on the responsiveness of cholinomimetic therapies (Burnett, 2000), as well as providing information about the causes of AD.

1.2.3.2 The Presenilins

Two related genes accounting for a number of cases of early onset familial AD are the *presenilins*, *PS1* and *PS2*. They are located on chromosome 14 and chromosome 1 (Citron, 2001; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). The *presenilins* belong to a family of genes with a high degree of interspecies conservation. *PS1* is composed of 12 exons, and is about 75kb in length, with open reading frames in exons 3 to 12. *PS2* is greater than 90kb in length, and is arranged into 10 exons (Citron, 2001). The *presenilin* genes, although sharing 67% sequence homology are not the product of recent gene duplication (Levy-Lahad et al., 1996).

The majority of cases of familial AD accounted for by the two presenilins are caused by mutations of *PS1* (Rogaev et al., 1995; Schellenberg, 1995; Sherrington et al., 1996; Sherrington et al., 1995). *PS1* encodes a 467 amino acid polypeptide, predicted to contain seven to nine transmembrane spanning domains (Sherrington et al., 1995). In mammalian cells it was found that the

polypeptide spans the cell membrane eight times. There are three hydrophilic domains, an N-terminus, a C-terminus and a loop, that project into the cytoplasm. The N-terminus and loop domains of PS1/2 have a consensus sequence for phosphorylation by cytosolic kinases (Arai et al., 2001). PS1/2 both undergo endoproteolytic processing which asymmetrically cleaves the proteins into C- and N-terminal fragments. Concentrations of these fragments do not increase in parallel with increases in mRNA and full length polypeptide (Citron et al., 1992; Haass and De Strooper, 1999; Mercken et al., 1996; Thinakaran et al., 1996). The 43kDa precursor protein is cleaved to a 27kDa N-terminal fragment and a 17kDa C-terminal fragment in various human organs, including the cortex, cerebellum, thyroid, lung, heart, liver, spleen, pancreas, kidneys, adrenal glands, testis and muscles (Arai et al., 2001). The cleavage occurs between amino acids 260 and 320. Familial AD-linked mutations result in alternative splicing which causes a deletion in exon 9, which codes for amino acids 290 to 319, and this prevents cleavage (Arai et al., 2001). With the exception of exon 9 mutants, there is little full length PS1 found, only fragments, and this is the only disease-linked mutation that dramatically alters proteolytic processing of *PS1*.

A gene with a high degree of sequence homology to *PS1* was identified on chromosome 1, and was identified as *PS2*. Mutations of *PS2* are a rare cause of familial AD, but links between familial AD and markers on chromosome 1 have been made in Volga German and Italian pedigrees (Burnett, 2000).

Presenilin 1 is a foetal protein, and is present in a much higher concentration in the cerebral cortices of foetuses compared to adults, but the concentration decreases rapidly during development (Okochi et al., 1998). In the human foetal cerebral cortex the concentration of PS1 is 300fmol / mg, but this drops to 14fmol / mg by adulthood (Okochi et al., 1998). Evidence of the importance of PS1 in development comes from the finding that PS1 null mice die at the foetal stage of development (Wong et al., 1997).

There is a high degree of homology between PS1/2 and the *Caenorhabditis elegans* protein SEL-12 (Arai et al., 2001). SEL-12 facilitates signalling mediated by the LIN 12 / Notch family of receptors (Levitan and Greenwald,

1995; Levy-Lahad et al., 1995). A decrease in SEL-12 leads to an egg-laying defect in *C. elegans*, which can be reversed by PS1/2, and it is thought that SEL-12 is interchangeable with the presenilins. SEL-12 also reveals that familial AD-linked *PS1* mutations do not eliminate PS1 function, as there are 6 independent familial AD-linked *PS1* mutations that can restore "missing" SEL-12 activity (Arai et al., 2001).

Transfection studies and transgenic mice (expressing PS1/2 and APP) experiments show that familial AD-linked PS variants influence APP processing (see section 1.2.3.3), which leads to an increase in the insoluble form of ß-amyloid, $Aß_{1-42}$ (Arai et al., 2001; Gracon and Emmerling, 2001). It has been suggested that PS1 and PS2 may be the γ-secretases that proteolytically cleave the amyloid precursor protein, and can influence the production of $Aß_{1-42}$ (De Strooper, 2000). More recent work has argued that ?-secretase is a complex of different proteins (De Strooper and Annaert, 2001; Esler and Wolfe, 2001; Octave et al., 2000), and that PS1 and PS2 are components of that complex, possibly even forming the active site of ?-secretase. It is believed that the ?-secretase complex is formed after leaving the endoplasmic reticulum (Maltese et al., 2001). Although evidence is starting to mount in favour of the theory that ?-secretase is a complex of proteins there is still debate over this. The main points of contention are as follows:

(i) The spatial paradox. The PS's predominantly localise to intracellular ER and Golgi-associated compartments, but PS cleavage of Notch and APP occurs at cell surface. How is it possible for a complex located in one region of the cell to cleave a protein found in another? (Cupers et al., 2001; Fortini, 2001)

(ii) Notch-1 processing is highly selective, APP processing is relaxed, allowing for the production of different products. If ?-secretase is a single entity, it must be extremely unusual to be able to generate different lengths of Aß (Sisodia et al., 2001).

Although these two points are difficult to address, they can be reconciled with the theory that the presenilins are involved in ?-secretase processing. The evidence in favour of this hypothesis is strong.

(i) *PS* mutations modulate Aß production, and alter ?-secretase activity. Primary neurones from *PS1* knockout mice embryos have been cultured. When transfected with *APP*, these cultures revealed a marked decrease in ?-secretase activity, the maturation and distribution of APP was not affected, nor was a- or ß- APP release. Substrates of ?-secretase increased dramatically in these cultures. Compared to their $PS1^{+}$ littermates, Aß production decreased 20%. *PS 1/2* double knockout mice were devoid of all ?-secretase activity (Esler and Wolfe, 2001).

(ii) There is evidence of direct interaction between PS1 and APP, as they co-immunoprecipitate. The substitution of conserved aspartate residues of presenilins reduces ?-secretase activity in the cell. Compounds that inhibit ?-secretase activity bind directly to presenilins (Cupers et al., 2001; Maltese et al., 2001)

(iii) Database comparisons have identified conserved PS motifs similar to bacterial proteases (Fortini, 2001).

(iv) The generation of ß-amyloids from a presenilin 1/APP fragment was blocked by a ?-secretase inhibitor (Shizuka-Ikeda et al., 2002).

1.2.3.3 Amyloid Precursor Protein

Beta-amyloid (Aß), one of the key components of senile plaques, is a 39 – 43 amino acid amyloidogenic peptide, derived from the proteolytic processing of the amyloid precursor processing (APP) gene, found on chromosome 21 (Wasco, 2001). The *APP* gene is composed of 18 exons, of which three can undergo alternate transcription, to produce different mRNA species, including APP_{695}, APP_{751}, and APP_{770}. The *APP* gene contains a Kunitz protein inhibitor (KPI) domain, and a domain with homology to the MRC Ox-2 antigen (Roberts et al., 1995; Wasco, 2001). The APP mRNA transcripts are widely, and possibly, ubiquitously, expressed, and display tissue specific and developmental regulation. Their transcription is low in the lungs and liver, but high in the heart, brain and kidneys. There is specificity to the transcript locations, but all APP transcripts are found in the brain (Wasco, 2001). Amyloid precursor proteins (APPs) show features of cell membrane receptor proteins. They have

extracellular, transmembrane and intracellular domains (Roberts et al., 1995), and can exist either as an integral membrane-associated type I or after proteolytic cleavage, a secreted protein (sAPP) (Wasco, 2001). Both forms of the protein exhibit extensive post-translational modifications. APP exists in three isoforms of 770, 751 and 695 amino acids, which may or may not contain a 57aa insert with homology to the Kunitz family of proteinase inhibitors. In addition, the cytoplasmic tail of APP undergoes Thr and Tyr phosphorylation, which can alter the affinity for APP with other proteins, such as growth factor receptor-bound protein 2 (Goldgaber et al., 1987; Kang et al., 1987; Oltersdorf et al., 1989; Podlisny et al., 1990; Tanzi et al., 1987; Van Nostrand et al., 1989; Zhou et al., 2004). The function of the amyloid precursor protein has not been determined, but recent research has suggested that the sAPP could be a transcription activator, since it forms a multimeric complex with the nuclear adapter protein Fe65 and the histone acetyltransferase Tip60 (Cao and Sudhof, 2001). The precursor protein can be metabolised by one of at least two pathways, which produce APP fragments. These fragments may, or may not contain Aß (Wasco, 2001). The pathways involve a-secretase, ß-secretase, and γ-secretase. The a-secretase pathway, which is the major pathway, involves cleavage at the Aß domain, and cleavage at this point precludes the formation of Aß (Howlett et al., 2000). The ß-secretase pathway leads to cleavage at the N-terminal and produces a C-terminal fragment, which can then be cleaved by γ-secretase, which produces Aß (Golde et al., 1992; Knops et al., 1992; Seubert et al., 1992) (Figure 1.2.2).

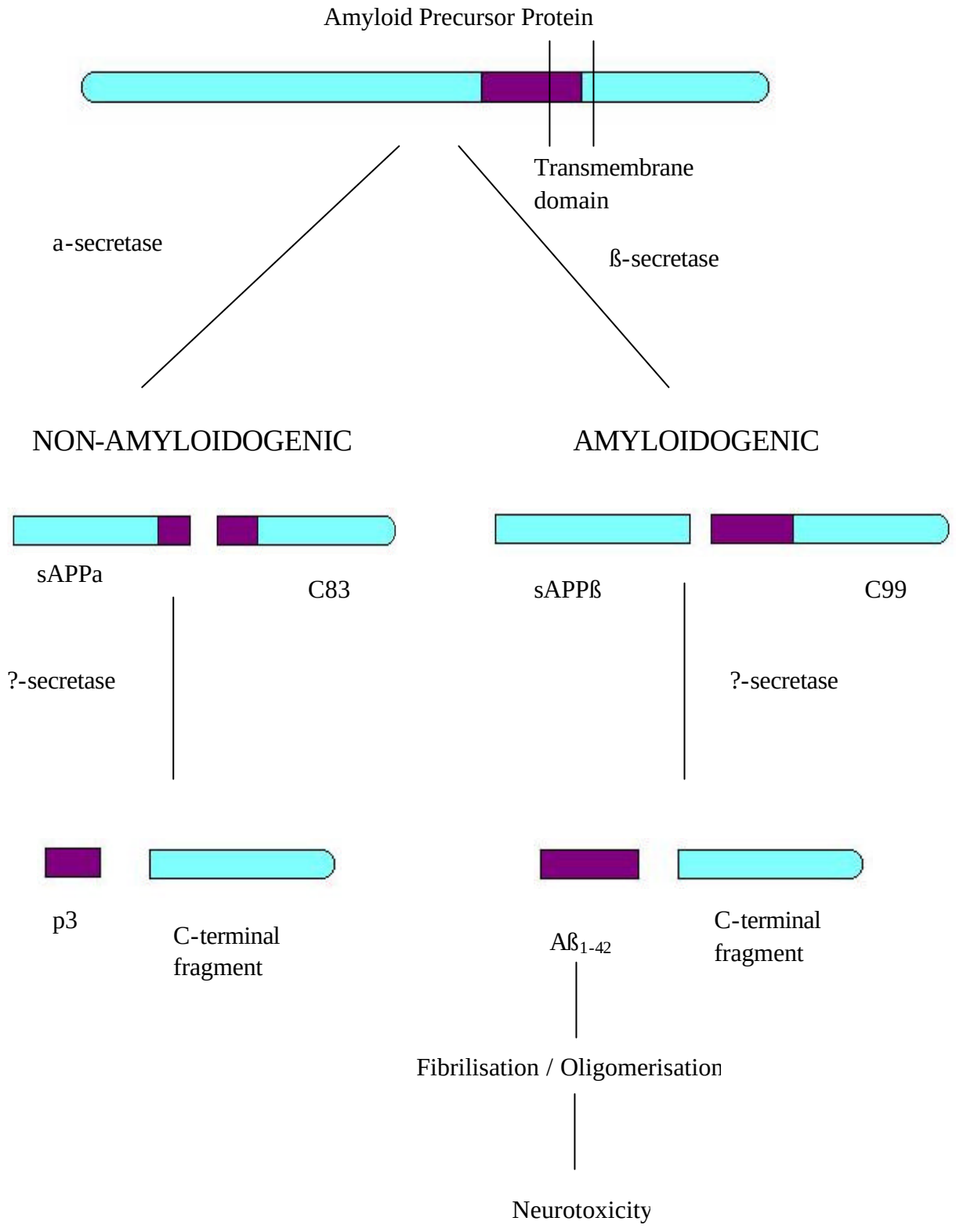

Figure 1.2.2: Cartoon showing processing of the Amyloid Precursor Protein by a-, ß- and ?-secretases. The amyloid precursor protein is shown in light blue, with the ß-amyloid domain shown in purple. Black lines bracket the position of the transmembrane domain. The approximate positions of the APP cleavage sites are shown.

1.2.4 Amyloid Precursor Protein and Amyloid ß Production

It is likely that the pathways shown in figure 1.2.2 need to be balanced to keep Aß below pathological levels, since both pathways occur in healthy brain (Burnett, 2000). Aß is a normal product of neuronal cells and is found in the CSF and plasma, although in AD it fibrilises to form insoluble deposits in parenchyma and vasculature of vulnerable brain regions (Howlett et al., 2000). 10% of the Aß produced is the relatively insoluble $Aß_{1-42}$, which preferentially aggregates into the ß-pleat configuration. Until recently, it had been thought that the insoluble aggregated form of Aß was toxic to neurones and caused apoptotic cell death and that the soluble form had little toxicity (Burnett, 2000; Howlett et al., 1995; Simmons et al., 1994). Newer evidence, however, points to smaller Aß chains, oligomers, as possessing neurotoxic properties, with aggregated Aß forming a reservoir of Aß molecules (Kayed et al., 2003; Lambert et al., 2001; Walsh et al., 2002a; Walsh et al., 2000). Mutations in the *APP* gene can increase Aß production (Howlett et al., 2000), and lead to a decrease in the potentially neuroprotective sAPPa (Mattson et al., 1992).

Mutations in the *APP* gene were the first familial AD gene defects to be reported (Chartier-Harlin et al., 1991; Goate et al., 1991; Hendriks et al., 1992; Levy et al., 1990a; Levy et al., 1990b; Mullan et al., 1992; Murrell et al., 1991), but these mutations cause a small proportion of early onset familial AD (Tanzi et al., 1992). There are at least twelve *APP* mutations, currently known to cause AD in an autosomal dominant manner in families with early onset AD (Wasco, 2001). All favour $Aß_{1-42}$ production over $Aß_{1-40}$, and all are located within exons 16 or 17 of the gene, exons which code for the Aß domain (see figure 1.2.3).

Finally, sufferers of Down's syndrome (trisomy 21), have three copies of the *APP* gene, and show Aß deposits as early as 12 years old (before developing other AD pathologies), and can go on to develop AD (Iwatsubo et al., 1995; Singleton et al., 2004).

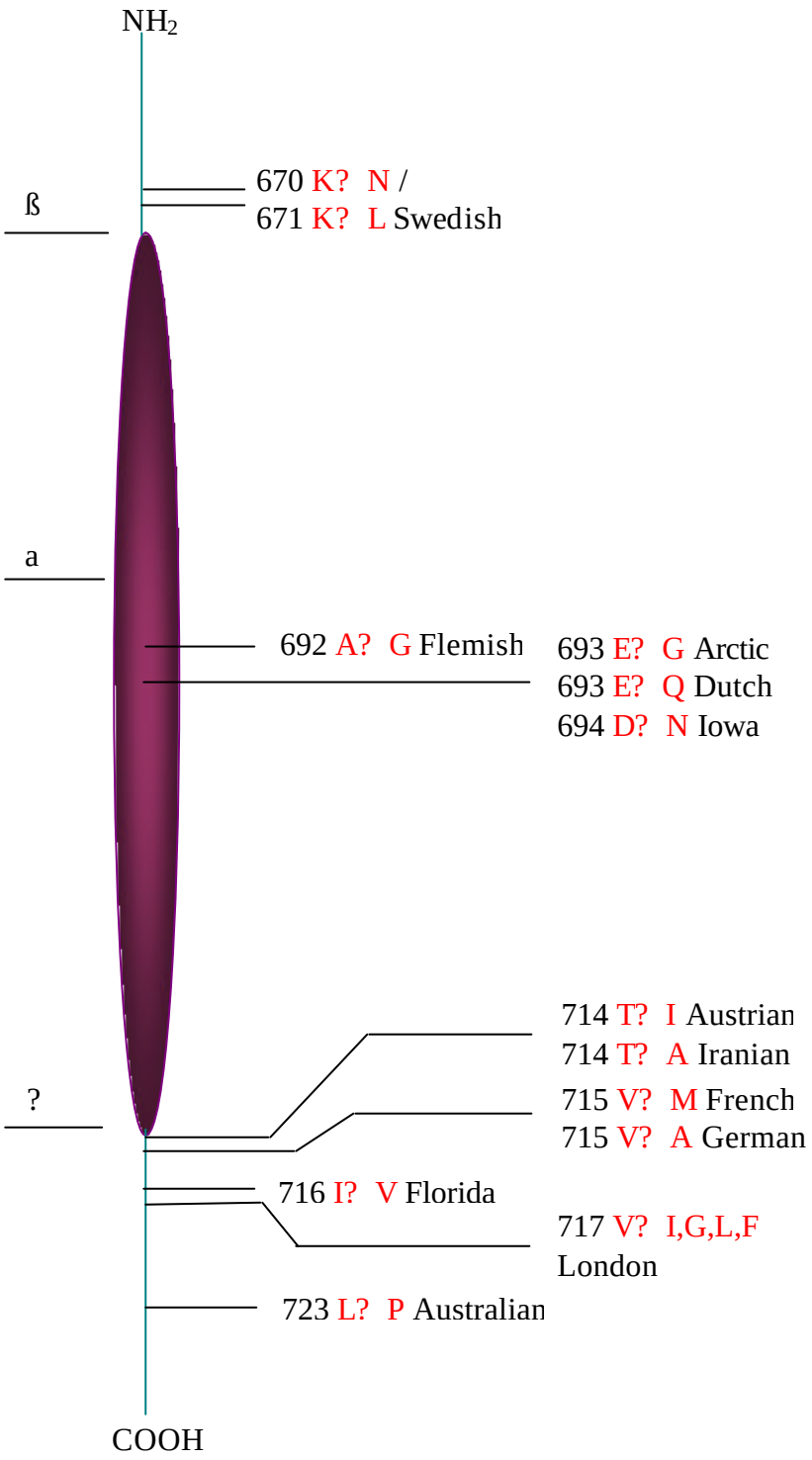

Figure 1.2.3: The Aß region of the Amyloid Precursor Protein. Purple section is the Aß domain, arrows show secretase (a-, ß-, and ?-) cleavage sites. The shaded box represents the membrane domain. Lines show positions of FAD associated coding mutations, with text to describe amino acid position, the name of the mutation and red letters describing amino acid substitution.

1.2.4.1 Amyloid ß Turnover

The role of Aß in the pathology of AD has meant that a good deal of attention has focussed on the metabolism of APP to Aß in the attempt to elucidate the mechanism of AD pathogenesis. There are several different ways believed to reduce the Aß load in the brain. These involve reducing the amount of Aß being produced, preventing Aß from causing harm, or clearing Aß from the brain before it can cause any damage.

The level of amyloid in the brain is the result of a balance between biosynthesis from APP, via the amyloidogenic pathway, and its catabolism by a number of proposed proteolytic enzymes (see figure 1.2.4) (Evin and Weidemann, 2002). Recently, research has focussed on the clearance of $Aß_{42}$ from the brain. This area had been somewhat neglected, in favour of learning about the processes of $Aß_{42}$ synthesis, but the discovery of a family of metalloendoproteases that degrade amyloid fibrils has led to increased interest in $Aß_{42}$ clearence. It has also been demonstrated that astrocytes in the brains of adult mice are attracted to Aß by monocyte chemoattractant protein-1, which is present in SPs, and can bind and degrade Aß *in vitro* (Wyss-Coray et al., 2003). The amyloid-degrading enzymes include insulin-degrading enzyme (IDE) (Chesneau et al., 2000; Vekrellis et al., 2000), metalloendopeptidase EC 3.4.24.15 (Yamin et al., 1999), neprilysin (Iwata et al., 2001) and endothelin-converting enzyme-1 (Eckman et al., 2001).

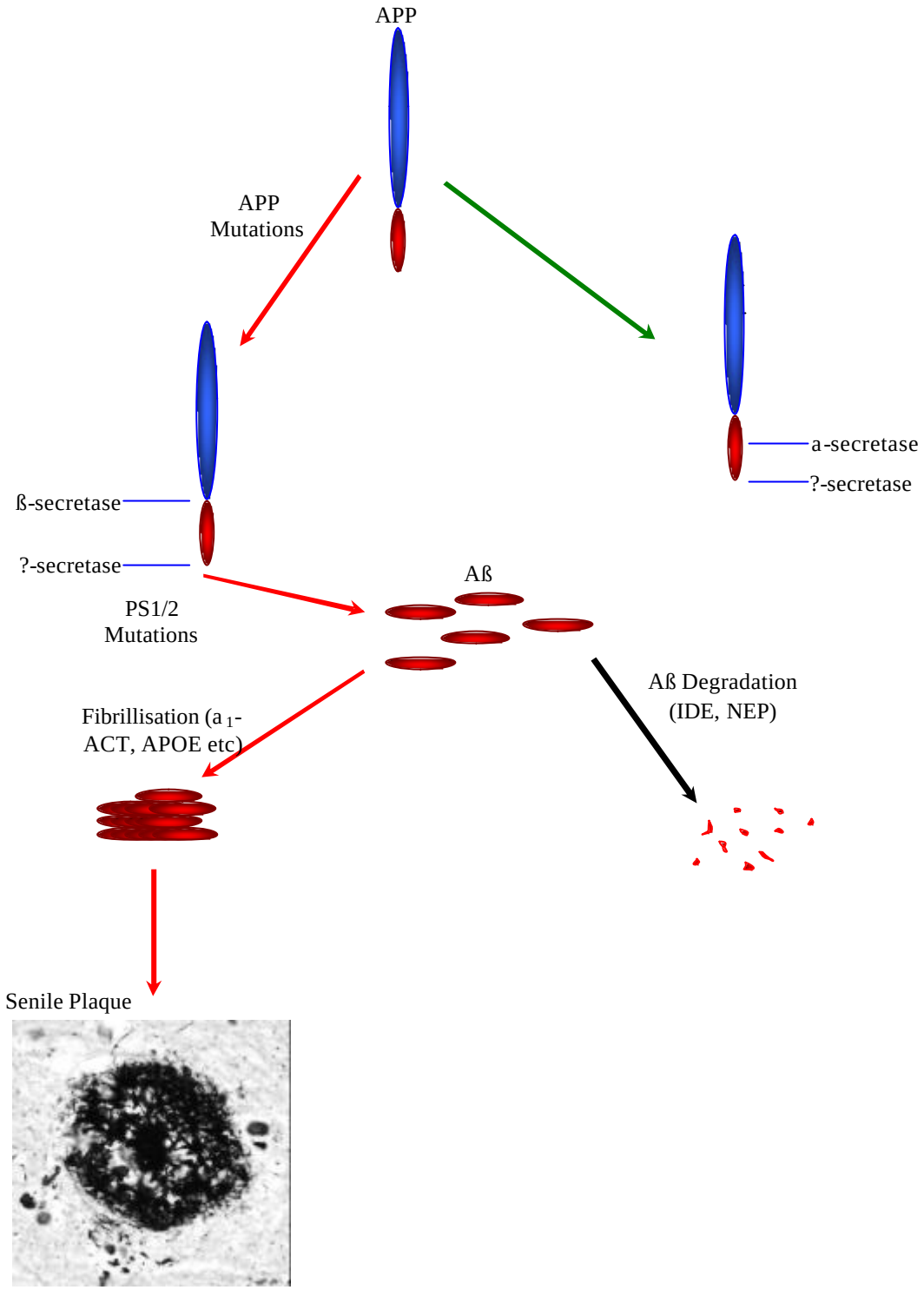

Figure 1.2.4: Generation and clearance of Aß. Red arrows show the amyloidogenic pathway, green arrows show the non-amyloidogenic pathway, blue arrows depict proteolytic cleavage. Red ellipses show Aß or the Aß domain in APP, and the red fragments represent degraded Aß.

Neprilysin (NEP) is a 90-110 k-Da plasma membrane glycoprotein, composed of a short N-terminal cytoplasmic region, a membrane-spanning section and a large C-terminal extracellular, catalytic domain, containing a zinc-binding motif (Carson and Turner, 2002). The active site of NEP is not accessible to large peptides and proteins, but it is able to interact with $Aß_{42}$. While NEP is primarily expressed in the kidney, it is also present in the brain, at low levels (Barnes et al., 1995), although expression in the brain of some members of the NEP family is modified after nerve injury, suggesting a neuroprotective role (Carson and Turner, 2002). NEP has been demonstrated to degrade amyloid *in vivo* (Mohajeri et al., 2002). Inhibition or deletion of the *NEP* gene results in ~25 to 35% decrease in Aß degradation by the membrane fraction of rat brain, suggesting that NEP degrades insoluble membrane-bound Aß (Suh and Checler, 2002). In the brains of young neprilysin-deficient mice, the steady-state levels of Aß are elevated, but not greatly, and there was no effect on plaque formation, which, given the rapid turnover of Aß in the brain, would indicate that neprilysin is not the major degrader of Aß. It is likely that other Aß-clearing pathways can compensate for a lack of neprilysin.

In the majority of cases of familial AD, mutations in genes encoding APP and the presenilins increase Aß production and deposition. Most cases of AD are sporadic, and in these cases consistently elevated plasma levels of Aß are not observed (Scheuner et al., 1996), which might indicate that a change in Aß degradation or clearance may be the cause of increased deposition.

Evidence to support the amyloid model of AD pathology, in which the generation of $Aß_{42}$ is an early and essential step in the pathological cascade which results in the neuronal damage characteristic of AD, comes from the discovery of cytokine-like properties of Aß. It has been shown that Aß is able to directly activate the alternative and classical complement pathway, trigger the formation of covalent complexes with the complement component C3, and generate the cytokine-like C5a fragment, as well as being able to mediate production of the proinflammatory C5b-9 membrane attack complex (Bradt et al., 1998). It has been known for some time that inflammation occurs in the brains of AD patients, and there had been speculation that Aß could trigger

inflammation, along with degenerating neuronal tissue. These ideas were taken further with the publication of a paper which demonstrated that a 105 aa carboxyl-terminal fragment (CT105) of APP, as well as Aß$_{42}$, can induce proinflammatory cytokines (Rah et al., 2001). At relatively low concentrations CT105 can induce IL-1ß, TNF-a and nitric oxide (NO), which are not induced by Aß. Carboxyl-terminal fragments of APP have been isolated from lymphoblastoid cells obtained from patients with early- or late-onset AD (Matsumoto, 1994).

1.2.5 Properties of Aß

In it's normal physiological role, unprocessed, full length APP is believed to function as a kinesin-1 membrane receptor, with a role in axonal transport (Kamal et al., 2001). The non-amyloidogenic proteolytic processing of APP generates C-terminal fragments, which can influence gene expression by acting as a transcription factor (Cao and Sudhof, 2001). In contrast, there has been a tendency to view Aß as a dangerous by-product of inappropriate APP processing, with only a pathological role. There is evidence, however, that this is not the case. The finding that Aß is present in the cerebrospinal fluid and plasma of healthy individuals throughout their lives has been known for more than 10 years (Seubert et al., 1992). A number of physiological roles have been proposed for Aß. One suggestion was that Aß could act as a physiological regulator of ion channel function in rat neurons (Ramsden et al., 2002; Ramsden et al., 2001). However, it is yet to be proven that endogenous Aß has a role on brain function after secretion from neurons. It has recently been shown that neuronal activity results in Aß secretion and Aß can downregulate excitory synaptic transmission. Previous work has shown that several factors, including neurotransmitters and neuronal depolarisation modulate the generation of APP secretory products (Mills and Reiner, 1999; Nitsch et al., 1993). It has been proposed that neuronal activity enhances ß-secretase-mediated cleavage of APP, leading to an increase in Aß secretion (Kamenetz et al., 2003). This is likely to be triggered by a signalling cascade initiated by the opening of NMDA

receptors, coupled with an influx of Ca^{2+} into the postsynaptic terminal (Esteban, 2004). Clinical studies have shown that benzodiazepines, which enhance inhibitory transmission, reducing excitatory drive, and memantine, an NMDA receptor agonist, protected against cognitive decline in Alzheimer's disease (Fastbom et al., 1998; Winblad and Poritis, 1999).

Aß is able to depress fast excitatory synaptic transmission, mediated by AMPA and NMDA receptors, but not inhibitory transmission, mediated by GABA receptors (Kamenetz et al., 2003). Additionally, Aß is able to affect not only the neuron producing Aß, but also neighbouring cells. Aß production impairs long-term potentiation (LTP), an indicator of learning and memory function. The effects of Aß on synaptic transmission were apparent at levels of Aß production well below those necessary for plaque formation, reinforcing the theory that the initial stages of AD are asymptomatic, and take place early in life.

It has also been shown that Aß and Aß fragments have cytokine-like and inflammatory properties. Aß peptides have been shown to activate the classical and alternative complement pathway, both directly and indirectly. Complexes of Aß with activation products of the complement component C3 can generate the cytokine-like C5a complement activation fragment, and mediate the formation of the proinflammatory C5b-9 membrane attack complex (Bradt et al., 1998; Halliday et al., 2000; McGeer and McGeer, 2001a). In cultured rat astrocytes, the ability of Aß to induce glial cell activation in concert with other proteins found in senile plaques is enhanced. Some of the proteins that can activate cultured rat glial cells when acting with Aß include ACT and interleukin-1ß (IL-1ß) (Hu and Van Eldik, 1999). This work has been taken further, and it has been demonstrated that in cultured human glioma cells Aß on its own can induce tumour necrosis factor-a (TNF-a), and IL-6. A mixture of ACT and Aß did not induce IL-6, but caused a much stronger induction of TNF-a (Sun et al., 2002). Other evidence suggests that it is not the enitre Aß peptide that induces a cytokine response, but only a fragment (see section 1.2.6.1) (Rah et al., 2001). This indicates that inflammation in AD is a self-perpetuating chronic state. It has been suggested that the cytokine-like properties of Aß

provide a link between the seemingly disparate pathologies of AD, plaque formation, tau protein fibril tangle formation, and neuroinflammation (Lukiw and Bazan, 2000).

Aß-induced oxidative stress has recently emerged as an important new theory. It is believed that Aß may be responsible for the generation of reactive oxygen species (ROS) (Hensley et al., 1994). There is evidence that Aß in proto- or fibrillar form disturbs the membrane environment of metabolic pathway enzymes. This could lead to increased leakage from redox chains that increase ROS toxicity (Goodman et al., 1994). There is a possible synergy between oxidative stress and apoptosis (Howlett et al., 2000), as APP production increases after neuronal injury and oxidative stress (Burnett, 2000). APP production also increases after ischaemic stress, and cytotoxic events that lead to increased intracellular calcium levels (Burnett, 2000).

The possibility that Aß has both a physiological and a pathological role is in accordance with the hypothesis that in familial forms of neurodegenerative diseases, variation occurs in the coding regions of genes, whilst in sporadic forms, genetic variations are found in the regulatory regions of genes. This theory explains that a normal protein that is overexpressed, or is not cleared at a sufficient rate will be deposited, in a way that may have pathological consequences (Singleton et al., 2004).

1.2.6 Therapeutic Strategies for Alzheimer's Disease

There are still many unanswered questions about Alzheimer's disease. For example, is there a specific 'trigger' that causes the disease, setting in motion the production of $Aß_{1-42}$, the inappropriate phosphorylation of tau protein, and from there the development of neurofibrillary tangles and senile plaques? What common pathway leads to the formation of senile plaques and neurofibrillary tangles? Would the clearance of $Aß_{1-42}$ fibrils be of benefit to sufferers of AD? On a more fundamental level, is AD an inflammatory condition, and if it is, would the use of anti-inflammatory drugs alleviate, or prevent neuronal damage in AD?

At present there is only one class of drug available for the treatment of AD, the acetylcholinesterase (AChE) inhibitors. These compounds are unable to halt, or reverse the progress of AD they can merely mask the symptoms, making their value limited. There are a large number of therapeutic strategies in different stages of design and testing, that have been proposed to either halt the progress of AD or to prevent it's occurrence altogether. Some of these therapies are already undergoing clinical trials, some are at the pre-clinical trial stage, and some are untested theories. Although therapies like inhibitors of protein kinase cdk-5, MAPK and cathepsins have been proposed that target tau protein phosphorylation, this section will focus mainly on strategies that disrupt the amyloidogenic pathway, as evidence is emerging that this is the earlier pathological process (Delacourte et al., 2002; Sergeant et al., 2002). The recent creation of a *C. elegans* model for studying AD-like neurological changes and the discovery that oxidative stress precedes Aß fibril formation also demonstrates the early impact of APP processing on AD pathogenesis (Drake et al., 2003; Link et al., 2003).

A number of AD therapies are available or undergoing trials. These therapies are listed in table 1.2.5, and those in the most advanced stages of development are described below.

Suppression of Symptoms		
Acetylcholinesterase Inhibitors	Improves mental state scores in short term	In use
Psychotropic drugs	Suppress behavioural symptoms; anxiety, apathy, agitation, aggressiveness, depression, serious delusions, hallucinations	In use
Neuroprotective Strategies		
Anti-oxidants	Delays time to entry of nursing home	Small clinical trials
Non-steroidal anti-inflammatories	Blunt neurotoxic effects of inflammatory and acute phase responses	Epidemiological studies and clinical trials
Calcium channel modulators	Limits neurotoxic effects of Aß	Theoretical
Free-radical scavengers	Reduces oxidative damage	Theoretical
Metal ion chelators	Inhibits Aß accumulation	Clinical trials
Inhibition of Aß Production		
ß-secretase inhibitors	Prevents secretion of Aß	Drug design stage
?-secretase inhibitors	Decrease formation of Aß oligomers	Animal trials
Aß clearance		
Active immunisation	Provoke T-cell response to Aß	Clinical trials halted
Passive immunisation	Provokes humoral response to Aß	Animal trials
Immunoconjugate immunisation	Provokes B-cell response to Aß	Animal trials
a-Secretase Stimulation		
Stimulation of neurotransmitter systems	Lowers cortical Aß, increase sAPPa secretion	Animal trials
Cholesterol-lowering agents	Inhibits Aß formation	Retrospective epidemiological studies, animal trials

Table 1.2.5: An overview of therapeutic strategies available or proposed for AD (Cherny, et al., 2001; Leissring, et al., 2000; Moller, 1998; Sramek and Cutler, 1999).

1.2.6.1 Acetylcholinesterase Inhibitors

It has been shown that cholinergic signalling and transmission in the cerebral cortex and hippocampus is disrupted in the brains of AD patients (Cutler and Sramek, 2001). These areas of the brain are associated with memory and intellect, and so damage to these areas is an obvious place to look to discover the cause of the memory and intellectual deficits found in AD. In addition to its role in transmission, cholinergic stimulation has also been shown to enhance the non-amyloidogenic pathway of APP processing, increasing production of sAPPa, which has a neuroprotective role (Suh and Checler, 2002). A number of compounds that restore or boost the function of cholinergic signalling have been considered for use in AD therapy, and some of these are licensed for use in the treatment of AD, such as tacrine hydrochloride (Cognex), which is rarely used, due to the severity of side effects, donepezil hydrochloride (Aricept), rivastigmine tartrate (Exelon) and galantamine (Reminyl) (Suh and Checler, 2002). These compounds are all acetylcholinesterase inhibitors, which inhibit the enzyme which hydrolyses acetylcholine (ACh), and therefore increase the amount of ACh available to neurones (Cutler and Sramek, 2001). These drugs slow progression of AD, but do not restore cognitive ability.

1.2.6.2 Antioxidants

There is evidence to suggest that oxidative damage to neurones is important in AD pathogenesis (Drake et al., 2003; Gahtan and Overmier, 1999; Guo et al., 1999; Lavrovsky et al., 2000). The use of antioxidants and free radical scavengers has been suggested to limit the damage caused by AD, and pre-clinical studies have shown some beneficial effects (Butterfield, 2001). Ginkgo biloba extract has been shown to protect neurones from oxidative stress, possibly caused by Aß effects on mitochondria (Luo et al., 2002). Another compound being investigated is melatonin, which is known to have both anti-oxidant and anti-amyloidogenic properties, which decreased neuronal damage in experimental models (Suh and Checler, 2002). Vitamin E has also been used in small clinical trials to attempt to limit the progress of AD (Behl and Moosmann, 2002). Vitamin E is an anti-oxidant and free radical scavenger. Clinical trials

showed no significant effect on cognitive ability, but it may have other beneficial health effects, as it delayed the time to entry to care homes.

1.2.6.3 Vaccines

The processing of APP to generate Aß is believed to be central to the pathogenesis of AD (Sergeant et al., 2002; Walsh et al., 2002a). AD patients accumulate Aß plaques at a greater rate than unaffected individuals, and the reasons for this are unclear. It has been suggested that AD patients either generate too much Aß peptide, or that they are less able to clear the peptide (Singleton et al., 2004). In other words the normal equilibrium of Aß is disrupted in AD patients resulting in excess Aß remaining in the brain tissues. If this hypothesis is correct, it can be assumed that reducing the synthesis of Aß or increasing its clearance will be beneficial to AD patients. Recently techniques to stimulate Aß clearance with anti-Aß antibodies have been proposed (Schenk et al., 2001).

There are two methods of introducing anti-Aß antibodies into the brain: active vaccination and passive immunisation. The first strategy, active vaccination, which involves introducing a fragment of Aß, or a synthetic Aß to provoke an immune response, has got as far as clinical trials. The other form, passive immunisation, where antibodies raised against parts of Aß are administered has mainly been used to study the mechanism of active immunisation. The results of experiments with passive immunisation had led some to believe that this would be a preferable therapeutic strategy for AD.

Active immunisation, whereby an immune response is triggered by introducing fragments of Aß, synthetic $Aß_{42}$ or other plaque-associated peptides, has been studied in transgenic mice models, and progressed to clinical trials. Early experiments used the PDAPP mouse model; a mini-*APP* gene coupled with the platelet-derived growth factor promoter, containing the FAD mutation Val717Phe. Experiments on mice given synthetic $Aß_{42}$ before and after developing Aß deposits showed a decrease in hippocampal plaques versus controls, a decrease in dystrophic neurites and astrocytosis (Games et al., 1995; Schenk et al., 1999; Selkoe and Schenk, 2003; Weiner et al., 2000).

In younger animals, the build up of Aß plaques was almost completely inhibited, and in older animals existing plaques were reduced (Schenk et al., 1999). It was demonstrated that mice that develop learning deficits as a result of mutant APP with accompanying Aß build up, showed an improvement in cognitive abilities after treatment with Aß vaccination (Janus et al., 2000; Morgan et al., 2000).

Aß production was not affected by active immunisation, but pre-existing plaques were cleared, and formation of plaques was inhibited in these experiments. Studies using different transgenic mouse models showed no learning deficits and improved cognitive performances, comparable to non-transgenic mice, when compared with transgenic mice in the control group (Janus et al., 2000; Morgan et al., 2000). No adverse effects to active immunisation were reported in mouse models. Immunisation with Aß results in antibodies (Ab) associated with extracellular dense-cored plaques and reduced Aß plaque deposition, while total brain Aß levels remain unchanged. Ab is directed against Aß in the ß-sheet conformation, not soluble forms of Aß (Selkoe and Schenk, 2003). The mechanism involved in clearing Aß from the brain is unknown. It has been suggested that Fc-mediated microglial phagocytosis is involved (Schenk et al., 1999). Some plaques in immunised mice were decorated with IgG, and co-localised with microglia. Microglia / monocytes were located near the remaining plaques. This mechanism is consistent with observations made after passive immunisation (Bard et al., 2000).

Passive immunisation involves generating anti-Aß monoclonal antibodies, and introducing these to transgenic mice. This approach has been used to gain an insight into the mechanisms involved in active immunisation, but could possibly be used in future as a treatment.

Experiments with 8-10 month old transgenic mice bred to produce Aß plaques were treated for 4-5 months with various anti-Aß antibodies. The antibodies used were 10D5, 21F12, polyclonal IgG and the mean cortical Aß was compared after 6 months treatment (table 1.2.6)

Antibody	10D5	21F12	IgG	Control
Target	$Aß_{1-5}$	$Aß_{33-42}$	Aß	-
Mean cortical Aß ng/g tissue	6,200	13,580	4,890	13,800

Table 1.2.6: Mean cortical Aß levels in PDAPP transgenic mice after 6 months from the start of peripheral antibody administration (Bard et al., 2000).

Following this slightly older mice (11.5 – 12 months) were treated with mAb 3D6 (anti-$Aß_{1-5}$) or 16C11 (anti-*tau* protein). The effects of 3D6 were similar to 10D5, but there was no effect shown with 16C11 (Bard et al., 2000).

mAb directed against $Aß_{13-28}$ does not bind to Aß plaques, but decreases brain Aß burden, shifting CNS-plasma Aß equilibrium and acting as a peripheral sink for amyloid. This effect was seen, although not as strongly, with 3D6 and 10D5 (DeMattos et al., 2001; DeMattos et al., 2002). In addition to this, experiments using agents with a high affinity to Aß that are unrelated to antibodies or immune-modulators, gelsolin and ganglioside GM1, were shown to reduce levels of Aß in the brain when administered peripherally to PDAPP mice (Matsuoka et al., 2003). These agents do not easily cross the blood-brain barrier, and add weight to the hypothesis that altering the equilibrium between CNS-plasma Aß could be an effective therapeutic strategy.

Another set of experiments that demonstrate a possible mode of action for immunisation therapies used antibodies raised against $Aß_{1-28}$, which prevented the fibrillar aggregation of Aß (Solomon et al., 1997; Solomon et al., 1996).

Another study to investigate the mechanism of Aß clearance after immunisation has raised the possibility of a way of providing a diagnostic test for AD. The antibody 10D5 was labelled with various fluorescent molecules such as Thioflavine-S and fluorescein, and directly applied to the cortex of transgenic mice. This permitted the direct observation of Aß plaques, and studying the same brain region over 3 days revealed the clearance of these plaques by microglial phagocytosis (Bacskai et al., 2001).

The mechanisms revealed by passive immunisation; prevention of fibrilisation, microglial phagocytosis, and removal of Aß from the CNS into the plasma are

not mutually exclusive, and one, or all of them could occur after active or passive immunisation.

In late 1999 development began on a human form of the vaccine. This was based on a synthetic human $A\beta_{42}$ peptide, and completed phase I trials with small numbers of patients. Phase II trials began in the USA and Europe, on a group of patients, in 2001. This was a double-blind, placebo-controlled study involving 360 patients, but after several months, in February 2002, the trial was halted when 15 patients that had received the vaccine developed encephalitis, with symptoms of CNS inflammation (Imbimbo, 2002a; Imbimbo, 2002b). While studies are underway to determine what caused this response (Imbimbo, 2002b), other vaccination strategies are being developed. These include other active vaccinations, as before, but with an altered route of administration (Weiner et al., 2000) or different adjuvants, (Levey, 2000), or using modified Aß epitopes (Imbimbo, 2002a; Sigurdsson et al., 2001), or fusion proteins, immunogenic conjugates of Aß (Schenk et al., 2001).

The mechanism by which the active Aß vaccination works is unclear, although there are theories as to what may happen. *Ex vivo* assays in brain sections from transgenic mice and AD patients demonstrated antibodies against Aß in plaques. The antibody triggered microglial cells to clear plaques through phagocytosis and peptide degradation, indicating that antibody can cross the blood-brain barrier (BBB) (Bard et al., 2000).

In vivo studies have shown that anti-Aß antibodies applied directly to the cortex of transgenic mice can provoke the clearance of Aß (Bacskai et al., 2001). Another proposal for the mechanism of Aß vaccination is that the antibodies sequester peripheral Aß, creating an Aß sink. This causes Aß to leave the CNS, resulting in a clearance of Aß oligomers and proto-fibrils from the brain (DeMattos et al., 2001; Vehmas et al., 2001).

The findings of these experiments has led to the possibility that the problems found in clinical trials of active immunisation may not have ended hopes of an immunisation therapy for AD. Modifying the antigen, altering the route of administration, or changing the adjuvant used in active immunisation may reduce the risks of adverse side effects. Alternatively, a passive immunisation

therapy may present a lower risk approach, especially if immunisation takes place long before development of AD in a younger, healthier population than that involved in the failed clinical trials. Finally, using agents that sequester Aß in the plasma, but are not immune-related molecules, might be a safer treatment. The story of AD immunisation is not over, although more work will be needed than might have been anticipated.

1.2.6.4 Anti-Inflammatory Drugs

Retrospective studies investigating non-steroidal anti-inflammatory (NSAID) use in AD patients have suggested a possible therapeutic benefit in delaying the onset of AD (Rich et al., 1995), and epidemiological studies have found that there is a reduced frequency of AD in leprosy and arthritis patients taking NSAIDs (Halliday et al., 2000; Mackenzie and Munoz, 1998; McGeer et al., 1996). It has been shown that people taking NSAIDs whilst showing clinical signs of AD later in life suffer from less severe AD (Cutler and Sramek, 2001; Suh and Checler, 2002). These findings agree with the hypothesis that inflammation is a key feature in the progression of AD. Many of the studies into the effects of NSAIDs on AD have been epidemiological, and therefore provide no evidence of the mechanism of how they work in alleviating the symptoms, or preventing the pathogenesis of the disease (Andersen et al., 1995; Flynn and Theesen, 1999; Halliday et al., 2000; McGeer et al., 1996; Rich et al., 1995; Sramek and Cutler, 1999; Stewart et al., 1997). The epidemiological studies indicate that long-term NSAID use reduces the risk of AD in subjects younger than 85 years of age (in 't Veld et al., 1998). Some studies have been carried out using different models to examine the effects of different NSAIDs on cell degeneration. Animal models have been carried out on rats that have been given chronic lipopolysaccharide (LPS) infusions to induce the effects of chronic neuroinflammation. These studies, using NO-flurbiprofen (NFP) suggested that NSAID therapy should start before inflammatory processes develop, and that NFP can reduce the density and active state of microglial cells in rats with chronic inflammation (Hauss-Wegrzyniak et al., 1999a; Hauss-Wegrzyniak et al., 1999b). Other studies have used transgenic mice that

overexpress APP. When treated with ibuprofen, a reduction was seen in the final levels of IL-1ß and glial fibrillary acidic protein. In addition, the number and area of Aß deposits and ubiquitin-labelled neurites was reduced (Lim et al., 2000). Another study, using the spice curcumin, which is not an NSAID, but has antioxidant properties, in the same strain of transgenic mice, showed a reduction IL-1ß, insoluble Aß, soluble Aß and plaque burden. The membrane-associated APP levels were not reduced (Lim et al., 2001). An NSAID with anti-oxidant properties, S-2474, has been investigated using primary cultures of rat cortical neurones. S-2474 is a COX-2 inhibitor, and was demonstrated to prevent Aß-induced cell death. In this study, it was shown that $Aß_{25-35}$ was generating prostaglandin D(2) (PGD(2)) and free radicals which cause cell death. S-2474 exhibits a protective effect either by suppressing PGD(2) or free-radical damage.

Several clinical trials with various anti-inflammatory drugs, such as indomethacin, dicloflenac and prednisone, have taken place, but these have shown mixed results (Aisen et al., 2000; Bruce-Jones et al., 1994; Mallet and Kuyumjian, 1998; Rogers et al., 1998; Rojas-Fernandez and Mallery, 1998; Scharf et al., 1999). A new generation of NSAIDs is under development, the COX-2 inhibitors, which are expected to target the dysfunctional inflammatory pathways of AD (McGeer and McGeer, 2001a).

It has been reasoned that since the formation of SPs is the result of chronic neuroinflammation, NSAIDs may prevent the build up of SPs. A study has compared brain pathology in post-mortem brain tissue of elderly non-demented patients with a history of chronic long-term NSAID use, with a control group of non-demented patients that had no history of any conditions that would necessitate the long-term use of NSAIDs (Mackenzie and Munoz, 1998). This study showed no difference in the mean number of plaques between the two groups, but did show that control patients with SPs had almost three times the number of activated microglia as patients with SPs that did take NSAIDs. This would suggest that NSAIDs suppress microglial activity in the brain, rather than suppress the formation of plaques. Animal models have shown NSAID treatment to be beneficial in several neurodegenerative diseases, but do not

make clear whether these effects are due to the actions of COX inhibitors or direct anti-inflammatory properties (Moore and O'Banion, 2002).

Following modest results in small-scale clinical trials, large multi-centre drug trials have been established to determine the efficacy of NSAIDs in the treatment of AD. These trials are not yet complete (Moore and O'Banion, 2002).

One proposed strategy for preventing Aß peptide triggered complement proteins from causing neuronal damage would be to introduce anti-sense mRNA to APP into the affected areas of the brain. Anti-sense mRNA can cross the blood-brain barrier, and reduces learning and memory deficits in aged mice, if it is coupled to an oligonucleotide transport system (Banks et al., 2001).

1.2.6.5 Oestrogen

The sex hormone oestrogen is known to affect intracellular signal transduction cascades, and can have a neuroprotective effect (Suh and Checler, 2002). Several studies have shown that being female is a risk factor for AD (Carr et al., 1997), and small scale trials have indicated that oestrogen replacement therapy can improve cognition in AD patients (Cutler and Sramek, 2001). Research has linked Parkinson's disease and AD with oestrogen levels, which decreases rapidly after the menopause (Suh and Checler, 2002), but the mechanism for it's actions is unclear. The effect, which seems to be preventative, is possibly due to anti-oxidant properties of oestrogen (Behl and Moosmann, 2002), but it might also be due to the role it plays in modifying inflammation, or by acting on IL-6, or signal transduction via the MAPK pathway (Suh and Checler, 2002). Other studies have shown that oestrogen can promote non-amyloidogenic processing of APP (Hooper and Turner, 2002). A number of large-scale trials are being undertaken, that may settle the question of the benefits of oestrogen replacement therapy in AD treatment (Cutler and Sramek, 2001).

1.2.6.6 Beta-secretase Inhibitors

In the amyloidogenic-processing pathway of APP, two factors are of critical importance: ß-secretase and ?-secretase. In this pathway, APP is first cleaved

by ß-secretase, then ?-secretase, resulting in the formation of Aß, so the suppression of either would theoretically prevent the pathogenesis of AD. The major enzyme responsible for ß-secretase cleavage has been shown to be ß-site APP cleavage enzyme 1 (BACE1) (Cai et al., 2001; Vassar, 2001; Vassar et al., 1999). Although a homologous enzyme, BACE2, has been found, inhibition of BACE1 appears sufficient to reduce AD pathology in mice (Luo et al., 2001; Vassar, 2001). The development of an effective BACE1 inhibitor for use by AD patients has become an important goal in the search for effective AD therapies, as it has been shown that BACE can produce toxic fragments of APP even if ?-secretase cleavage of APP has been blocked (Suh and Checler, 2002). Several BACE1 inhibitors have been found, and one class is capable of inhibiting both ß- and ?-secretase cleavage. These inhibitors have been shown to cross the blood-brain barrier, and are undergoing pre-clinical trials to determine toxicity and tolerance (Suh and Checler, 2002).

1.2.6.7 Gamma-secretase Inhibitors

Attention has focussed on ?-secretase, as a potential target for therapy (Beher and Shearman, 2002; De Strooper et al., 1998; Esler et al., 2000; Schenk et al., 2001; Selkoe, 1998; Tsai et al., 2002; Zhang et al., 2000), but there have been suggestions that since it may not be involved in normal cell signalling, ß-secretase may be a better target (Howlett et al., 2000; Selkoe, 2001). It has been discovered, however, that compounds can be developed that would inhibit ?-secretase cleavage of APP, but maintain Notch signalling (Beher and Shearman, 2002; Petit et al., 2001), as it appears that the Notch signalling and APP processing mechanisms of ?-secretase are not entirely homologous (Beher et al., 2001; Lichtenthaler et al., 2002; Rozmahel et al., 2002).

An off-shoot into research looking at ?-secretase/presenilin activity in AD has been the discovery that reduced PS1 activity alters calcium channel modulation (Yoo et al., 2000). This corresponds to earlier findings, that associated Aß with the destabilisation of calcium homeostasis, resulting in neuronal damage (Mattson et al., 1992). A number of compounds that inhibit amyloidogenic APP processing, but maintain a sufficient level of Notch processing are currently

being investigated for efficacy and toxicity in cultured cell and animal models (Suh and Checler, 2002).

Another method of decreasing amyloidogenesis would be to increase the activity of a-secretase. The protease plasmin seems to increase APP processing at the a-secretase cleavage site, and degrade APP fragments, which if verified would suggest another target for AD therapy (Carson and Turner, 2002; Ledesma et al., 2000; Periz and Fortini, 2000).

1.2.6.8 Other Therapeutic Strategies

Some drugs that are licensed for the treatment of other conditions have been shown to have beneficial effects in the treatment of AD, in addition to the anti-inflammatory drugs, discussed above. These include cholesterol-lowering drugs, such as simvastatin and lovastatin, which have been shown to lower the levels of $A\beta_{42}$ and $A\beta_{40}$ in the CSF and brain homogenates from guinea pigs (Fassbender et al., 2001). The finding that cholesterol-lowering drugs may be beneficial in the treatment of AD is related to the cholesterol transport protein apolipoprotein E being a recognised risk factor for AD (Pappolla et al., 2002; Poirier, 2003; Poirier et al., 1995; Puglielli et al., 2003). Clinical trials have begun on treating AD with cholesterol-lowering drugs (Puglielli et al., 2003; Suh and Checler, 2002). Another drug believed to have therapeutic benefits in AD is the antibiotic clioquinol (Helmuth, 2000). This antibiotic is a metal ion chelator, which is believed to be able to have a neuroprotective effect, although the mechanism of this is unclear. Several metal ions have been shown to promote plaque formation (Cherny et al., 2001; Chong and Suh, 1995; Dominguez and Strooper, 2002; Kawahara et al., 2001), but as it is believed that Aß plaques are the result, not the cause, of neural degeneration in AD, this is unlikely to be the mechanism by which metal ions cause neuronal damage.

In addition to therapeutic interventions that are intended to halt, or prevent AD, a number of treatments to ameliorate or temporarily suppress certain debilitating symptoms are available. AD can result in behavioural disturbances, including anxiety, apathy, agitation, aggressiveness, depression, delusions and hallucinations (Mayeux and Sano, 1999). Various anxiolytics, such as

benzodiazepines, can be used to suppress these symptoms, but a worsening of dementia often outweighs their benefits. Non-phenothiazine antipsychotic agents, such as haloperidol, are more usually used. For patients with depression, low doses of selective serotonin re-uptake inhibitors are sometimes prescribed, although these may temporarily heighten cognitive defects. In cases of marked apathy, stimulants, such as methylphenidate, can be used. Anticonvulsants, such as carbamazepine, are administered for anxiety and agitation.

Another possible strategy for AD therapy would be to utilise the Aß degrading properties of neprilysin. This strategy does not interfere with the processing of APP or signalling pathways (see figure 1.2.4). The restricted location of the active site limits potential substrates to less than 50 k-Da, reducing the number of inappropriate interactions that could result in side effects that could occur with inhibiting APP processing. In addition, neprilysin preferentially degrades abundant substrates, such as the large amounts of Aß found in pathological conditions (Fukami et al., 2002).

1.2.7 Evidence of Inflammation in Alzheimer's Disease

There is evidence to support the view that inflammatory-like, if not actual inflammatory, processes take place during the pathology of Alzheimer's disease. Strong evidence comes from the observations of several proteins involved in inflammation, such as a_1-antichymotrypsin and apolipoprotein E, being associated with amyloid plaques (Durany et al., 2000; Rebeck et al., 1995; Styren et al., 1998). In addition, a number of epidemiological studies revealed that anti-inflammatory drug treatment correlates to a negative risk of AD (Andersen et al., 1995; McGeer et al., 1996; Rich et al., 1995). These studies were followed up by animal experiments and clinical trials of non steroidal anti-inflammatory (NSAID) treatments (Hauss-Wegrzyniak et al., 1999a; Lim et al., 2001; Lim et al., 2000; Scharf et al., 1999; Yagami et al., 2001). It has also been demonstrated that Aß plays a role in initiating inflammatory responses (Cotman et al., 1996; Kalaria et al., 1996). The study of inflammation is

detailed and complex, and the processes of neuroinflammation in AD are bewilderingly complex, even though the humoral immune response appears to take little or no part. Of the pathways that are involved in AD, the complement, cytokine, chemokine, acute phase response and so on, most research has focussed on the acute phase response, which will be investigated more closely as part of this thesis. The acute phase is a highly sophisticated and complex response, involving numerous reactions and interactions, and only those relevant to this body of work will be detailed here. There are a number of reviews that summarise the major research into neuroinflammation, e.g. Akiyama, *et al.*, 2000.

There is evidence to show that neuroinflammation is a prominent feature of Alzheimer's disease, which has important implications for therapy. In addition to the epidemiological studies that identified a reduced risk for AD in patients treated with anti-inflammatory drugs (Andersen et al., 1995; Halliday et al., 2000; McGeer et al., 1996) and longitudinal studies showing that consumption of anti-inflammatory drugs lowers the risk of AD in younger patients (Halliday et al., 2000), other lines of evidence support this theory. Degenerating tissue, which is a feature of AD is a classical stimulant of inflammation (Akiyama et al., 2000). Cultured microglia and astrocytes, which localise to SPs (Eikelenboom and Veerhuis, 1996; Halliday et al., 2000; McGeer and McGeer, 1995) are known to produce inflammatory molecules, such as cytokines, complement factors and acute phase proteins (Eikelenboom and Veerhuis, 1996; McGeer and McGeer, 1995). There is also evidence of increased platelet activation in AD (Halliday et al., 2000). Polymorphisms in genes for inflammatory molecules, including IL-1a, IL-1ß, a2-macroglobulin and ACT are known to modulate the risk of AD (Franceschi et al., 2001; McGeer and McGeer, 2001b; Morgan et al., 2001; Rocchi et al., 2003; Scacchi et al., 2001), IL-1, for instance, has been shown to upregulate the translation of *APP* (Potter et al., 2001).

1.2.8 The Acute Phase Response and Inflammation

As a result of an insult, such as injury, trauma or infection of a tissue a series of tightly regulated responses is initiated by the body in an attempt to limit the damage caused, and restore normal homeostasis. These responses are collectively known as inflammation, and the early and immediate set of reactions induced is known as the acute phase response. The acute phase response begins in the region of the insult, but can lead to the activation of genes in organs far from the original site (Baumann and Gauldie, 1994; Trowbridge and Emling, 1997).

The acute phase response is made up of many systemic and metabolic changes, and is initiated in response to tissue damage, infection, malignant disease or immunological disorders. These traumas unbalance the normal homeostasis and the purpose of the acute phase response is to restore homeostasis (Slaviero et al., 2003). To achieve this the acute phase response increases local neutrophil levels, which activate complement and coagulation pathways, and upregulates the production of acute phase proteins (Baumann and Gauldie, 1994). The symptoms of the acute phase response include fever and fatigue, and it normally lasts for a few days, until homeostasis is restored (Slaviero et al., 2003). If inflammation is chronic or recurring, the acute phase response can persist, and contribute to the pathology of disease (Balkwill and Mantovani, 2001).

The acute phase response is the result of complex phagocyte-derived endogenous mediators, in particular the cytokines, a family of over 80 proteins. Cytokines are small (8-40kDa) and soluble and are crucial to the activation, development and determination of the extent of the immune response. They act at pico- or femtomolar concentrations, and their production is generally transient and inducible (Slaviero et al., 2003). A number of cytokines are involved in inflammation and the acute phase response, the proinflammatory cytokines, which includes tumour necrosis factor a (TNFa), some interleukins (IL-1, IL-6) and interferons (Trowbridge and Emling, 1997). The inflammatory response to stimuli involves the synthesis and release of a number of factors, including cytokines, inflammatory mediators, e.g., histamine, bradykinin and prostagladins, and hormones, such as cortisol and corticotrophin (Slaviero et al.,

2003). These factors activate inflammatory cells, such as macrophages, which can initiate the acute phase response through the release of TNFa and IL-1 (Baumann and Gauldie, 1994). These cytokines act locally and distally, and result in more factors being synthesised, such as second phase cytokines, like IL-6, chemokines, for instance IL-8, macrophage inflammatory protein and monocyte chemoattractant protein (Baumann and Gauldie, 1994; Slaviero et al., 2003). These factors circulate to other tissues and modulate the expression of acute-phase proteins, such as fibrilogen, a_1-acid glycoprotein, a2-macroglobulin, a_1-antitrypsin and a_1-antichymotrypsin (See table 1.2.7). Some acute phase proteins can increase in concentration by as much as 200 times during the acute phase, like C-reactive protein and serum amyloid A (Slaviero et al., 2003). In addition to these other proteins, like phospholipase A, cyclooxygenase 2 and inducible nitric oxide synthase can also be upregulated.

Acute Phase Protein or Family	Role in inflammation
Ceruloplasmin	Scavenges oxygen radicals generated by leukocytes
Protease inhibitors	Inhibit proteases responsible for wide ranging reactions; i.e. degrading collagen, re-modelling tissue
C-reactive protein	Binds to bacteria and produces capsular swelling
Transferrin	A major iron transport protein

Table 1.2.7: Examples of acute phase proteins, from (Trowbridge and Emling, 1997).

In the brain microglia, which can perform a similar function to macrophages, respond to interferon-? and TNF by expressing the major histocompatibility complex (MHC) class I and II molecules, which allows the microglia to function as antigen-presenting cells. The microglia are the first cells to respond to injury and infection in the brain, and recruit astrocytes by secreting acute-phase proteins, especially complement proteins, but also cytokines, such as IL-1ß and TNF-a (Halliday et al., 2000). The astrocytes react by releasing other complement proteins and acute-phase proteins, including ACT and a2-

macroglobulin, neural growth factors and cytokines, including IL-6 (Halliday et al., 2000).

The major pathway for transcriptional activation of acute phase proteins involves the Janus kinases (JAK) and signal transduction and activation of transcription (STAT), especially STAT3. Another pathway is the mitogen-activated protein kinases, which induce various nuclear factors including CCAAT enhancer binding proteins (C/EBP), also called nuclear factor (NF) interleukin 6a (NF-IL6a), NFkB and hepatocyte NF's, HNF1 and HNF4. The C/EBP family includes C/EBPß, C/EBP? also called NF-IL6ß, and C/EBPa. C/EBPß, one of the factors that upregulates a_1-acid glycoprotein (Chiu et al., 2002) is, like STAT3 and STAT5, a pro-inflammatory signal transcription factor. Some cytokines are known to have an anti-inflammatory effect, such as IL-2, IL-4 and IL-10, and these counter the pro-inflammatory cytokines to maintain homeostasis (Jeschke et al., 2002). There are also anti-inflammatory signal transduction factors, such as the suppressors of cytokine signalling (SOCS), such as SOCS-1, -2, -3, or regulated on activation, normally T-cell expressed and secreted (RANTES) (Jeschke et al., 2002). SOC-3, for instance, suppresses IL-6 (Croker et al., 2003). No single signalling pathway is solely responsible for upregulating acute phase proteins, and there is a substantial overlap and co-operation between signal pathways (Slaviero et al., 2003). As an example, IL-6 receptors include a sub-unit, gp130, that is capable of signal transduction via the JAK pathway, creating docking sites for cytoplasmic proteins with a Src homology domain-2, such as the STAT factors. STATs then form homo- and heterodimers which migrate to the nucleus and upregulate gene transcription via STAT responsive elements in promoters (Croker et al., 2003). STATs may also be activated by receptors that are unrelated to cytokines, like the activation of STAT3 by hepatitis type C virus core protein, or angiotensin II which activates the STAT/JAK pathway (Minoguchi et al., 2003).

1.3 Serpins

1.3.1 Molecular Biology of Serpins

Serine Protease Inhibitors (serpins) are a protein superfamily, found in higher eukaryotes and viruses (Gettins, 2000; Irving et al., 2000), composed mainly of enzymes that use serine residues for nucleophilic catalysis of their target molecule (Turgeon and Houenou, 1997). There are several hundred members of this superfamily (Gettins, 2000; Irving et al., 2000). There are some molecules classified as serpins, that have no protease inhibitory function, such as ovalbumin, maspin and HSP47, due to their homology with the inhibitory serpins (Whisstock et al., 1998). Alpha-1-antichymotrypsin is a typical serpin, with inhibitory activity against proteases.

Serpins are variably glycosylated single chain proteins (Potempa et al., 1994), between 350 – 400 amino acids in length (Irving et al., 2000), with a conserved domain structure of between 370 – 390 amino acid residues (Potempa et al., 1994) that form three ß-sheets and nine a-helices (Whisstock et al., 1998). The inhibitory serpins interact with their target protease at a reactive site loop (RSL), 30 – 40 amino acids from the carboxyl terminus. This area is exposed on the surface of the serpin, and is susceptible to proteolysis (Potempa et al., 1994).

As mentioned earlier, the main function of most of the serpins is the inhibition of serine proteases. They are involved in various complex physiological processes, and play a vital role in the control of events associated with connective tissue turnover, coagulation, fibrinolysis, complement activation, and inflammatory reactions, amongst others, in humans (Gettins, 2000; Potempa et al., 1994). They can be found in both intra- and extracellular environments (Gettins, 2000).

Serpins do not inhibit their target molecules by cleavage, but instead use a process that is analogous to a mouse trap (Carrell and Lomas, 1997). In the stressed state, the serpin can be likened to a primed mouse trap, with the RSL as bait (Carrell and Lomas, 1997). The inhibition involves profound conformational change by the serpin, and is initiated by reaction of the active

serine with the serpin's RSL (Huntington et al., 2000). This results in the cleavage of the RSL, which upon cleavage moves 71Å, to the opposite pole of the serpin, taking the protease with it, trapping the target (Carrell and Lomas, 1997; Huntington et al., 2000). This is possible because serpins can exist in two states; the stressed labile configuration (S), or an ordered, heat stable, relaxed form (R). The molecule can move between these forms due to a "hinge" in the RSL (Potempa et al., 1994). This manoeuvre results in a 37% loss of protease structure (Huntington et al., 2000), but is an irreversible form of inhibition (Gettins, 2000). Once cleaved, ACT contains two chains, A and B, of 337 and 35 amino acids respectively (see figure 1.3.1)

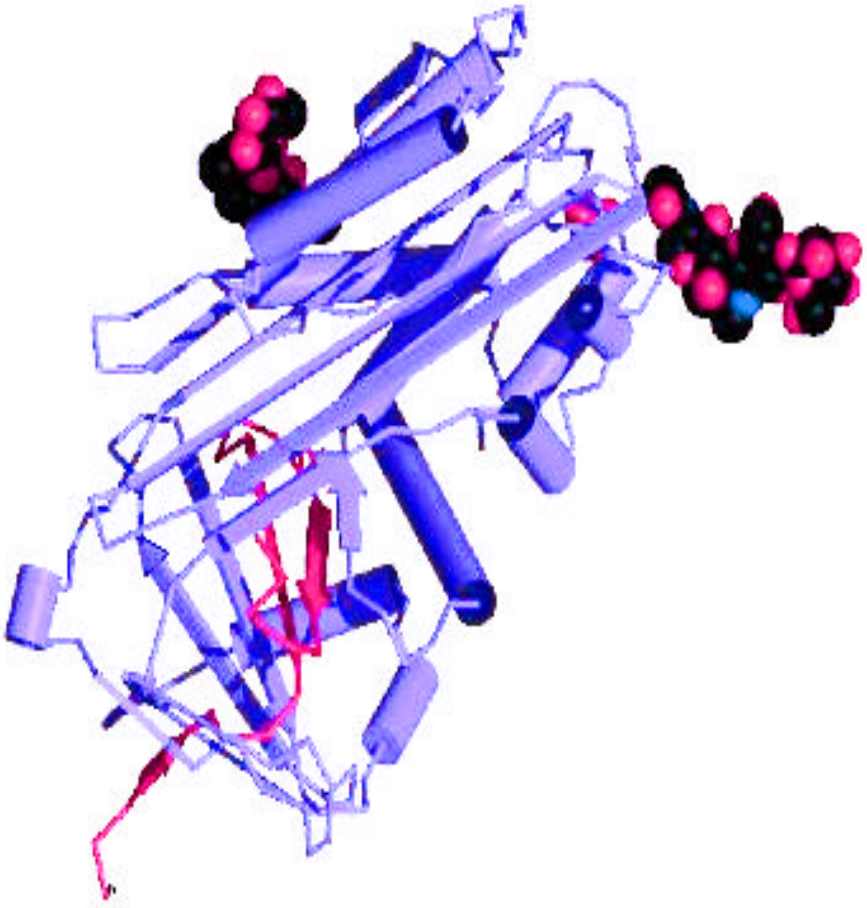

Figure 1.3.1: The structure of the mature, cleaved a_1-antichymotrypsin protein. Purple sections are the 337 amino acid A chain, pink sections are the 35 amino acid B chain. Diagram obtained from National Center for Biotechnology Information, with permission

In AD, ACT can act as a "pathological chaperone", catalysing the formation of fibrils of Aß, increasing the rate of polymerisation by 10 fold (Ma et al., 1994). Amino acids 1-12 of $Aß_{42}$ closely resemble the conserved active site of serine proteases, which is the target of the RSL of ACT. ACT is able to form a stable complex with Aß by interacting with these residues (Ma et al., 1996), moving from the stressed to the relaxed conformation. This increases the thermostability of ACT as the residues N-terminal to the cleaved bond insert into the major ß sheet of Aß (Whisstock et al., 1998). It is also possible for the ß-sheet of another Aß molecule to insert into ACT (Ma et al., 1996). During

this process ACT moves through four conformations, from a native, uncleaved state through two intermediates into an unfolded form. This occurs relatively quickly given the size of ACT (Pearce et al., 2000), and results in the loss of inhibitory activity (Whisstock et al., 1998).

1.3.2 Alpha-1-Antichymotrypsin as a Serpin

Alpha-1-Antichymotrypsin (ACT), also called SERPINA3 (Silverman et al., 2001) is a member of the serine protease inhibitor (serpin) family of acute phase proteins. It has a molecular mass of 55 – 66 kDa (Bao et al., 1987; Kalsheker, 1996; Travis and Salvesen, 1983) of which approximately 25% is carbohydrate. The mature protein consists of 394 amino acid residues. ACT has a very well ordered three-dimensional structure, consisting of eight well-defined alpha helices and three large ß-pleated sheets (Kalsheker, 1996). ACT is present in the blood at relatively low concentrations in the normal physiological state, but during the acute phase the levels in the blood increase dramatically and rapidly. During inflammation the increase in concentration is five fold (Kalsheker, 1996), and the concentration can double within eight hours of trauma (Travis and Salvesen, 1983).

The *ACT* gene (Fig. 1.3.2), located on chromosome 14q32.1 (Billingsley et al., 1993), is approximately 12,000 base pairs in length, and organised into five exons and four introns (Bao et al., 1987), and shows sequence homology with a_1-*antitrypsin*, and *antithrobin III*, suggesting a common ancestry (Bao et al., 1987; Chandra et al., 1983; Kalsheker, 1996; Travis and Salvesen, 1983).

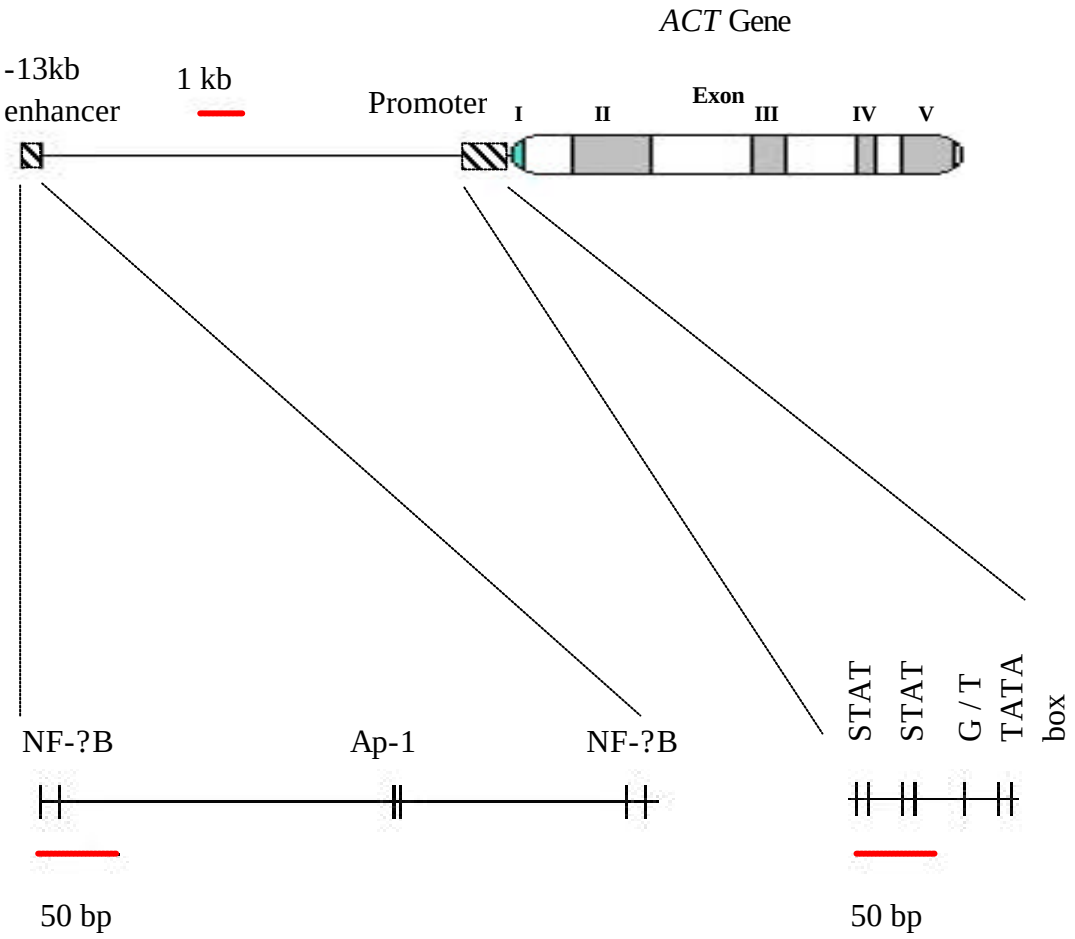

Figure 1.3.2: A scale diagram of the *ACT* gene and 5' regulatory regions. Scale bars shown in red. Hatched boxes indicate the positions of regulatory regions in the 5' region, and are shown in large scale at the bottom of the diagram. Shown in the enhancer region are the two nuclear factor-?B (NF-?B) sites and the Activating protein-1 (Ap-1) site. The promoter region shows the positions of the Signal Transducer and Activator of Transcription (STAT) binding sites and TATA box in relation to the –51bp promoter polymorphism, shown as G/T. Shaded regions within the coding sequence of the *ACT* gene represent exons The product of exon 1, the signal sequence, shaded blue, does not feature in the mature protein.

ACT is mainly synthesised in the liver, but it's mRNA is also expressed in a variety of other organs, such as the heart, lung, kidney, brain, breast and prostate (Kalsheker, 1996). There are three known biological functions of ACT. The first is the inhibition of mast cell chymases, neutrophil cathepsin G and proteases that convert angiotensin I to the biologically active vasoconstrictor angiotensin II (Bao et al., 1987; Kalsheker, 1996; Travis and Salvesen, 1983).

This means that one of the main functions of ACT may be the regulation of angiotensin II production. As mentioned above, ACT accelerates the formation of Aß fibrils (Ma et al., 1994), and uniquely amongst serpins it is able to bind DNA. This is achieved at a stretch of three basic lysine residues 212 – 214, but the significance of this is as yet unknown (Kalsheker, 1996).

The inhibition of neutrophil cathepsin G and mast cell chymases means that ACT plays a part in protecting the lower respiratory tract from damage by proteolytic enzymes, and it has been found in high concentrations in the bronchial secretions of patients with chronic bronchitis (Travis and Salvesen, 1983). The signal sequence polymorphism of *ACT* has been shown to associate with lung disease in smokers (Ishii et al., 2000). Complexed ACT is removed from circulation by receptor-mediated pathways, but cleaved ACT is a powerful chemoattractant for neutrophils. Cleaved and complexed ACT can induce the synthesis of IL-6, which may provide a mechanism for it's rapid production during the acute phase (Kurdowska and Travis, 1990), since IL-6 upregulates the synthesis of ACT.

1.3.3 Regulation of the a_1-Antichymotrypsin Gene

The expression of ACT in the liver is enhanced by IL–6 and glucocorticoids, and to a lesser extent by IL-1 (Baumann et al., 1987; Castell et al., 1989). ACT is also found in the brain, but as it is too large to pass the blood brain barrier it is instead synthesised by astrocytes (Kordula et al., 1998; Lieb et al., 1996). Subsequently it was found that expression of ACT in astrocytes is induced most potently by oncostatin M (OSM) and IL-1, while tumour necrosis factor-a (TNF–a) has a modest effect (Machein et al., 1995). On its own IL-6 is ineffective in inducing ACT expression, but co-treatment of astrocytes with soluble IL-6 receptor (sIL-6r) and IL-6 is a potent activator of ACT expression in astrocytes (Kordula et al., 1998; Lieb et al., 1996). This suggests that astrocytes may express specific OSM receptors. The expression of *ACT* mRNA in astrocytes is further enhanced by glucocorticoids working in concert with IL-1 and IL-6 without sIL-6r (Kordula et al., 1998; Nilsson et al., 2001b; Potter et

al., 2001). In lung epithelial cells OSM, IL-1 and the glucocorticoid analogue, dexamethasone have been shown to stimulate ACT production individually, but particularly, together, and it appears that the OSM II receptor may be utilised in these cells. This is due to the fact that leukaemia inhibitory factor (LIF) which binds to the OSM I receptor does not alter production of ACT (Cichy et al., 1995; Cichy et al., 1998)

OSM was found to activate two Signal Transducer and Activator of Transcription (STAT) elements, that are located at –125bp to –117bp and at -96bp to –87bp away from the *ACT* transcription start site. These STAT elements were identified as STATA and STATB, with the STATB element responding first, followed later by STATA (Kordula et al., 1998). The STAT elements do not appear to interact with IL-1 or TNF-a, suggesting that other regulatory elements must be able to influence the *ACT* gene. A 413bp-long element that conferred a response to IL-1 and TNF-a was identified 13kbp upstream of the transcription start site. This site, the 5' distal IL-1/TNF-responsive enhancer, contains three transcription factor-binding sites, two nuclear factor ?B (NF-?B) sites, at –13213bp to –13202bp and at –12831bp to -12820bp and an activating protein 1 (AP-1) site at –12985bp to –12979bp (Kordula et al., 2000). Investigation of signal transduction pathways (Lieb et al., 1996) and mutational analysis of these sites indicates that the 5' NF- ?B element contributes the most to the responsiveness of the *ACT* gene, the AP-1 site contributes to the response of both IL-1 and TNF, but the effect of the 3' NF-?B site is minimal (Kordula et al., 2000). In hepatoma HepG2 cells, IL-1 and TNF has only a slight effect on ACT mRNA synthesis, indicating that the -13kb enhancer element is not fully functional in hepatoma cells (Kordula et al., 2000). It is interesting to note that the *APP* gene flanking sequence also contains two NF- ?B binding sites that respond to IL-1ß, resulting in the upregulation of APP (Grilli et al., 1996; Grilli and Memo, 1999; Grilli et al., 1995). IL-1ß is upregulated in AD brains and interacts with a number of proteins associated with AD in addition to ACT and APP, such as ApoE and a_2-macroglobulin (Mrak and Griffin, 2000).

1.4 Alpha-1-Antichymotrypsin in Alzheimer's Disease

1.4.1 Beta-Amyloid Fibril Formation

As mentioned above, one of the characteristics of AD are the senile plaques observed in the brain. The major component of senile plaques is amyloid-ß (Aß), which is a proteolytic product of the amyloid precursor protein (Eriksson et al., 1995; Fraser et al., 1993; Mucke et al., 2000). The Aß surrounds a core of degenerating nerve endings. However, Aß is not the sole protein associated with senile plaques in AD. In addition a number of other proteins are found in association with senile plaques, such as apolipoprotein E, extracellular matrix proteins, amyloid P component, complement factors and cytokines (Atwood et al., 2002; Bradt et al., 1998; Luterman et al., 2000; Mucke et al., 2000; Namba et al., 1991; Ohm et al., 1995; Rebeck et al., 1995). The acute phase protein, a_1-antichymotrypsin is also associated with senile plaques, as it binds tightly to Aß, and specifically co-localises to AD plaques and blood vessels in the brain of patients with AD (Fraser et al., 1993; Janciauskiene et al., 1996; Janciauskiene et al., 1998; Licastro et al., 2000a; Ma et al., 1994; Pasternack et al., 1989). There are several conditions that result from the dysfunction or inappropriate regulation of acute phase proteins, such as a deficiency of the serpin a_1-protease inhibitor, which can result in cirrhosis or emphysema (Trowbridge and Emling, 1997). ACT is an acute phase protein, and the potential role of ACT in the pathogenesis of AD has been the subject of considerable interest.

Aside from the presence of ACT at the site of amyloid deposits in AD, there is further evidence to implicate ACT in AD, for instance Aß is known to activate microglia, which then secrete a variety of cytokines, including interleukin (IL)-1ß and IL-6, and IL-1ß is known to stimulate ACT expression (Abraham et al., 2000; Kordula et al., 1998; Licastro et al., 2000b; Vandenabeele and Fiers, 1991). It is also known that while many of the proteins that associate with Aß in senile plaques bind to other forms of amyloid, ACT is associated primarily with Aß amyloidosis (Mucke et al., 2000).

There is also evidence that ACT is produced in the region of senile plaques, rather than in the liver and then migrating to the brain. Northern analysis of the grey matter of AD brains revealed ACT mRNA expression. In addition, *in situ* radiolabelled mRNA probing and astrocyte specific immunostaining has demonstrated that ACT mRNA was originating from astrocytes in AD brains, something that rarely happens in the brains of control subjects (Pasternack et al., 1989). In addition, studies using double transgenic mice, that is mice that produce both human APP and human ACT in their brains, and mice that express hACT or hAPP alone generate evidence to link ACT to plaque production. Mice expressing hACT alone do not develop senile plaques, whereas mice expressing hAPP alone, or hAPP and hACT together, do develop AD-like plaques in the hippocampus. The plaque burden of mice expressing hACT and hAPP was greater than that of mice expressing hAPP alone (Mucke et al., 2000). Further studies revealed that amyloid was being deposited particularly in the hippocampus, where ACT expression was at its highest, and Aß peptide levels were raised from an early age, before amyloid was being deposited (Nilsson et al., 2001a). Taken together, these models provide compelling evidence for the involvement of ACT in AD.

It has been demonstrated that ACT has a strong stimulatory role in the polymerisation of Aß (Ma et al., 1994). Polymers of Aß form Aß fibrils, something which happens spontaneously, but in the presence of ACT and apolipoprotein E this will happen more rapidly, within hours, rather than days. There is a variation in the catalytic effect of apolipoprotein E between its isoforms. The greatest effect is seen with *apolipoprotein E* ε4, which is also known to be the strongest genetic risk factor for AD. *Apolipoprotein E* e2 when combined with e4 has an inhibitory effect on the formation of amyloid fibrils, and *apolipoprotein E* ε2 is believed to be a negative risk factor for AD (McGeer and McGeer, 1995).

The amino- and carboxyl-termini of Aß strongly resemble the conserved active site of serine proteases, and this region is recognised specifically by ACT, and can apparently act as a pseudosubstrate (Fraser et al., 1993; Janciauskiene et al., 1998; Potter et al., 1992). It is these regions that interact with ACT, and they

insert into one of two ß-sheets, destroying the inhibitory function of the serpin. This also causes ACT to undergo a transition from the stressed to the relaxed state, and is accompanied by an increase in thermostability (Eriksson et al., 1995).

The role of ACT in fibril formation, is however, somewhat complicated by the finding that it may play a part in destabilising Aß fibrils (Eriksson et al., 1995; Fraser et al., 1993; Janciauskiene et al., 1998). At a molar ratio of 1 : 10 (ACT to Aß) the serpin inhibits the formation of ß-amyloid fibril formation, and at an equimolar level ACT promotes the rapid disaggregation of pre-formed Aß fibrils (Eriksson et al., 1995; Fraser et al., 1993). It would appear that there is a threshold ratio of ACT to Aß below which aggregate formation is stimulated (Janciauskiene et al., 1998). There are at least two explanations for the discrepancies between the findings that ACT destabilises and disaggregates Aß fibrils at high concentrations, but that ACT is found in higher concentrations in AD brains compared with normal brains (Janciauskiene et al., 1996). One is that these studies were *in vitro*, using synthetic ACT, and that *in vivo* ACT undergoes some processing that does not occur with the synthetic form, or that there are other factors in the brain that have a bearing on the behaviour of ACT, such as the length of the amyloid fibril or the pH at which the reactions occur. It has been demonstrated that fibrils form faster at lower pH (Eriksson et al., 1995). The second explanation is that when ACT destabilises or disaggregates Aß fibrils it exposes new domains within the amyloid fibril that may undergo additional proteolysis, or interact with cell-surface receptors (Fraser et al., 1993).

Another mechanism by which ACT may affect the concentration of Aß in the brains of AD patients involves the clearance of Aß. It has been reported that Aß clearance is dependent on metallopeptidase E.C.3.4.24.15, or MP24.15 (Yamin et al., 1999). MP24.15 appears to activate a serine protease that cleaves Aß (Yamin et al., 1999). $Aß_{1-42}$, in comparison with $Aß_{1-40}$, is degraded slowly by the MP24.15 pathway and aggregated $Aß_{1-42}$ showed almost no degradation. In the presence of serine protease inhibitors Aß degradation is partially or

completely inhibited. ACT achieves a 60% inhibition of this process (Yamin et al., 1999).

Another report has suggested a further mechanism by which ACT and $Aß_{42}$ can interact in AD. The exact role of Aß in AD pathology is unclear, and it has been speculated that its neurotoxic effects may be a result of interacting with other molecules to produce complexes with biological activities that promote neuroinflammation. Microglia and astrocytes in brain regions exhibiting abundant AD plaques over-express both pro- and anti-inflammatory reactants. The ACT-Aß complexes that are found in AD plaques are known to have lost the protease inhibitor activity of ACT, however, when ACT is incubated with Aß it has been shown to demonstrate pro-inflammatory properties (Sun et al., 2002). These experiments showed that Aß alone induced TNF-a and IL-6 levels in glioma cells, but a 1:10 molar ratio mixture of ACT:$Aß_{42}$ mixture under the same experimental conditions showed no effect on IL-6 levels, but had an even greater effect on TNF-a induction in the same cells. With $Aß_{42}$ alone TNF-a levels rose by 55% compared to controls, but with the mixture of ACT and $Aß_{42}$ the TNF-a activity rose by 190% against the controls.

Studies have shown that AD patients have an increased concentration of ACT in the CSF and plasma (Janciauskiene et al., 1996; Licastro et al., 2000a). This has led to an examination of the factors that cause overexpresion of ACT, to see if these factors have an effect on the risk of developing AD. IL-1ß is known to stimulate the expression of acute phase proteins in the liver, and it promotes overexpression of ACT mRNA in activated astrocytes (Kordula et al., 1998; Lieb et al., 1996). Common polymorphisms of the *ACT* and *IL-1ß* genes affect the plasma levels of ACT or IL-1ß. Patients who had the *ACT* signal sequence or *IL-1ß* T/T genotypes were shown to have the highest plasma levels of ACT or IL-1ß in plasma, and the presence of these genotypes increased the risk of AD (Licastro et al., 2000a; Licastro et al., 2000b). It has also been reported that the A (threonine) allele of this *ACT* polymorphism, a biallelic (A/G) mutation at position –17, can work in concert with the *apolipoprotein E* ε4 allele (Kamboh et al., 1995) which agrees with the earlier findings that ACT and apolipoprotein E together have a stimulatory effect on fibril polymerisation (Ma et al., 1994). A

further biallelic polymorphism has been located in the promoter region of *ACT* (-51, G/T), which has an effect on the rate of ACT expression (Licastro, 2002; Morgan et al., 2001). The T allele of the *ACT* promoter polymorphism was shown to be in 95% linkage disequilibrium with the signal sequence threonine.

1.4.2 Genetics of a_1-Antichymotrypsin in Alzheimer's Disease

In the search for genes that might predispose to AD, an early candidate was the *apolipoprotein E* (*APOE*) gene. Antibodies to ApoE bind to the amyloid plaques of Alzheimer's disease (Namba et al., 1991) and ApoE was soon found to bind to synthetic Aß (Strittmatter et al., 1993). It was found that the e4 allele of *APOE* correlated with amyloid plaque density, both in AD and normal brains (Berr et al., 1994). Although further studies demonstrated that *APOE* e4 is neither necessary, nor sufficient for AD to develop (Polvikoski et al., 1995) it is a risk factor (Kamboh et al., 1995; Roses, 1996; Saunders et al., 1993). The search began for other genes associated with AD, which might work in conjunction with, or independently of *APOE*.

Previously, it had been demonstrated that ACT had been found associated with amyloid deposits in AD (Abraham et al., 1988), and it had also been shown that Aß fibrils form more rapidly in the presence of ApoE and ACT (Ma et al., 1994). Studies of the *ACT* gene have revealed a number of polymorphisms in coding and non-coding regions (Byth and Cox, 1993; Morgan et al., 2001; Poller et al., 1993; Samilchuk and Chuchalin, 1993; Tsuda et al., 1992; Wang et al., 2002). (See figure 1.4.1 and table 1.4.1). In mouse models, as well as in AD brains the *APOE* e4 allele has been linked with higher levels of ACT (Licastro et al., 1999a).

Most attention has been focussed on the signal sequence polymorphism located 17 bp upstream of the *ACT* start site. This is an A? G substitution that results in the substitution of a threonine with an alanine at amino acid -15, which has a more hydrophobic character than threonine. This, it has been suggested, may enhance ACT secretion (Wang et al., 2002). The A-17G polymorphism is unable to affect ACT function, as the signal peptide it generates is cleaved from ACT. Several studies have attempted to prove an association between either

allele of the *ACT* signal sequence polymorphism and AD, either alone (Wang et al., 2002), or acting in concert with *APOE* e4 (DeKosky et al., 2003; Kamboh et al., 1997; Licastro et al., 1998; Licastro et al., 1999b; Nacmias et al., 1996; Scacchi et al., 1999), with *IL-1ß* T (Licastro, 2001; Licastro et al., 2000b), *presenilin 1* allele 1 (*PS1**1) (Wang et al., 1998). These studies have failed to show a consistent association with the signal sequence polymorphism and AD, either alone or in concert with other genes. Less attention has been given to the reported associations between *ACT* –17G and *IL-1ß* T or *PS1**1, but a number of reports have been published that contradict the finding that *APOE* e4 and *ACT* –17G are associated with AD (Didierjean et al., 1997; Ezquerra et al., 1998; Fallin et al., 1997; Haines et al., 1996; Helisalmi et al., 1997; Lamb et al., 1998; Muller et al., 1996; Murphy et al., 1997; Tysoe et al., 1997; Yoshizawa et al., 1997). It cannot be argued that the contradictions between these reports are due to differences in the populations studied, as studies reporting an association, and those that showed no association have been performed on a wide range of populations, from Europe (United Kingdom, Italy, Germany, Spain, Finland, and France), North America (United States of America), Asia (Japan) and Africa (Nigeria). There were no regional differences between the findings of an association between *APOE* e4 and *ACT* -17G. Experimental methodologies, such as sample size, and classification of AD, differed between studies, but the majority of reports used the NINCDS-ADRD method of diagnosing AD, and samples sizes varied between all of the papers. It is possible that the effect of the *ACT* –17G and *APOE* e4 haplotype is to lower the age of onset of AD, and therefore in a comparison of AD patients against controls would show no association with this haplotype (Talbot et al., 1996). If this is correct, patients would have to be age-matched with controls, and results stratified by age.

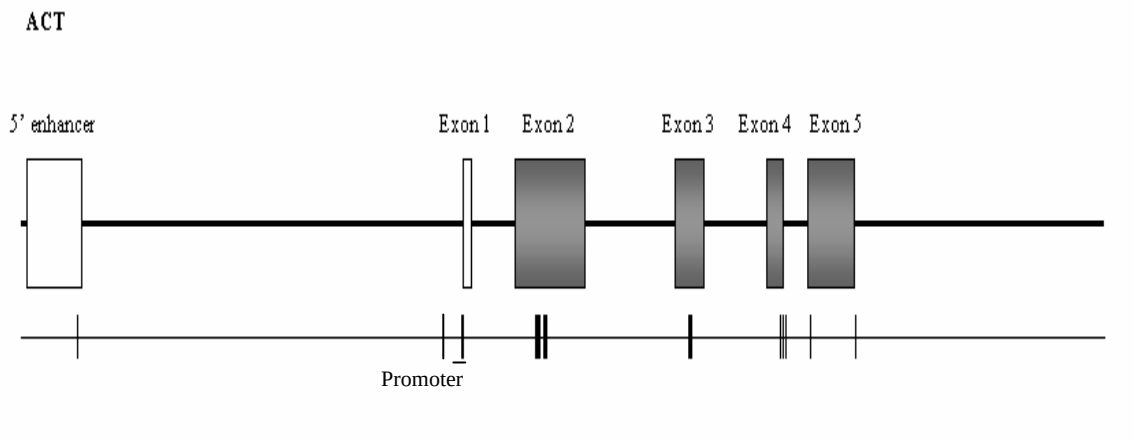

Figure 1.4.1: A cartoon showing the *ACT* gene with positions of known polymorphisms (excluding microsatellite) shown as vertical lines underneath.

Region	Nucleotide Position	Base Substitution	Amino Acid Position	Amino Acid Substitution	Reference	Population
Enhancer	-12779	G/T	N/A	N/A	*	Caucasian
Promoter	-596	G/A	N/A	N/A	*	Caucasian
Promoter	-501	G/A	N/A	N/A	*	Caucasian
Promoter	-51	G/T	N/A	N/A	Morgan, 2001	Caucasian
Exon 1	-17	A/G	-15	Thr ? Ala	Thome, 1995	Caucasian
Exon 2	2398	A/G	25	Alu? Asn	Meng, 2000	Han
Exon 2	2440	G/A	39	Leu? Leu	Meng, 2000	Han
Exon 2	2484	T/C	53	Leu? Pro	Poller, 1993	Caucasian
Exon 2	2552	A/G	76	Lys? Lys	Wang, 2002	Caucasian
Exon 2	2701	G/A	128	Asp? Asn	Wang, 2002	Caucasian
Exon 2	2745	C/G	142	Ala? Gly	Poller, 1993	Caucasian
Exon 3	7505	C/G	227	Pro? Ala	Poller, 1993	Caucasian
Exon 3	7559	G/A	241	Leu? Leu	Wang, 2002	Caucasian
Exon 3	7580	C/T	250	Ser? Ser	Wang, 2002	Caucasian
Intron 4	7698	G/A	N/A	N/A	Wang, 2002	Caucasian
Exon 4	10804	T/C	301	Leu? Pro	Wang, 2002	Caucasian
Exon 4	11071	A/G	324	Thr? Thr	Wang, 2002	Caucasian
Exon 5	12794	A/G	389	Met? Val	Muramatsu, 1996	Japanese
3' UTR	11998	C/A	N/A	N/A	Wang, 2002	Caucasian
3' UTR	12013	C/T	N/A	N/A	*	Caucasian

Table 1.4.1 : Previously discovered SNPs in the *ACT* gene, and the population in which they were first discovered. * indicates SNPs discovered in our laboratory, as yet unpublished.

Another variable sequence of the *ACT* gene is the polymorphic microsatellite located in the 5' flanking region, 2.5kb from the start of the *ACT* gene. This microsatellite consists of a variable number of (TA)n(GA)n repeats (Byth and Cox, 1993). Although no significant difference in microsatellite allele frequency was reported between AD cases and controls, when the *APOE* allele was taken into account it was shown that with the *APOE* e4 allele, the A10 (called B10 in Byth and Cox, 1993) allele frequency was lower in controls, compared to AD cases (Morgan et al., 1997). This study also found no association between the *APOE* e4 allele and the *ACT* –17G allele of the signal sequence polymorphism. Another study, on a smaller sample, with younger sporadic AD patients, again showed an association between the *APOE* e4 allele and AD, but did not show an increased risk for AD in individuals possessing both *APOE* e4 and *ACT* A10 (Durany et al., 1998). This may be due to a number of factors. Although AD was confirmed histologically by the CERAD criteria (Mirra et al., 1993) in both cases, and the control groups were of equivalent age (72.8±9.0 years, (Morgan et al., 1997) against 72.2±8.8 years, (Durany et al., 1998)), the ages of AD patients were different (80.8±9.8 years, (Morgan et al., 1997) against 72.4±9.2 years, (Durany et al., 1998)). It has been suggested that the *ACT* –17G allele of the signal sequence polymorphism may be associated with a lower age-at-onset of AD (Talbot et al., 1996). It may be possible that a similar effect could account for the differences in the two studies. Another possible reason could be the difference in sample sizes. The Durany study contained less than a third of the number of AD cases compared to the earlier Morgan study. It may be that the combined effects of the *APOE* e4 and *ACT* –17G alleles are too small to be detected with a sample size as small as in the Durany study.

Another study (Bass et al., 1998), looking at the same combined effect of *APOE* e4 and *ACT* –17A in AD casts doubt on the possibility of an interaction between these alleles in AD. Although this study did not examine the brains of AD patients histologically, and probably examined a different population, it seems to contradict the previous findings (Morgan et al., 1997). There was no association found between the microsatellite A10 allele and the *ACT* signal

sequence –17A allele to lower the age of onset of Alzheimer's disease. In addition, there was no significant difference found in microsatellite allele frequencies between AD cases and controls, either alone or with *APOE* e4.

It has been reported that ACT regulates the formation of Aß-fibrils (Eriksson et al., 1995), and that there may be an increase in ACT levels in the brains (Licastro et al., 1998), and blood (DeKosky et al., 2003; Gabriel et al., 1998; Hinds et al., 1994; Lieberman et al., 1995; Matsubara et al., 1990; Wang et al., 2002) of AD patients. In cerebrospinal fluid some studies have shown a correlation between ACT levels and AD (Matsubara et al., 1990), others have not, but suggest that there may be an involvement between AD and ACT (Pirttila et al., 1994). Studies of the *ACT* coding region have not provided conclusive evidence of a mutation that might enhance the secretion of ACT from cells producing ACT. Some laboratories have begun looking at non-coding, regulatory regions of the *ACT* gene. A promoter and an enhancer for *ACT* have been found, and functional activity has been determined as described above.

A polymorphism has been discovered in the promoter region of the *ACT* gene, located 51 bp upstream of the starting codon (Morgan et al., 2001). This polymorphism, a G ? T transversion, is located 21 bp upstream of the putative *ACT* TATA box, and 36 bp downstream of the STATB site. TATA boxes bind nuclear proteins, specifically transcription factors, and transcription initiation complexes form around them (see figure 1.4.2). It has been demonstrated that that the T allele results in a greater expression of *ACT* with and without stimulation by OSM. This effect was shown in the human liver cell-line, Hep G2 as well as in a mixed population of astrocytes and glial cells, T98G (Morgan et al., 2001). In a sample of elderly subjects with a clinical diagnosis of AD, patients with a GG genotype were found to have lower plasma ACT concentration than those with a GT genotype, which again was lower than those with a TT genotype. This study found that there was a significant difference in plasma ACT concentration between G allele carriers and T allele carriers. A third finding of this study was that the *ACT* promoter polymorphism G allele is in almost complete linkage disequilibrium with the *ACT* –17 signal sequence G

allele, and the T allele of the promoter polymorphism with the A allele of the signal sequence polymorphism.

These findings are interesting for a number of reasons. Firstly, it provides a mechanism for the elevated ACT levels seen in AD brains. In contrast to the theories about the signal sequence polymorphism having an effect on the rate of secretion of ACT, the promoter polymorphism demonstrates that more ACT is being produced, which would have an effect on the rate of fibrilisation of Aß in AD brains. In addition, several studies looking at polymorphisms in the *ACT* gene have suggested that alleles reported in some studies have an association with AD, and others contradict that. It has been suggested that these alleles could be in linkage disequilibrium with an allele that does directly affect ACT release, and it appears that the promoter polymorphism fits this description.

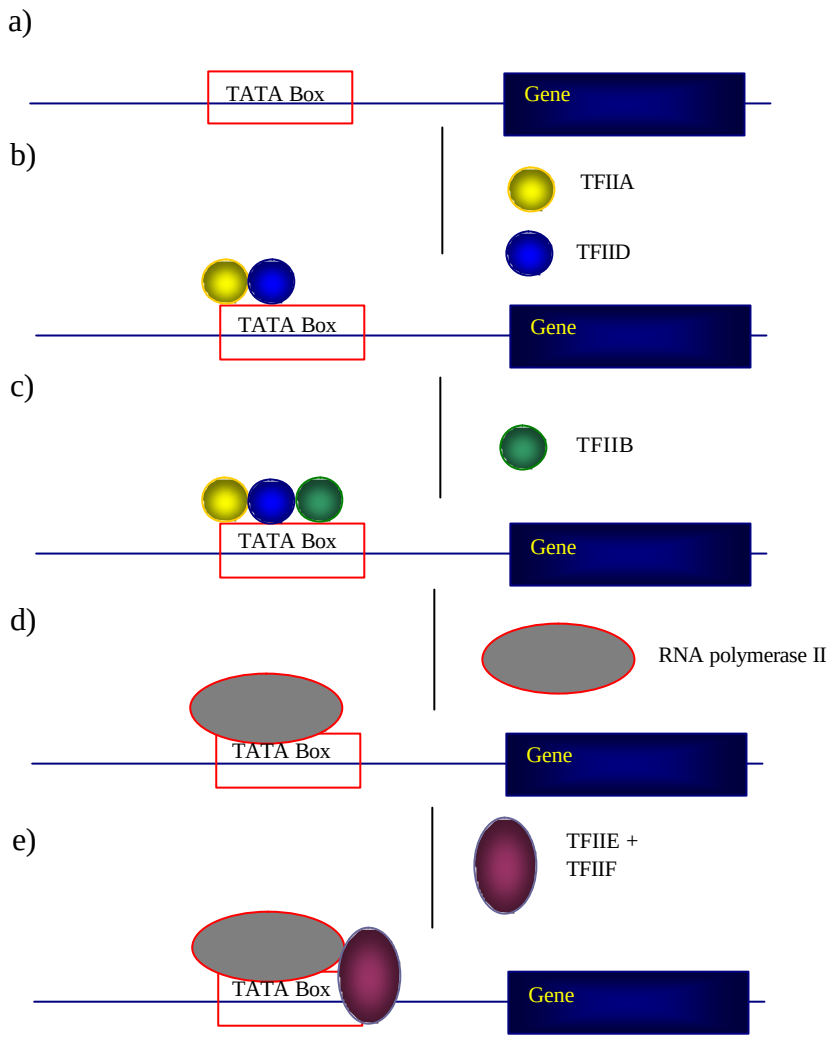

Figure 1.4.2: Cartoon outlining initiation of transcription in eukaryotic cells. Upstream TATA boxes bind transcription factors to form a pre-initiation complex. a) The TATA box is located upstream of the gene. b) Two transcription factors (TF), TFIIA and TFIID bind to the TATA box. c) TFIIB binds to the TATA box and proteins. d) RNA polymerase II binds to the complex. d) With the addition of TFIIE and F the pre-initiation complex is complete. (Brown, 1993)

1.4.3 Alpha-1-Antichymotrypsin and it's Potential for Therapy

As discussed above, a_1-antichymotrypsin plays a role in modulating Aß fibril formation in Alzheimer's disease in a concentration-dependent manner. If lowering the amount of Aß fibrils is proved to be of benefit to patients with AD, then ACT would be an extremely useful target for therapy. Using a competitor or inhibitor of ACT would mean less ACT would be available to catalyse the fibrillisation of Aß. This alone makes it worthy of study, but other reports have suggested that ACT may have a role to play as a biomarker in the diagnosis of AD (DeKosky et al., 2003; Sun et al., 2003). There is a clear need to develop better diagnostic tools for the study of AD. At present AD can only be diagnosed reliably *post mortem*, so many studies recruit patients with dementia that may ultimately prove not to be Alzheimer's disease. This has undoubtedly hampered progress in the understanding of this condition. If a reliable clinical test for AD can be developed, then research will be more conclusive, and the treatment of this disease should benefit as a result.

The criteria of a good biomarker have been defined by Gracon and Emmerling, 2001. In brief, it should measure a fundamental contributor to AD neuropathology, be clinically accessible, be relevant to the clinical outcome, and must be validated in cross-section and longitudinally. The potential sources of such a marker are the brain, CSF, plasma, serum, platelets, lymphocytes, or other cellular components of the blood or other tissues. Potential markers include APP, or APP secretases, total Aß, or $Aß_{1-40}$, or $Aß_{1-42}$, tau protein, apolipoprotein E, cholesterol, inflammatory cytokines, or antioxidant capacity.

ACT fits many of the criteria for a good biomarker. It is clinically accessible, as it can be measured in the serum or CSF and it has been postulated to play a fundamental role in AD (DeKosky et al., 2003; Lieberman et al., 1995; Sun et al., 2003). There have been several proposals for the use of ACT as a biomarker (Brugge et al., 1992; Hinds et al., 1994; Matsubara et al., 1990). Several studies have been completed in order to test the efficacy of ACT as a biomarker of AD. It has been reported that a concentration of 60 mg/dL of serum ACT can separate putative AD patients from control groups (Lieberman et al., 1995), and

taking several measurements of serum or CSF ACT over time was effective in differentiating AD-type patients from controls and patients with other dementias and degenerative disorders (Matsubara et al., 1990). This study also revealed that CSF ACT levels tended to correlate with the degree of dementia being suffered. Other reports have not been able to demonstrate the effectiveness of ACT as a biomarker, however, it would appear that those that did not support the evidence of ACT being a suitable biomarker utilised different methodologies in measuring ACT levels. When radial immunodiffusion assays (RIA) were used they were able to support this theory (Furby et al., 1991; Lieberman et al., 1995; Matsubara et al., 1990; Pirttila et al., 1994).

The problem with using ACT as a biomarker is that it tends to produce an unacceptably high level of false positives, making it unsuitable for population screening (Lieberman et al., 1995). RIA does not appear to be extremely well suited for individual diagnosis either, as it is somewhat expensive and time consuming to use on a small scale. Having to repeat the assay over a period of time, to observe persistently elevated ACT, which appears characteristic of AD (Matsubara et al., 1990) further reduces the suitability of this test. At present, serum ACT levels can be used only to add weight to a putative diagnosis of AD made with the help of clinical observation and testing, such as with the Mini Mental State Examination (MMSE).

1.5 Summary

Central to the pathology of AD is the peptide ß amyloid. The physiological role, if any, of this peptide is currently unknown. It is derived from the proteolytic processing of the membrane associated amyloid precursor protein. Recent evidence suggests that the development of the neurotoxic form of Aß is critical in the aetiology of AD. This results from cleavage by ?-secretase, a protein complex that includes the presenilin 1 and 2 proteins. Most mutations associated with the familial form of AD alter the cleavage site of APP, or the presenilins.

Evidence from various sources supports the theory that a chronic neuroinflammation develops during AD. Brain tissue from AD patients have elevated levels of inflammatory molecules, such as cytokines, complement factors and serpins. Drugs that are used in the treatment of inflammation may have some effect in slowing the onset of AD. If this is the case, controlling neuroinflammation through drugs may help to slow the onset and progression of the disease.

To develop therapies that will prevent or halt the damage caused by AD it will be necessary to learn more about the disease. More research is needed to explain what causes AD, how it progresses, and precisely how it results in cell death in neurones. Much of this research has centred on senile plaques. These plaques are composed of fibrils of Aß, which had been thought to be responsible for the neurological damage seen in AD. More recently, it has been shown that smaller Aß molecules seem to be responsible for the pathology of AD. These Aß oligomers are the focus of a great deal of attention, but the wider issues of Aß generation and clearance should also be addressed. If AD is the result of an imbalance between Aß generation and clearance, it is possible that the senile plaques provide a reservoir of Aß, caused by too little Aß clearance relative to generation. The molecule that binds amyloid in the fibrillar form is the serine protease inhibitor, a_1-antichymotrypsin.

ACT is synthesised in the brain as part of the inflammatory response. It is able to bind to two Aß molecules, which can result in the formation of insoluble fibres, which are found in senile plaques. There have been conflicting reports that polymorphisms in the *ACT* gene are associated with AD. If it can be demonstrated that polymorphisms in the *ACT* gene do associate with AD then this could be useful in developing a diagnostic tool for AD.

1.6 Research Aims

The aim of this project is to further the understanding of the function of the a_1-*antichymotrypsin* promoter: specifically the effect the G? T transversion has on that function and its role in disease. Achieving these aims will involve an investigation of the functional activity of the promoter and the production of mRNA in different cell lines and also a characterisation of the mechanism of promoter activity. In addition the relevance of ACT as a risk modifier of AD has not been firmly established, and this is to be explored in more detail.

2 MATERIALS AND METHODS

2.1 Dual-Luciferase Reporter® Assays

The Dual Luciferase Reporter® (DRL) assay provides a way of determining the functional activity of any DNA segment. It can be used to demonstrate that a putative regulatory region does have a functional effect, to determine if a regulatory region responds to a cytokine, or, as in this case, to compare the effect of two variant promoters. The putative promoter is coupled to the *luciferase* gene, from the firefly, *Photinus pyralis*, and transfected into cultured cells. Three commercially available constructs were used for this work, pGL3 enhancer, pGL3 control and pRL (Promega). The pRL construct does not contain the *luc* reporter gene, instead it contains the *Renilla* gene, taken from the sea pansy *Renilla reformis.*

pGL3 control is used as a positive control in the DRL assays. The *luc* gene is flanked by the SV40 promoter and enhancer, ensuring that the *luc* gene is constitutively active at a high level. The pGL3 enhancer construct is used to determine the "background" level of expression, as the *luc* gene is active at a low level with this construct. The pRL construct contains all the regulatory elements to activate the *Renilla* gene at a steady level, and acts as a transfection control. This construct is co-transfected with the other constructs.

In addition, two experimental constructs were prepared pGL3E 'G' and pGL3E 'T', containing the G and T alleles of the *ACT* promoter. These were based on the pGL3 enhancer construct, but the *ACT* promoter was inserted just before the *luc* gene (see figure 2.1.1). This should enable the reporter gene to be activated by the same mechanism as the *ACT* gene *in vivo*, allowing the *luc* gene to act as a surrogate for *ACT*. The luciferase protein emits light, allowing it to be measured more conveniently than ACT.

The cells used for the DRL assays were grown in 12-well plates, and typically one experiment required at least three wells each to be transfected with control, enhancer, pGL3E 'G' and pGL3E 'T', all co-transfected with pRL (see figure

2.1.1). Once they were transfected, the cells could then be stimulated, as required, with human recombinant oncostatin M, OSM (R and D Systems), incubated for the time required, then harvested. The harvesting lysed the cells, allowing light output to be measured. Both luciferase and Renilla were measured separately on the same luminometer.

The light measured by the luminometer was dependent on the amount of luciferase present, which was dependent on the activity of the *luc* gene. The firefly luciferase reading was normalised by the *Renilla* luciferase, to allow for differences in transfection efficiency. The luminescence ratio of both *ACT* constructs is then normalised to the enhancer activity alone, to remove the effect of the SV40 enhancer present in the *ACT* promoter constructs. This ratio gives an idea of how active each *ACT* promoter allele is under basal conditions, or after stimulation with OSM in a variety of different cell lines.

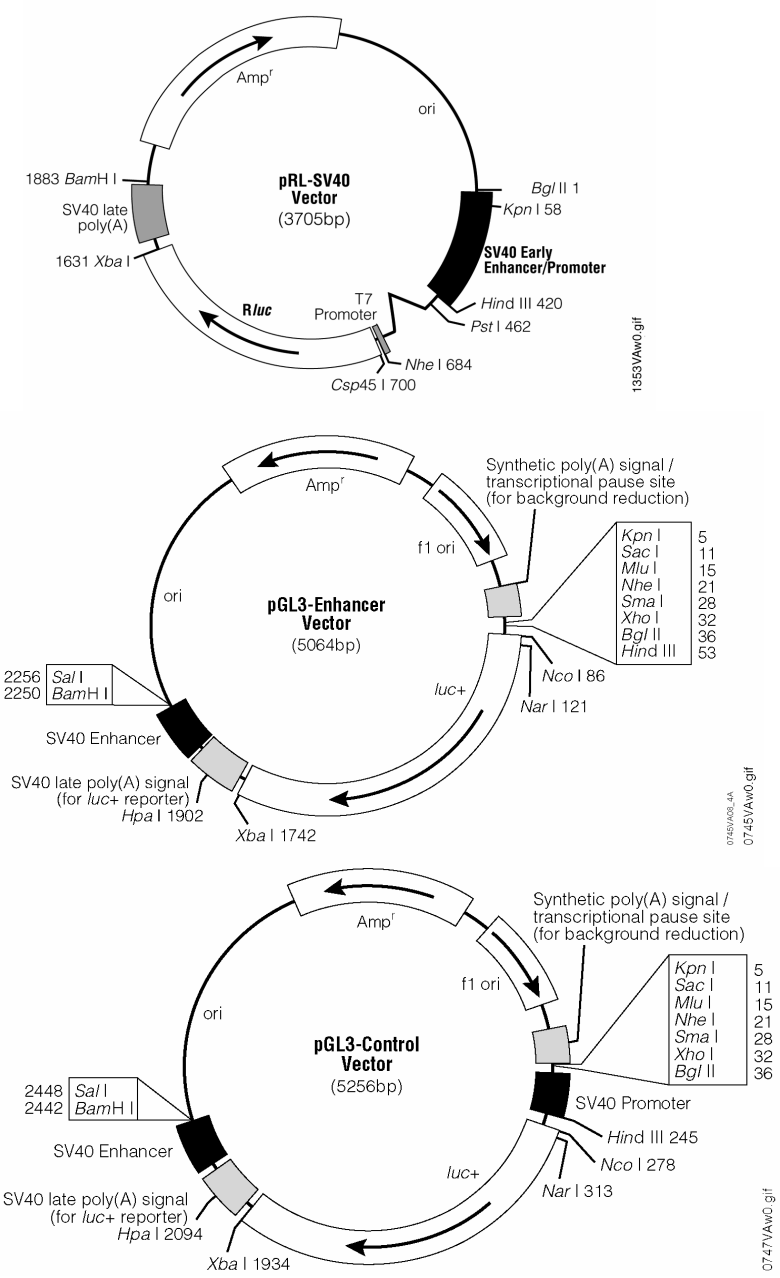

Figure 2.1.1: Maps of the constructs used in the DLR assays, showing functional regions, luciferase genes, antibiotic resistance genes and restriction sites. The pGL3 Enhancer vector was modified to include the *ACT* promoter thereby generating constructs pGL3E 'G' and 'T'. Figure is reproduced with kind permission of www.promega.com, the suppliers of these constructs.

2.1.1 Tissue Culture

2.1.1.1 General Cell Culture Materials and Methods

The growth of cells for the DLR assays has a number of common elements, which will be described here, to avoid unnecessary repetition later.

There were four cell lines used in the DLR assays. HepG2 (ECACC), human liver hepatoma, isolated from a liver biopsy of a 15 year old male Caucasian, T98G, human glioblastoma, a mixed glial / neuronal cell line derived from a multiform tumour from a 61 year old Caucasian male (ECACC), U373 MG, astrocytes (ECACC) and SH-SY5Y, a neuronal cell line, derived from a human neuroblastoma. The SH-SY5Y cells were a gift from Dr. Ellen Billett of Nottingham Trent University. Cells were always grown at 37ºC, in a humidified atmosphere containing 5% CO_2. Unless specifically mentioned, they were grown in complete media, although the media varied between cell lines. Complete media contains a number of additives not included by the manufacturer, listed in table 2.1.1.

Additive	Stock Concentration	Dulbeco's Modified Essential Medium, DMEM (Invitrogen)	Eagles Minimum Essential Medium, EMEM (Sigma)
Cell Type		T98G	Hep G2, U373 MG
Foetal Bovine Serum (Invitrogen)	100%	50ml	50ml
L-Glutamine (Invitrogen)	200mM	5ml	10ml
Penicillin – Streptomycin (Invitrogen)	10,000 U/ml	5ml	5ml
Amphotericin B (Invitrogen)	250µg / ml	5ml	5ml
Tetracycline	10µg / ml	250µl	250µl
Non-essential amino acids (Sigma)	100x	-	5ml
Sodium Pyruvate (Sigma)	100mM	-	5ml

Table 2.1.1 : Table showing the additives required to make up 500ml of complete media.

Serum-free media contains all the above additives except FBS.

In addition, the SH-SH5Y cells were cultured in complete Ham's F12/EMEM. This contained the following media and supplements, at the same concentrations given in table 2.1.1.

Supplement	Volume
EMEM	214.88ml
Ham's F12 (Sigma)	214.88ml
FBS	50ml
L-Glutamine	5ml
Penicillin - streptomycin	5ml
Ampicillin	5ml
Tetracyline	250µl
Non-essential amino acids	5ml

Table 2.1.2: Supplements added to make complete Ham's F12 / EMEM

All media was filtered through a 0.5µm filter, stored at 4°C, and warmed to 37°C before use.

2.1.1.2 Thawing Cells

The DLR assays were performed on the cell lines listed above (section 2.1.1.1). Whilst some of these cells were supplied commercially, others were taken from liquid nitrogen storage, from our own stocks, or from other laboratories. In the case of cells stored in liquid nitrogen, before use they needed to be defrosted in such a way as to minimise damaging the cells.

Cells that had been frozen in liquid nitrogen were usually stored in aliquots of 1 – 1.5ml, in freezing media. The cells need to be warmed as quickly as possible to prevent damage, so after ensuring the cryo-vials were sealed, they were incubated at 37°C until defrosted. After this the cell suspension was mixed with 5ml complete medium (see table 2.1.1) and 5ml FBS. This suspension was transferred to a 25cm^2 flask and incubated at 37°C.

2.1.1.3 Maintaining Cell Lines

To keep cultured cells growing, the same techniques were used for all cell lines, although cells were grown in the appropriate medium. This involved removing the old medium, washing the cells, and replacing the medium. When the cells reached 90% confluency, they were removed and allowed to grow on a surface area three times bigger, so cells in a 25cm^2 flask would be moved to a 75cm^2 flask, or cells from one 75cm^2 flask would be divided into three 75cm^2 flasks.

Cells were fed every two to three days with complete medium. The old medium was removed, the cells were washed twice with 3ml filter sterilised phosphate buffered saline (PBS, Oxoid) and then an appropriate volume of medium was added. For cells growing in 25cm^2 flasks, this was 10ml, for 75cm^2 flasks, 25ml, then the cells were incubated at 37°C. When cells were nearly confluent, they were split. The first time this was done after defrosting the cells, they were split into three 25cm^2 flasks. When the cells in these flasks had almost reached confluence they were transferred to 75cm^2 flasks.

To split cells the medium was removed, and the cells were washed twice in PBS. 3ml of 1x trypsin-EDTA were added to the cells, which were then incubated for 5 minutes. The cells were observed through a microscope to confirm that they had detached from the flask after this time. If they had not, they were incubated for longer. Once the cells had detached, the trypsin was stopped by the addition of 10ml complete medium. The suspension was thoroughly mixed, and 5ml of it added to one of three new flasks. These were topped up with 20ml of complete medium, and gently mixed, before being incubated.

2.1.1.4 Preparing Tissue Cultured Cells for Transfection

Each experiment was carried out at least three times, and each experiment obtained three measurements under the same conditions. The cells were transferred to 12-well tissue culture plates, which were arranged as three rows of four columns. Cells in each column were transfected with the same constructs, and were incubated for the same time before harvesting.

Cells, grown to approximately 70% confluence, in 75cm^2 flasks were used to seed four 12-well plates. The cells were washed twice with PBS, then 3ml 1x trypsin-EDTA was added. The cells were incubated at 37°C for five minutes, and then the trypsin was stopped with 10ml complete medium. This cell suspension was mixed thoroughly, and divided into four 3.8ml aliquots, which were then made up to 14.4ml with complete medium. The aliquots were again mixed thoroughly, and 1.2ml was added to each well of the 12-well tissue culture plates. The plates were then incubated for 24 hours at 37°C.

2.1.2 Preparing Reporter Gene Constructs

2.1.2.1 Amplifying DNA for Plasmid Construction

To investigate the effects of the *ACT* promoter T and G alleles in different cell lines, it was necessary to introduce reporter genes, coupled with the *ACT* promoter (T/G), into the cells. To achieve this, the two forms of the *ACT* promoter were cloned into the pGL3 enhancer vector. *ACT* promoter DNA from homozygous TT and GG samples was therefore amplified to provide enough material to clone into the vectors.

To prepare a 365bp fragment of sufficient quality, a template was amplifed from genomic DNA. This DNA template was a 646bp PCR product, that had previously been amplified with the primers F1, 5' TGT CTC TAC GAA AAA TAC AA 3' and R1, 5' AGG ATG TAA AAC ATC ATG G 3'. An aliquot of this product was taken and digested with the restriction enzyme *Van*91I, this digestion product was used to verify the promoter genotypes (see figure 2.1.2). GG and TT genotypes were used for generating the pGL3E 'G' and pGL3E 'T' constructs, respectively.

The PCR reactions (646bp product) were carried out in a final volume of 25µl containing 200ng genomic DNA; 0.5µM of each primer, 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1 unit *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC, 30 seconds at 57ºC, 60 seconds at 72ºC, followed by 10 minutes at 72ºC.

A 345bp fragment of the *ACT* 5' flanking region (see Appendix 1), containing known regulatory elements (Kordula et al., 1998) and each allele of the polymorphism was amplified, and cloned into the pCR®-2.1 TOPO vector (Invitrogen). This region was then sub-cloned into the pGL3E vector, so that two experimental constructs, pGL3E 'T' and pGL3E 'G', differing by a single base, were produced. These constructs were verified by sequencing.

The 345bp fragment of the *ACT* gene promoter region was amplified using primers F2, 5' GGA GGT ACC CAC ATG TTA GCT 3' and R2, 5' AAG CTT ATT TAT TCG TGA GA 3'. These primers were designed with engineered restriction sites for the enzymes *Kpn*I and *Hind*III , respectively.

The genotypes of the PCR products were verified by restriction digest. The PCR products (7.5µl) were incubated, with 5U of *Van*91I, at 37ºC for 2 hours, then resolved through a 2.5% agarose gel stained with ethidium bromide. The G allele lacks a site for the restriction enzyme while the T allele is cleaved into fragments of 511bp and 135bp.

To produce the 345bp fragment for insertion into the pCR®-2.1 TOPO vector 50ng of undigested PCR product (646bp) was amplified with 0.8µM each of F1 and R1, 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1 unit *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC, 30 seconds at 58ºC, 60 seconds at 72ºC, followed by 10 minutes at 72ºC.

2.1.2.2 Cloning PCR Products into pCR®-2.1 TOPO Vectors

Approximately 10ng of the 345bp PCR products were cloned into the pCR®2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) according to manufacturer's protocol in XL1-Blue *E. coli* (Stratagene). CG-agar plates, containing 50 µg / ml ampicillin, were prepared and pre-warmed to 37°C. To enable blue/white screening, 40µl of 40mg/ml X-gal was spread onto these plates. Approximately 10µl transformation mix, containing the pCR®2.1-TOPO vector was spread onto a plate and incubated overnight at 37°C. Positive clones (white colonies) were selected and cultured overnight in CG broth containing 50µg/ml ampicillin.

Plasmid DNA was extracted by the modified QIAfilter method (see section 2.1.2.3) and analysed by restriction digest with 5 units *Kpn*I and 5 units *Hind*III restriction enzymes in 1x One-Phor-All Buffer *PLUS* (Pharmacia; 10mM Tris-acetate, pH 7.5; 10mM magnesium acetate; 50mM potassium acetate) in a final volume of 10µl at 37°C for 2 hours.

The products of the restriction digest were run on a 2% agarose gel, and visualised by staining with ethidium bromide. Confirmation of successful cloning came by the appearance of a 330bp insert band.

2.1.2.3 Isolation of Plasmid by Modified QIAFilter (QIAgen) Technique

A 15ml overnight culture, containing XL-1 blue cells transformed with pCR®2.1-TOPO plasmid of each *ACT* promoter allele was centrifuged at 14,000g for 5 minutes at 4°C. The resulting supernatant was discarded, and the cell pellet was gently resuspended in 4ml resuspension buffer, P1. To this was added 4ml lysis buffer, P2, and the solution was mixed by gently inverting 6 times, before incubation at room temperature for 5 minutes. During this incubation, a QIAfilter cartridge was prepared, by closing one end with a removable cap. Next, 4ml of chilled neutralisation buffer, P3 was added, and the lysate was mixed by gentle inversion 6 times. The lysate was then added to the QIAfilter cartridge, and incubated at room temperature for 10 minutes. During this incubation the QIAgen-tip 100 was prepared by the addition of 4ml endotoxin-free buffer QBT. This buffer was allowed to pass through the tip by gravity flow. Once the lysate had been incubated, the plunger was added to the QIAfilter cartridge, and the lysate was gently filtered into the QIAgen-tip. The filtered lysate was allowed to pass through the QIAgen cartridge by gravity flow. Once the tip had cleared, it was washed twice with 10ml endotoxin-free buffer QC. After washing the DNA was eluted by the addition of 5ml endotoxin-free buffer QN. The DNA was precipitated by adding 3.5ml isopropanol and mixed. This solution was centrifuged at 4,600g for 90 minutes at 4°C, and the resulting supernatant was carefully decanted. The pellet that remained was washed with 2ml 70% endotoxin-free ethanol, and centrifuged again at 4,600g, for 30 minutes, at 4°C. The supernatant was carefully decanted

and the pellet was air-dried for 10-15 minutes. The DNA was re-dissolved in 100µl endotoxin-free 1x TE buffer (pH 8.0) for 10 minutes. The yield was determined by spectrophotometry.

2.1.2.4 Preparing *a_1-Antichymotrypsin* Insert for Cloning into pGL3 Enhancer Vector

The TA clone DNA prepared as mentioned in section 2.1.2.3 was digested in a final volume of 15µl containing 15 units *Kpn*I and 15 units *Hind*III in 1x One-Phor-All-Buffer *PLUS* at 37°C for approximately 5 hours. 3 digests of 15µl were prepared for both the G and T alleles of the TA clones. The fragments of DNA resulting from the restriction digest were electrophoresed on 2% agarose, stained with ethidium bromide, and the resulting 330bp insert bands were removed from the gel. Insert DNA was extracted from the gel slices using the QIAquick gel extraction kit (QIAgen), according to the manufacturer's protocol, pooling 3 gel slices per column. DNA was eluted from the column in 30µl Tris-HCl (pH 8.5). An estimation of the DNA concentration was made by electrophoresis of gel-purified insert DNA on 2% agarose stained with ethidium bromide.

2.1.2.5 Preparing pGL3 Enhancer Vector for Cloning

Approximately 2µg of pGL3E vector was digested with 5 units each of *Kpn*I and *Hind*III in 1x One-Phor-All-Buffer *PLUS* in a final volume of 10µl at 37°C for at least 2 hours. After this, the restriction enzymes were inactivated by heating them to 85°C for 15 minutes. The linearised vector was then precipitated in 2.5 volumes of 100% ethanol, and chilled to –20°C for 1 hour. The DNA was then pelleted by centrifugation at 10,000g for 10 minutes, followed by a wash with 70% ethanol, to remove the excess salt. The pellet was air-dried, before being resuspended in 17µl distilled H_2O.

There is a risk that the cut pGL3E vector will re-circularise, preventing ligation of cut vector fragments. To prevent this the vector was dephosphorylated after ethanol precipitation. This was achieved using shrimp alkaline phosphatase, SAP (USB), which removes the 5' phosphate groups from the DNA. The presence of 5' phosphate groups on the insert DNA allows for efficient ligation.

The dephosphorylation reactions were performed with 1U SAP enzyme made up to a final volume of 20µl with 1x SAP buffer (USB). The reaction was carried out by incubating at 37°C for 30 minutes, after which the enzyme was de-activated by heating to 85°C for 15 minutes. An estimation of the concentration of dephosphorylated linear vector was made by electrophoresis of a 3µl sample through a 2% agarose gel with ethidium bromide staining.

2.1.2.6 Ligations

The linear pGL3E vector and T/G inserts, prepared as described above were ligated in a final volume of 20µl. The solution contained 200ng pGL3E vector, 200ng insert and 5 units of T4 DNA ligase enzyme. Ligation reactions were set up to prepare vectors containing the T allele and the G allele, and a control reaction was performed with water replacing the inserts. This control reaction was to assess the level of background ligation, in the absence of insert. All ligation reactions were incubated at 22°C for 1 hour.

2.1.2.7 Transformation of XL-1 Blue Cells with pGL3E Vectors

The plasmids prepared by ligation were transformed into XL-1 Blue super competent *E. coli* cells (Stratagene). For each transformation 40µl of competent cells were required, and 1µl of ligation reaction was inoculated into the cells. The cells were chilled on ice for 30 minutes after inoculation, and were then heat shocked at 42°C for 45 seconds. After this, they were put back on ice for a further 2 minutes, and then 450µl SOC medium (20g Bacto-tryptone, 5g Bacto-yeast extract, 0.5g NaCl, 10ml 250mM KCl, 5ml 2M $MgCl_2$, 20ml 1M glucose per litre) was added. The transformation mix was incubated at 37°C for 1 hour, with 225 rpm shaking, before being spread onto a pre-warmed CG-agar plate containing 50µg / ml ampicillin. The plates were cultured at 37°C overnight. Two plates were cultured for each transformation, one plate was spread with 50µl undiluted transformation mix, the other with 50µl of a 1 in 10 dilution, with SOC medium being used to dilute the solution. After overnight incubation, single colonies were picked from plates inoculated with neat or diluted transformation mix, whichever was most convenient.

2.1.2.8 Analysis of Transformed XL-1 Blue Cells

After being picked, single colonies were inoculated into 5ml CG broth containing 250µg ampicillin, and incubated at 37°C overnight with 225rpm shaking. The plasmids were extracted using a modified version of the QIAfilter protocol described above. Presence of the insert was determined by restriction in a final volume of 10µl, containing 5 units each of *Kpn*I and *Hind*III, 1x One-Phor-All-Buffer *PLUS* at 37°C for 2 hours. After digestion, the DNA fragments were separated by electrophoresis through a 2% agarose gel stained with ethidium bromide. The presence of a 330bp insert band determined which were the positive clones (see figure 2.1.2). Positive clones were preserved as glycerol stocks by combining an equal volume of glycerol with overnight culture and thoroughly, but gently, resuspending.

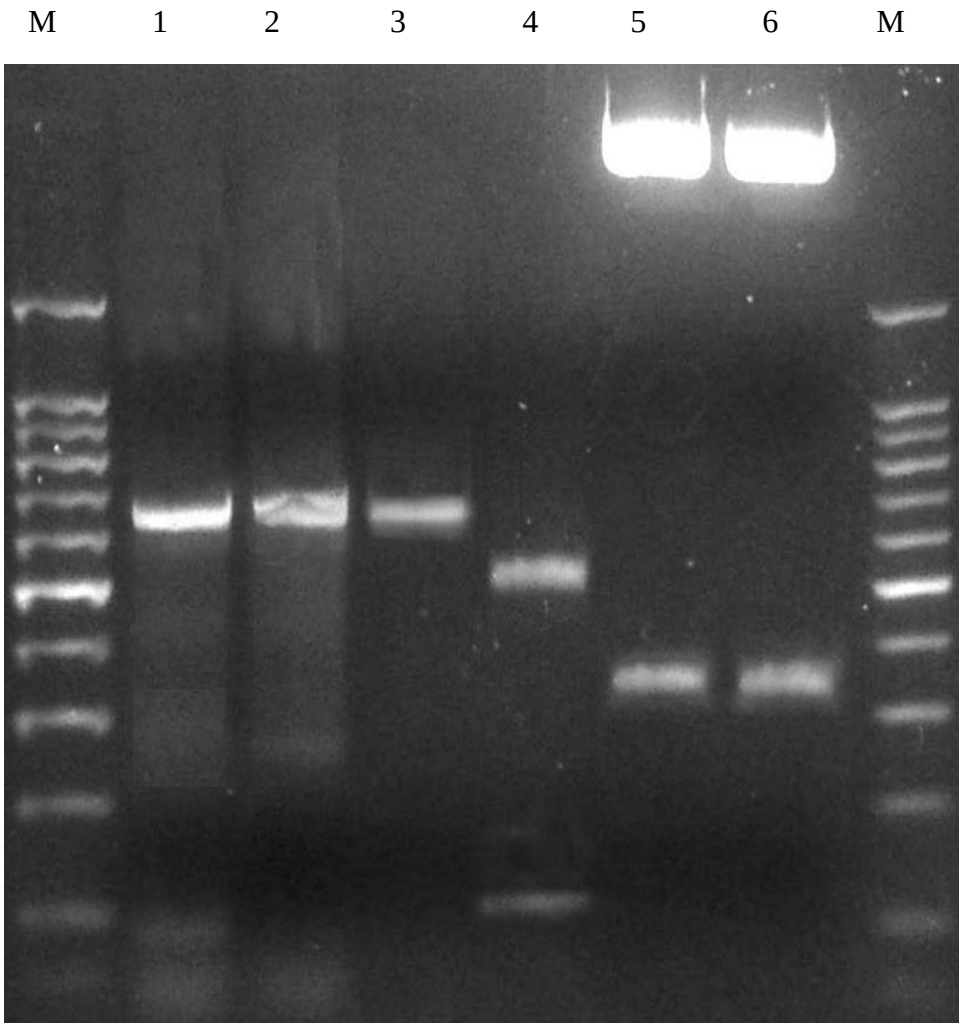

Figure 2.1.2: Verifying the insertion of the *ACT* promoter region into pGL3E vectors. M = 100 bp ladder, brightest band is 500bp. Lanes 1 and 2; 646bp PCR product of *ACT* promoter region PCR. Lanes 3 and 4; products from lanes 1 and 2 were digested with *Van*91I to genotype. Lane 3 shows no digestion, confirming GG genotype. Lane 4 has been cleaved to give fragments of 511bp and 135bp, confriming TT genotype. Lanes 5 and 6; after 330bp PCR product amplified from product shown in lanes 1 and 2 were inserted into pGL3E vectors the vector was digested with *Kpn*I and *Hind*III to show vector at the top of the lane and insert. To verify the allele inserted vectors were sequenced.

2.1.2.9 Determining pGL3E Clone Sequences

It was important to confirm that the clones held in glycerol contained the correct sequences, which differed by a single base (see figure 2.1.3 and figures 6.1.2 and 6.1.3, Appendix 1.). Plasmid DNA was sequenced by PCR using the RV3 primer (Promega) which anneals to the pGL3E vector. The reactions were performed in a final volume of 10µl, which contained 4µl Big Dye Ready Reaction mix (Applied Biosciences) approximately 500ng plasmid DNA and 1.5 pmol RV3. Reactions took place over 25 thermal cycles of 30 seconds at 96°C, 15 seconds at 50°C, 4 minutes at 60°C, followed by 1 minute at 28°C. As the sequencing dyes are photo-labile reactions were carried out in the dark.

After cycling the products were cleaned by adding 124µl of 25:5:1 ethanol : 3M sodium acetate : water solution. This was mixed, then incubated on ice for 10 minutes, the mixture was centrifuged at 13000g for 15 minutes, and the supernatant was discarded. The pellet was dried by heating to 94°C for a couple of minutes, then stored, in the dark, at -20°C before being automatically sequenced on an ABI sequencer. Once confirmed the reporter gene constructs could be stored at –20°C, or used immediately for Dual-Luciferase Reporter® assays (see section 2.1.3).

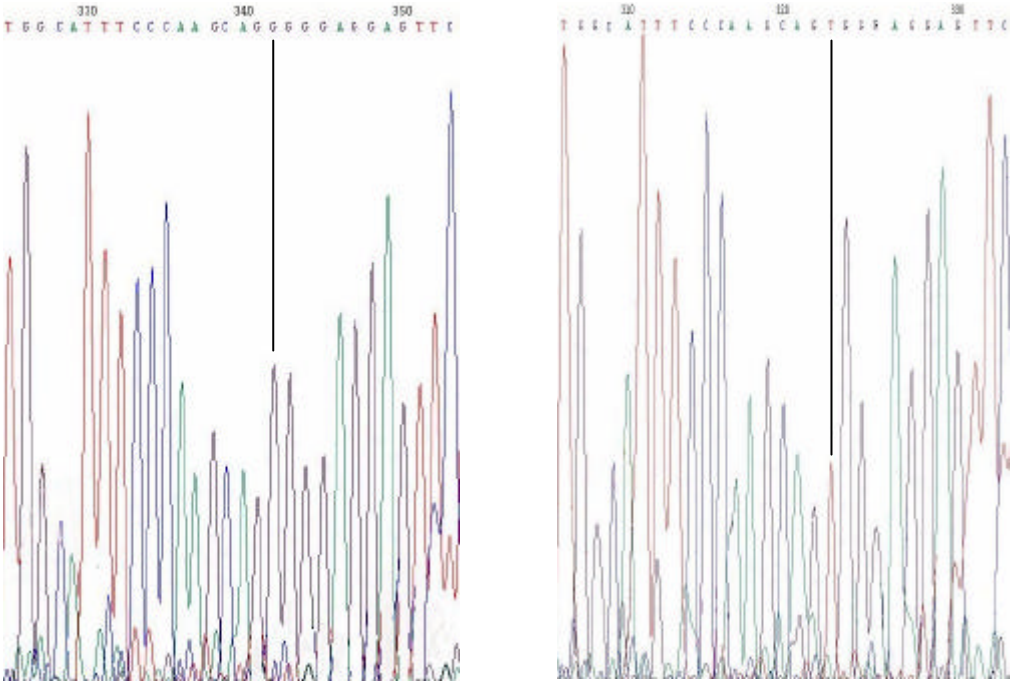

Figure 2.1.3: Confirmation of the correct allele in pGL3E constructs. Arrow shows position of polymorphism. For longer sequences, showing position of primer R2 site see Appendix 1.

2.1.3 Dual-Luciferase Reporter® Assays

2.1.3.1 Co-transfecting Cells with Reporter Gene Constructs

Plasmid DNA (pGL3 and pRL) was diluted in serum-free medium and mixed with a transfection reagent, either Tfx-20 or Tfx-50 (Promega). These are cationic lipid based reagents which allow DNA to associate more closely with the cell membrane as they form a lipid / nucleic acid complex with anionic DNA. The optimum Tfx reagent and charge ratio was determined for each cell line prior to use. For T98G, U373 MG and SH-SY5Y cells, Tfx-20 was the appropriate choice, while Tfx-50 allowed efficient transfections for Hep G2 cells. For T98G cells a 4:1 charge ratio was used, for the U373 MG cells and SH-SY5Y cells and Hep G2 cells a 2:1 ratio was used.

For each experiment, two 12-well plates of cells at 60-70% confluence were used. A master mix was made up containing 400µl serum-free medium and 20ng pRL for each well. This was mixed well and divided into four aliquots, to which were added 200ng per transfection of pGL3 enhancer, pGL3 control, pGL3E 'G' and pGL3E 'T'. The appropriate amount of transfection reagent was added to these transfection mixes, which were thoroughly mixed and incubated at room temperature for 15 minutes. While the transfection mixes were incubating, the cells were prepared. The medium was removed and the cells were washed twice with PBS. To each well in the plates, 400µl of transfection mix were added. The cells were incubated at 37ºC for one hour and 800µl complete medium was added. The cells were then incubated at 37ºC for 24 hours.

2.1.3.2 Cytokine Stimulation

24 hours after transfection the cells were stimulated with 50ng/ml OSM. In different experiments, cells were exposed to OSM for different periods of time. Unstimulated cells, those that were not exposed to OSM, were used to represent the basal level of luciferase production, and therefore *ACT* promoter activity.

The transfection mixes were removed from the cells, which were then washed twice with PBS, to remove all traces of growth medium, taking care to remove

medium from the walls of the wells. Once all traces of PBS had been removed from the wells, 1ml of serum-free medium containing 2.3pmoles OSM for the cells to be stimulated, or 1ml serum-free medium for cells used to measure basal activity was added. The cells were then allowed to grow at 37ºC for the required time (3 – 48 hours) prior to harvesting.

2.1.3.3 Harvesting Cells for Dual-Luciferase Reporter® Assay

After the cells had been stimulated with cytokine, they were harvested. Growth medium was removed and the cells were washed twice with PBS. Again, it was important to remove all traces of medium and PBS from the wells. Once this had been done 200µl 1x Passive Lysis Buffer, PLB (Promega) was added to each well. The cells were incubated at room temperature for 15 minutes, and the resulting lysates were transferred to 1.5ml eppendorf tubes. Whilst it is possible to store this lysate at -20ºC at this point, the DLR assays were performed immediately.

2.1.3.4 Dual Luciferase® Reporter Assays

Luciferase Assay Reagent II, LARII, and 1x Stop & Glo® reagent (Promega) were prepared according to the manufacturer's instructions. DLR assays were performed on a Turner Designs Model TD-20/20 Luminometer, as recommended by Promega, allowing sequential readings of firefly and *Renilla* luciferases. The luminometer was programmed to allow a 2 second delay period prior to quantification of light output over 10 seconds for both firefly and *Renilla* luciferases.

One tube per reaction was prepared containing 50µl LARII and 20µl cell lysate was added. The firefly luciferase activity was quantified and then quenched by the addition of 50µl 1x Stop & Glo®, allowing the *Renilla* luciferase activity to be determined.

The luminometer output reading is a measurement of light emitted, expressed as relative luminescence units (RLU), and is a measurement of firefly and *Renilla* luciferase activities. The ratio of firefly to *Renilla* luciferase activity represents firefly activity normalised for transfection efficiency.

2.1.4 Statistical Analysis

The values used to compare functional effects of promoter polymorphisms are the fold increase over the pGL3 enhancer alone, and have no units. Log_{10} of the fold increases were used to compare reporter gene activity between different transfected vectors in order to obtain normally distributed values. The comparison between alleles was made using the 2-tailed paired Student's T-test, with statistical significance being defined as $p<0.05$. The comparison of activity after different periods of stimulation with OSM were made by univariate analysis of variance, using SPSS v11.0 to provide and overall measure of statistical significance, followed by post hoc 2-tailed paired Student's T-test. For ANOVA and post hoc T-testing statistical significance was defined as $p<0.05$.

2.2 Reverse-Transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) is a technique that demonstrates the presence of specific mRNA sequences in a sample. The technique involves creating DNA from mRNA, which can then be amplified by PCR. The production of this complementary DNA, is a reversal of the normal eukaryotic transcription process, and relies on reverse transcriptase, an enzyme produced by retro-viruses to insert the viral RNA-based genome into the host's DNA based genome.

The technique can be quantitative, to give a measure of how much mRNA is present, semi-quantitative, giving a comparison of mRNA levels between samples, or qualitative. In these experiments, qualitative RT-PCR was used, to demonstrate that *in vivo* the cells used for the DLR assays and in the EMSA and supershift assay do actively synthesise ACT mRNA.

2.2.1 mRNA Isolation

Cultured cells were grown to 90% confluence, as described above. The cells used were Hep G2, U373 MG, SH-SY5Y and T98 G cells. The growth media

was removed, and the cells were washed twice with PBS. 10ml of serum-free growth medium, with or without 2.3 pmoles OSM as appropriate, was added and the cells were incubated at 37°C for the appropriate length of time. After this, the medium was removed and the cells were again washed twice with PBS. The cells were then removed from the flasks by adding 10ml PBS, containing 1.5ml 1xtrypsin-EDTA, and incubating at 37°C for 5 minutes. The cell solution was then transferred to a 15ml tube and centrifuged at 1000g for 5 minutes at 4°C. The supernatant was removed, and the total mRNA was isolated using the SV Total mRNA Isolation kit (Promega) according to the manufacturer's instructions. Some care was taken to ensure that the mRNA, which degenerates rapidly, was used immediately, but where this was not possible it was stored at –80°C before being used. Prior to use as the template for cDNA synthesis, the concentration of mRNA was measured with a spectrophotometer.

2.2.2 cDNA Synthesis

In order to amplify the *ACT* region, the mRNA needs to be converted to cDNA. The method used produced cDNA counterparts to all of the mRNA isolated.

The cDNA synthesis was a two-stage reaction, performed in a reaction mix containing 200ng random hexamers (Promega), 1µg mRNA and water to a final volume of 26µl. This reaction mix was incubated at 70ºC for 10 minutes then cooled on ice. The volume was increased to 40µl by the addition of the following: 50mM Tris-HCl, pH 8.0; 50mM KCL; 4mM $MgCl_2$; 10mM DTT; 1mM dNTPs and 20U ribonuclease inhibitor (MBI Fermentas). This was incubated at 25°C for 5 minutes before the addition of 200U RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas). The reaction was incubated at 25ºC for 10 minutes, 42ºC for 1 hour and 70ºC for 10 minutes, and finally chilled on ice. As cDNA is more stable than mRNA the cDNA could be stored at -20°C, if not used immediately.

2.2.3 RT-PCR

Two PCR reactions were set up for each cDNA sample. One reaction amplified the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT*), which is constitutively active in all cell lines. This is used as an internal control, and also serves to demonstrate that cDNA has been synthesised, which in turn proves that mRNA was successfully extracted. The second PCR amplifies the *ACT* gene. The primers must be different to those used to amplify the promoter polymorphism region, as this region is not transcribed into mRNA.

The *HPRT* gene was amplified with primers F3; 5' GAC CAG TCA ACA GGG GAC AT 3' and R3; 5' CGA CCT TGA CCA TCT TTG GA 3'. PCR reactions were carried out in a final volume of 25µl containing cDNA; 0.5µM of each primer; 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1U *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC, 60 seconds at 60ºC and 60 seconds at 72ºC followed by 10 minutes at 72ºC.

The *ACT* gene was amplified with primers F4; 5' CAG AGA TTC TCA AGG CCT CGA GTT 3' and R4; 5' GAC CCC CAA GAT ACT CAT CAG T 3'. PCR reactions were carried out in a final volume of 25µl containing cDNA; 0.5µM of each primer; 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1U *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds 94ºC, 60 seconds at 61ºC and 60 seconds at 72ºC followed by 10 minutes at 72ºC.

Following amplification, PCR products were run through a 2% agarose gel, and stained with ethidium bromide.

2.3 Electrophoretic Mobility Shift Assays (EMSA)

The electrophoretic mobility shift assay is designed to demonstrate the ability of specific DNA sequences to form complexes with nuclear proteins. Having previously demonstrated the functional effect of the alleles with the DLR assays, the EMSA was used to provide an insight into the mechanisms involved in generating the functional effect. Short DNA probes of each *ACT* promoter allele were radiolabeled, in order to permit visualisation. They were then allowed to interact with nuclear proteins extracted from cultured cells. If the DNA sequence is recognised by DNA-binding proteins, a complex will form. The proteins were then separated by electrophoresis, and the gel exposed to an X-ray film. This film was then developed, and the positions of the free DNA probes and DNA-protein complexes were revealed. Large molecules (DNA / protein) are retarded by the molecular structure of the gel to a greater extent than the smaller molecules (probes alone). EMSA autoradiographs can be compared to see if the same complexes occur when the nuclear proteins are isolated from stimulated or unstimulated cells, or between different cell lines. It is also possible to see if certain proteins bind more strongly to one DNA sequence than another.

The supershift assays are a variation on this technique. An antibody that is expected to bind to the protein likely to bind DNA is introduced to the nuclear extract. The labelled probe is then added to the nuclear extract, where it binds with the antibody-protein complex. When these complexes are run through a gel the antibody-protein-DNA complex is much larger than the protein-DNA complexes, and so does not move as rapidly through the gel. On the autoradiograph, the protein recognised by the antibody appears “shifted”; that is, its position has changed relative to the complex not involving antibody. In this way it is possible to identify the DNA-binding proteins associated with the DNA sequence under investigation.

2.3.1 Preparing Probes for EMSA

2.3.1.1 Amplifying the *ACT* Promoter Region

Two samples, homozygotes for the *ACT* promoter polymorphism, representing each allele, were amplified by PCR over the promoter region. The PCR product was 80bp long, with the polymorphic site 29bp downstream of the start of the amplicon (see Appendix 1).

The 80bp fragment of DNA from the 5' flanking region of the *ACT* gene was amplified, using a forward primer, F5: 5' CTT GGT TGT CCT GGC ATT TC 3' and reverse primer, R5: 5' TGG ATT TTC ATG AAT GCT GA 3'. PCR reactions were carried out in a final volume of 25µl containing 200ng genomic DNA; 0.5µM of each primer; 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1U *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC, 60 seconds at 55ºC and 60 seconds at 72ºC followed by 10 minutes at 72ºC. After amplification, the PCR product was cleaned by gel extraction, using the QIAquick Gel Extraction kit (QIAgen) following the manufacturer's instructions. A small amount of the resulting product (5µl) was mixed with the same amount of 15% Ficoll and run on a 2% agarose gel.

2.3.1.2 Radiolabeling DNA Probes for EMSA

The probes to bind nuclear protein were end-labeled with ?^{32}P-ATP. The following solution was prepared; ~1.8pmol allele-specific probe, 1µl of 10x T4 polynucleotide kinase reaction buffer (Forward reaction) (45.5mM Tris-HCl (pH7.6 at 25°C), 9mM $MgCl_2$, 4.5mM DTT, 0.1mM spermidine, 0.1mM EDTA, MBI Fermentas), 3.6pmol ?^{32}P-ATP (20µCi), 5 units polynuclear kinase (MBI Fermentas), made up to 10µl with de-ionised water. This was incubated at 37°C for 30 minutes, before 40µl water and 50µl phenol:chloroform was added. This solution was centrifuged briefly and the aqueous phase was run through a Sephadex G50 spin column. The eluant was retained and if it not used immediately, the labelled probe was stored at –20°C, for no more than one week.

2.3.1.3 Isolation of Cell Nuclear Proteins

Cells were grown in the appropriate medium, as detailed above (section 2.1.1.3). Once the cells were 70 – 80% confluent they were washed twice with PBS, then covered with 10ml serum-free medium, with or without 2.3pmoles OSM as appropriate. The cells were incubated with OSM for a length of time determined by the maximum response seen in the DLR assays. Nuclear proteins were extracted using the NucBuster Nuclear Extraction kit (Novagen). The protocol was modified as described below, and the cells, reagents and nuclear extracts were kept on ice throughout the procedure.

After cytokine stimulation, the cells were harvested. The medium was removed, and the cells were washed twice with PBS. The cells were then trypsinised by the addition of 10ml PBS containing 1.5ml 1x trypsin-EDTA followed by incubation at 37°C for 5 minutes, or until the cells had detached from the flasks. Once the cells detached the solution of PBS and trypsin, containing the cells was transferred to a 15ml tube. This was centrifuged at 4°C, for 5 minutes at 500g. The supernatant was removed, and the pellet was resuspended in 150µl NucBuster Reagent A per 50µl of cell pellet. After gentle resuspension, the solution was mixed by vortex for 15 seconds, then incubated on ice for 5 minutes. After another 15 seconds vortexing, the solution was centrifuged, at 4°C, for 5 minutes at 14,000rpm. The supernatant was removed, and for each 50µl of the original cell pellet 75µl NucBuster Reagent B was added, and the cell pellet was gently resuspended, then vortexed for 15 seconds. Once the cell solution was homogeneous, it was incubated on ice for 5 minutes, vortexed for 15 seconds, and then centrifuged at 4°C, for 5 minutes at 14,000rpm. The supernatant, containing nuclear proteins, was placed in labelled tubes, previously cooled on ice, in 8µl aliquots. The aliquots were snap frozen on dry ice, and stored at –80°C. The nuclear proteins degrade fairly rapidly, even at -80°C, so the nuclear extracts were used within 4 weeks of preparation, at the latest, but where possible, they were used in gel-shift assays as soon as they were extracted.

2.3.1.4 EMSA

The radiolabeled probes were allowed to interact with proteins from nuclear extracts. The following solutions were prepared; 0.03pmol end-labeled probe, 8.5µl low salt binding buffer, LSB (100mM NaCl, 30mM $MgCl_2$ and 50mM Tris base), 1µg poly(dI):poly(dC) (Sigma), and 0.25 - 3µl of nuclear extract was titrated. The solution was mixed by gentle pipetting and incubated on ice for 30 minutes then the reaction was stopped by adding 2µl 6x loading dye (MBI). DNA-protein complexes were separated by running through a 4% polyacrylamide gel at 20mA for 17 – 20 minutes in 0.25x TAE buffer (10mM Tris-HCL (pH8.0), 10mM acetate, 0.25mM EDTA). The gel was then dried at 80°C and exposed to an X-ray film.

2.3.1.5 Supershift EMSA

Since the ACT promoter region under investigation contained a putative binding site for transcription factor II B (TFIIB), an antibody against this protein was used.

DNA probes and nuclear proteins were prepared as above. The following solution was made up; 8.5µl LSB, 1µg poly(dI):poly(dC), and a titration of 0.25 – 3µl nuclear extract, 2µg anti-TFIIB (Autogen Bioclear). This was incubated on ice for 30 minutes, then 0.03pmol ^{32}P end-labeled probe was added, and the solution was incubated on ice for a further 30 minutes. The reaction was stopped by adding 2µl 6x loading dye (MBI). The sample was electrophoresed through a 4% polyacrylamide gel at 20mA for 17 – 20 minutes in 0.25x TAE. The gel was then dried at 80°C and exposed to an X-ray film.

2.4 Genotyping the a_1-Antichymotrypsin Gene

The genotyping was performed at the MRC's Geneservice facility in Cambridge, and the data was confirmed by restriction enzyme digestion or sequencing of a selected number of samples. The samples used were from two centres, the University of Nottingham and the University of Oxford. The assistance of Professor James Lowe at the University of Nottingham's Histopathology Department and Dr. Donald Lehmann of the OPTIMA study (Oxford) was invaluable in recruiting patients, making clinical and pathological observations and supplying material. Dr. Louise Tilley extracted DNA from the Nottingham samples for an earlier study.

Genotyping polymorphisms by restriction enzyme digestion is a relatively quick and cheap two-step process. It relies on the polymorphism under investigation altering the digestion site such that each allele produces a different pattern of DNA bands when run on an agarose gel. For the *ACT* promoter polymorphism, the G allele has no restriction site that can be recognised by the enzyme *Van*91I, while the T allele can be cleaved.

The first step in this process is to amplify the region of DNA incorporating the polymorphism. The PCR product is then digested with the restriction enzyme, so that the DNA can be cut completely at the correct site. The PCR is designed in such a way that a clear distinction can be made between digested and undigested DNA. If a sample is homozygous for the allele that retains the restriction site, one larger band will appear when run through an agarose gel. Heterozygotes will appear as two bands, and homozygotes for the other allele appear as one smaller band.

Aside from producing data about the frequency of different alleles, genotyping was used as a way of selecting DNA to make constructs for the functional assays, and probes for the EMSA and supershift assays.

2.4.1 Alpha-1-Antichymotrypsin Promoter Polymorphism

One third of the samples were randomly selected for repeat genotyping to ensure accuracy of the MRC data. The DNA used came from 729 subjects, divided into three groups, control (n=335, mean age 75.00±9.39 years), probable AD (n=86, mean age 69.50±9.17 years) and confirmed AD (n=308, mean age 77.31±10.09 years). Of the 729 samples, 240 (32.9%) were genotyped by RFLP, prior to receiving data from MRC Geneservice. Discrepencies in genotypes obtained using the two methods was found in 11 (4.6%) cases. On sequencing, genotypes from all 11 cases agreed with data provided by the MRC. Confirmed AD status was determined following the CERAD criteria (Mirra et al., 1993), probable AD status was determined by the Mini Mental State Examination.

A 646bp region of the 5' flanking sequence of the *ACT* gene was amplified with primers F1; 5' TGT CTC TAC GAA AAA TAC AA 3' and R1; 5' AGG ATG TAA AAC ATC ATG G 3' (see Appendix 1). The template was genomic DNA and PCR reactions were carried out in a final volume of 25µl containing 200ng DNA; 0.5µM of each primer; 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1U *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC,

30 seconds at 57ºC and 60 seconds at 72ºC followed by 10 minutes at 72ºC. To determine that the amplification was satisfactory, 5µl of PCR product was run on a 2% agarose gel and visualised with ethidium bromide.

To genotype the PCR products 10µl was digested with 5U *Van*91I at 37°C overnight. The digestion product was run through a 2.5% agarose gel and stained with ethidium bromide. Genotypes were verified by two observers. The undigested DNA was 646bp long and could be distinguished from the cut DNA that had fragments of 511bp and 135bp (see figure 2.4.1).

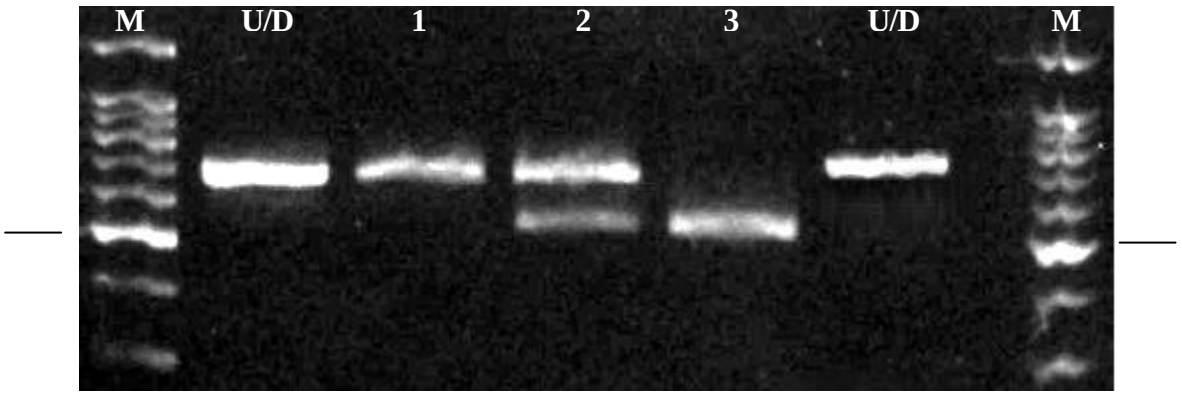

Figure 2.4.1 Genotyping *ACT* -51 Promoter Polymorphism by RFLP. M shows 100bp ladder, U/D is undigested PCR product 646bp in length, lane 1 contains a known GG genotype, lane 2 contains a known GT genotype and lane 3 contains a known TT genotype. Arrow shows position of 500bp.

2.4.2 Apolipoprotein E Genotyping

A 172 bp region of the *APOE* gene was amplified with primers F6; 5' TCC AAG GAG CTG CAG GCG GCG CA 3' and R6; 5' ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A 3'. PCR reactions were carried out in a final volume of 25µl containing 200ng DNA; 0.5µM of each primer; 200µM dNTPs; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM $MgCl_2$: 0.08% Nonidet P40 and 1U *Taq* polymerase. Reactions were overlaid with mineral oil and subjected to 35 cycles of 30 seconds at 94ºC, 30 seconds at 65ºC and 60 seconds at 72ºC followed by 10 minutes at 72ºC. To determine that the amplification was satisfactory, 5µl of PCR product was run on a 2% agarose gel and visualised with ethidium bromide.

To genotype the PCR products 10µl was digested with 5U *Hin*61 at 37°C overnight. The digestion product was run through a 10% polyacrylamide gel and stained with ethidium bromide and two observers verified genotypes. The e2 allele generated bands of 91bp and 81bp; the e3 allele, bands of 91bp, 48bp and 33bp; the e4 allele, bands of 72bp, 48bp and 33bp. The 33bp bands were not visible on the 10% polyacrylamide gels.

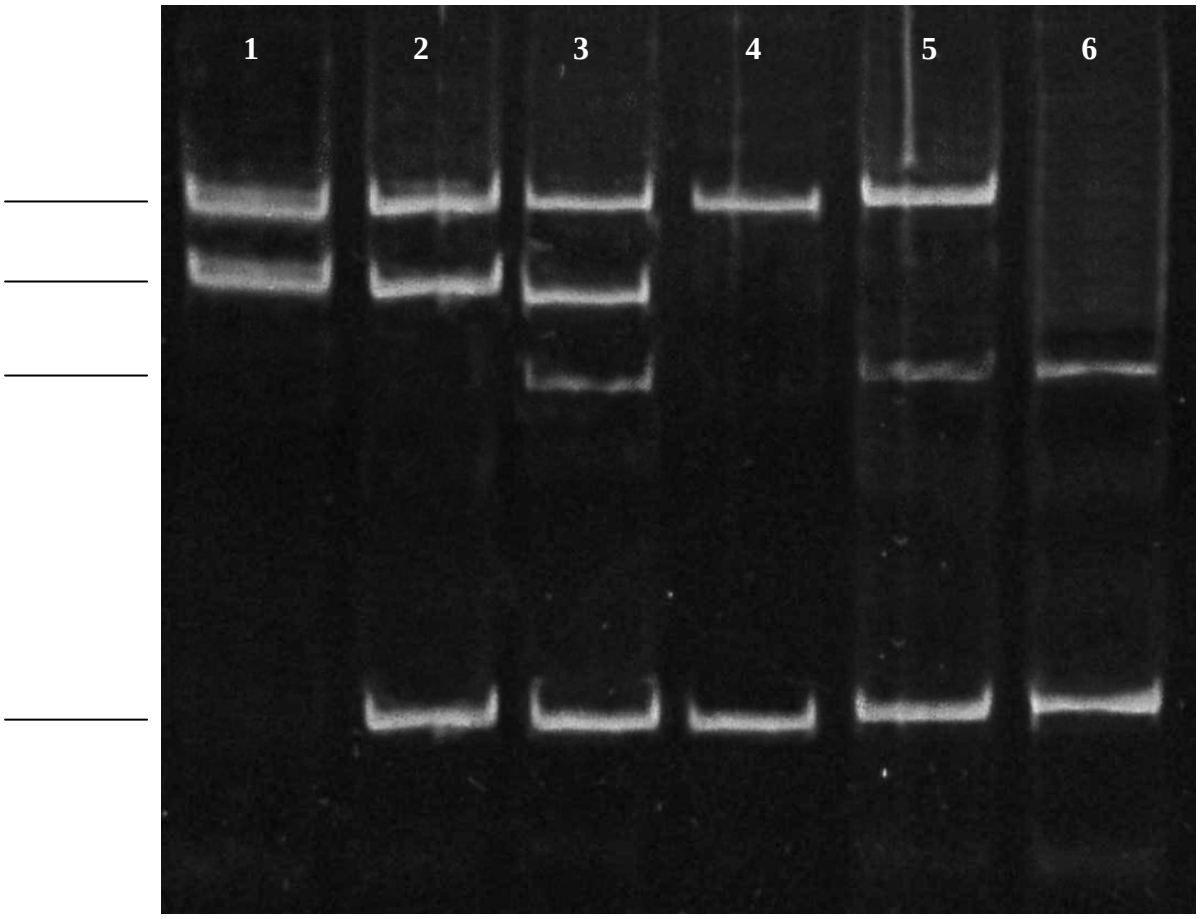

Figure 2.4.2: ApoE genotyping. Lane 1 contains a known e2/e2 genotype, lane 2 contains a known e2/e3 genotype, lane 3 contains a known e2/e4 genotype, lane 4 contains a known e3/e3 genotype, lane 5 contains a known e3/e4 and lane 6 contains a known e4/e4. Arrows show, from top, 91bp, 81bp, 72bp, 48bp. Known e2/e4 was used as a marker when scoring unknown genotypes.

2.4.3 Automated Sequencing of the a_1-Antichymotrypsin Gene Promoter Polymorphism

In addition to confirming the *ACT* genotyping results by "in house" restriction analysis, two samples of each genotype were sequenced across the *ACT* promoter polymorphism site. One reason for doing this was to confirm that the results obtained by restriction enzyme genotyping were correct, but more importantly, to verify that the sequence was as expected. These samples were used later, to produce constructs for the functional assays, and probes for the EMSA. It was important that the samples chosen did not contain unreported polymorphisms that might interfere with these experiments, as well as being absolutely sure that the correct genotypes were used.

2.4.3.1 Cleaning PCR Products for Sequencing

PCR products for the 646bp 5' flanking sequence of the *ACT* gene were obtained, as described above. The PCR products (0.2µg) were then cleaned with 5U exonuclease I, exoI (USB), 1U shrimp alkaline phosphatase, SAP (USB), in a final volume of 6µl. This was incubated at 37°C for 15 minutes, and then the enzymes were inactivated by incubating at 80°C for 15 minutes. Following this, the products were chilled on ice and centrifuged briefly at 15,000rpm.

2.4.3.2 Sequencing Reaction

The reverse primer, R1 (see section 2.4.1, or Appendix 1) was used to sequence the *ACT* flanking region using the Big Dye protocol (ABI).

Sequencing reactions took place in a final volume of 10µl which contained 4µl Big Dye Ready Reaction mix version 3.1 (Applied Biosciences), 1.5pmol primer, and approximately 50ng enzyme treated PCR product. This was overlaid with mineral oil and centrifuged, then subjected to 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C, 240 seconds at 60°C, after which there was 60 seconds of incubation at 28°C.

The sequencing reaction was purified by filtration through AGTC Spin Columns (VH Bio) following the manufacturer's instructions. The cleaned

product was heated to 90°C for approximately 5 minutes, or until the liquid had evaporated, leaving dry DNA at the bottom of the tube. The tubes were then delivered to a sequencing laboratory.

2.5 Statistical Analysis

The alleles were counted to provide an estimation of allele frequencies. The frequencies could then be compared between AD and control groups by χ^2 testing. Due to the small sample sizes, Yates continuity correction was used. To determine if genotypes were in Hardy-Weinberg equilibrium, the following equation was used:

$$1 = p^2 + 2pq + q^2$$

where p and q are the observed frequencies of the two alleles, determined by counting. This equation gave the expected genotype frequencies, which could be compared against the observed frequencies by ?^{2} testing. In both cases, the level of statistical significance was defined as $p<0.05$.

Comparisons between discrete variables, such as allele frequencies and AD status were made using the ?^{2} test, and comparisons between ranges of values, such as mean age, were made using ANOVA. These statistical analyses were performed using the statistical software package SPSS for Windows v11.01.

Power calculations were performed using Quanta 0.5 software, assuming a recessive inheritance of the allele under investigation, with a 5% error rate.

Odds ratios were calculated using the equation:

$$\frac{a \times d}{b \times c}$$

where a is the number of cases possessing the allele investigated, b is the number of controls possessing the same allele. c is the number of cases not possessing the allele under investigation, and d is the number of controls not possessing this allele. 95% CI was derived using the equation:

$$95\%\ CI = LnOR \pm 1.96\ SE(LnOR)$$

where $SE(LnOR) = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}$

3 RESULTS

3.1 Functional Assays

The functional assays were performed using four different cell lines, Hep G2 (hepatocyte), T98G (mixed population), U373 MG (astrocytes), and SH-SY5Y (neuronal cells). The experiments were performed over a defined time course, to demonstrate each allele's response both with and without exposure to oncostatin M (OSM). Each experiment was performed at least three times, in triplicate.

3.1.1 Effect of the ACT *Promoter Polymorphism on Reporter Gene Activity in Hep G2 Cells*

Reporter gene activity was higher in Hep G2 cells transfected with the T allele by an average of 187% compared with the G allele when data from stimulated and unstimulated cells were combined; this was to be expected from previous observations. The greatest difference in reporter gene activity between the two promoters occurred after twenty-four hours, when the difference in reporter gene response was 427% (see figure 3.1.1 and table 3.1.1). Under basal conditions there was a significant difference in activity between alleles. The T allele showed a 90% higher response over the G allele. After three hours of OSM stimulation, the T allele response was only 36% greater than the G allele, and this difference was not significant. When the cells had been stimulated with OSM for six hours, the difference in reporter gene activity was significant. The difference in reporter gene activity between the two promoters at this time was 289%. After twelve hours of OSM stimulation, the difference in reporter gene activity between the two promoters was 92% although the difference was not significant.

The liver is the main site of ACT synthesis (Kalsheker, 1996), and functional assays were carried out in hepatocytes, to gain an insight into how circulating ACT levels might vary with OSM stimulation. It can be seen that the functional response to 50ng/ml OSM is large and rapid (see table 3.1.2.). In Hep G2 cells, when the 'T' allele was present, there was an immediate response to OSM. The reporter gene response increases 10 fold in three hours. After six hours of OSM stimulation, this response had risen by more than 20 fold, before reaching a peak of activity, more than 40 times the basal activity, after twelve hours had elapsed. When the G allele was present, the response was slightly different. There was an initial rise in activity, with reporter gene activity 14 fold greater after three hours of OSM stimulation compared with the basal level. After six hours the response had fallen, but was still over 10 fold higher than basal. As with the T allele response, the G allele saw reporter gene activity peak after twelve hours, with a response 40 times higher than basal. The response dropped to basal levels after 24 hours.

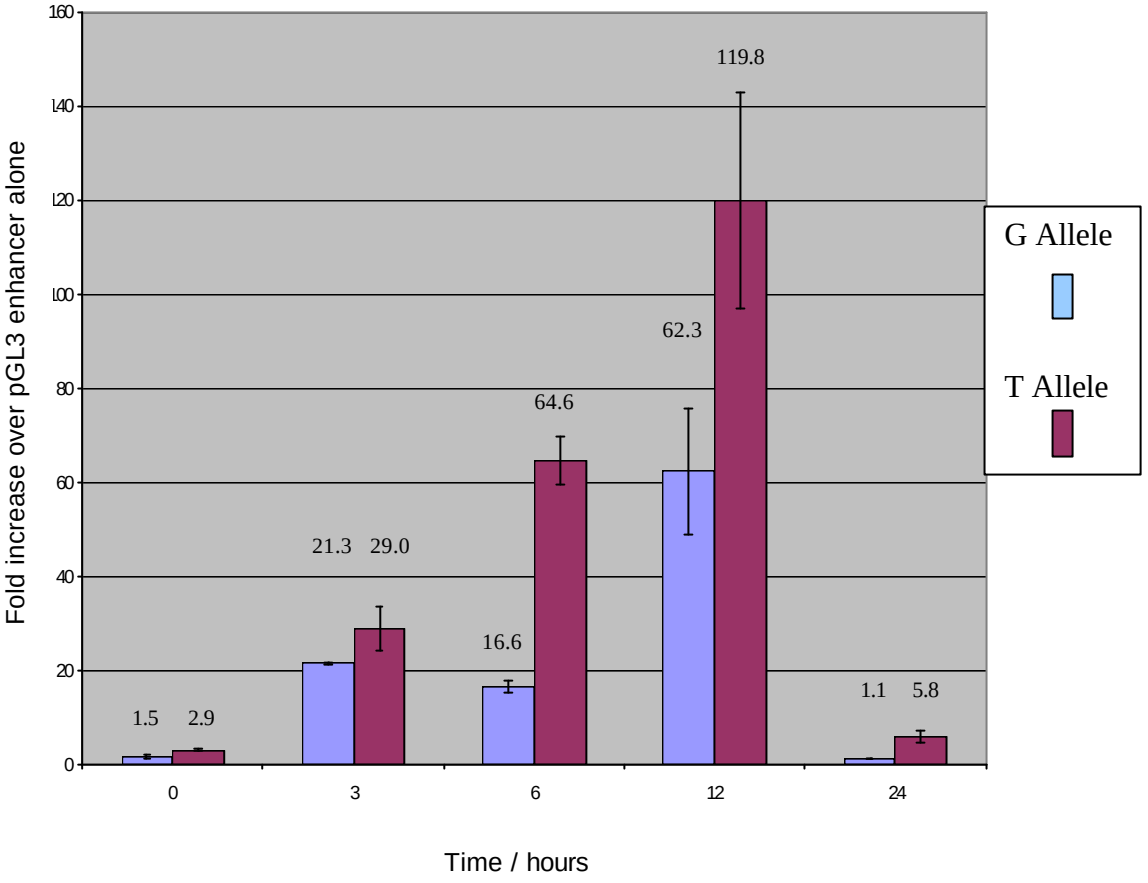

Figure 3.1.1: Functional response of the *ACT* promoter polymorphism to OSM stimulation in Hep G2 (hepatocyte) cells. Results of three experiments are shown as mean ± standard error of the mean (error bars).

	Exp1	Exp2	Exp3	Mean	Std. Deviation	% Change	p value
T Basal	3.2	2.7	2.9	2.9	0.3	90.3	0.042
G Basal	1.6	1.9	1.1	1.5	0.4		
T 3 hrs	35.2	31.9	19.8	29.0	8.1	36.2	0.250
G 3 hrs	17.8	22.5	23.7	21.3	0.3		
T 6hrs	72.8	65.6	55.3	64.6	8.8	289.2	<0.001
G 6 hrs	14.1	18.2	17.5	16.6	2.2		
T 12 hrs	103.3	165.3	90.9	119.8	39.9	92.3	0.103
G 12 hrs	75.2	35.3	76.3	62.3	23.3		
T 24 hrs	4.9	4.2	8.2	5.8	2.1	427.3	0.011
G 24 hrs	1.2	1.0	1.1	1.1	0.1		

Table 3.1.1: Difference between activity of the pGL3E 'G' and 'T' constructs, shown as % change in Hep G2 cells basally and following OSM treatment. The fold increases of luciferase activity, over the pGL3 enhancer vector alone are given as mean ± standard deviation. p values are derived using the Student's t-test on the log of the fold increases.

	T Allele			G Allele		
Time	Mean increase over pGL3 enhancer alone	Fold increase over basal	p value	Mean increase over pGL3 enhancer alone	Fold increase over basal	p value
Basal	2.9±0.3	-	-	1.5±0.4	-	-
3 hrs	29.0±8.1	10.0x	0.006	21.3±0.3	13.9x	0.006
6 hrs	64.6±8.8	22.3x	0.001	16.6±2.2	10.8x	0.006
12 hrs	119.8±39.9	41.3x	0.004	62.3±23.3	40.6x	0.011
24hrs	5.8±2.1	2.0x	0.085	1.1±0.1	0.7x	0.242

Table 3.1.2: OSM Response of *ACT* promoter alleles. Response of the pGL3E 'T' and 'G' constructs in Hep G2 cells, comparing basal response to different OSM exposure times. Results (mean ± SD) are the mean of three experiments; % change is the percentage difference between activity of the pGL3E 'G' and 'T' constructs. Univariate analysis of variation of both variation over each time point provided a p value of <0.001. p values shown in table are calculated by performing post hoc Student's t-test on the log of the fold increase over the pGL3 enhancer alone.

3.1.2 *Effect of the* ACT *Promoter Polymorphism on Reporter Gene Activity in T98G Cells*

The T98G cell line is a mixed population of astrocytes and neuronal cells. Once again, the T allele always provoked a greater functional activity in comparison to the G allele (see figure 3.1.2 and table 3.1.3). The difference was greatest after twenty-four hours stimulation with OSM at 115%, and the smallest difference, 19% was seen after three hours of stimulation. The mean difference in functional activity between the two promoters when transfected into T98G cells was 49%.

The comparison of functional activity of cells stimulated and unstimulated with OSM revealed a trend to increasing activation with longer exposure to OSM (see table 3.1.4). When the G allele was present the mean rise in activity was 3 fold, a rise that began at three hours, increased after six hours, and declined, but not to the basal level, after twenty-four hours. Of these changes, only that observed after six hours was statistically significant, according to the t-test. When the T allele was present, the mean rise in activity was 4 fold, and once more there was a 3-fold rise at three hours. The peak OSM response of 4-fold occurred at six hours. At twenty-four hours the increase in functional activity was 3-fold compared to the basal level. In each case, the difference was statistically significant.

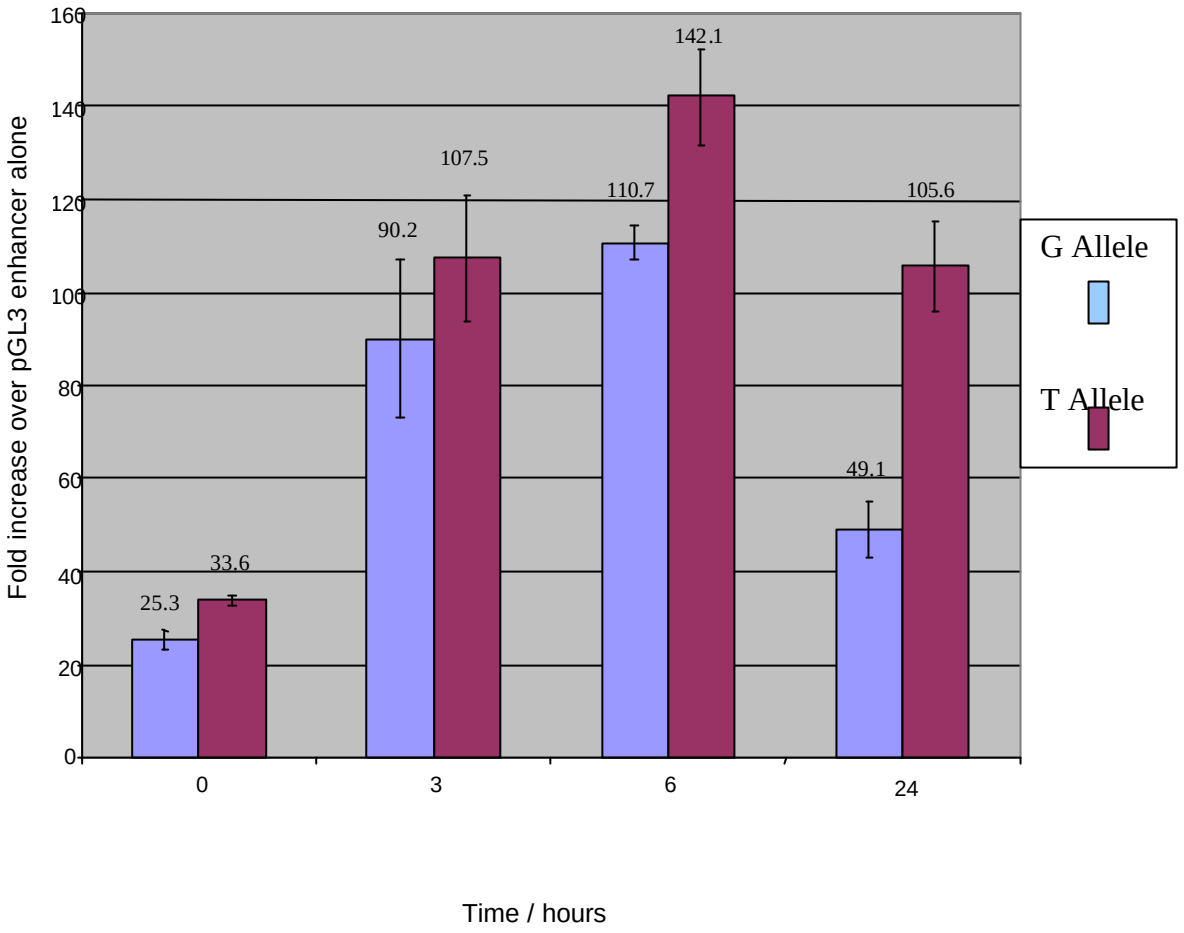

Figure 3.1.2: Functional response of the *ACT* promoter polymorphism to OSM stimulation in T98 G (mixed population) cells. Results of three experiments are shown as mean ± standard error of the mean (error bars).

	Exp1	Exp2	Exp3	Mean	Std. deviation	% Change	p value
T Basal	33.5	31.8	35.7	33.6	2.0	32.8	0.044
G Basal	23.6	23.0	29.2	25.3	3.4		
T 3 hrs	80.2	120.4	121.9	107.5	23.7	19.2	0.484
G 3 hrs	106.0	108.3	56.4	90.2	29.3		
T 6 hrs	148.4	155.9	121.9	142.1	17.9	28.4	0.066
G 6 hrs	117.8	106.0	108.3	110.7	6.3		
T 24 hrs	88.3	121.6	107.0	105.6	16.7	115.1	0.007
G 24 hrs	61.0	44.8	41.4	49.1	10.4		

Table 3.1.3: Difference between activity of the pGL3E 'G' and 'T' constructs, shown as % change in T98G cells basally and following OSM treatment. The fold increases of luciferase activity, over the pGL3 enhancer vector alone are given as mean ± standard deviation. p values are derived using the Student's t-test on the log of the fold increases.

	T Allele			G Allele		
Time	Mean increase over pGL3E enhancer alone	Fold increase over basal	p value	Mean increase over pGL3E enhancer alone	Fold increase over basal	p value
Basal	33.6±2.0	-	-	25.3±3.4	-	-
3 hrs	107.5±23.7	3.2x	0.032	90.2±29.3	3.6x	0.075
6 hrs	142.1±17.9	4.2x	0.011	110.7±6.3	4.4x	0.003
24 hrs	105.6±16.7	3.1x	0.019	49.1±10.4	1.9x	0.083

Table 3.1.4: OSM Response of *ACT* promoter alleles. Response of the pGL3E 'T' and 'G' constructs in T98G cells, comparing basal response to different OSM exposure times. Results (mean ± SD) are the mean of three experiments; % change is the percentage difference between activity of the pGL3E 'G' and 'T' constructs. Univariate analysis of variation of both variation over each time point provided a p value of 0.001. p values shown in table are calculated by performing post hoc Student's t-test on the log of the fold increase over the pGL3 enhancer alone.

3.1.3 Effect of the ACT *Promoter Polymorphism on Reporter Gene Activity in U373 MG Cells*

Since ACT is too large to pass through the blood-brain barrier, it must be synthesised by the brain. The cells that produce ACT in the brain are the astrocytes, and U373 MG cells were chosen to study represent a pure astrocyte population in this study.

There was again a difference in the functional response produced between the different alleles of the *ACT* promoter polymorphism (see figure 3.1.3 and table 3.1.5). Once again, the T allele provoked higher activity compared to the G allele at each time interval. When the astrocytes were transfected a mean difference between the promoter alleles of 167% was seen. The greatest difference in functional effect between the alleles was seen at twelve hours, when the difference was 320%, a highly significant difference. Under basal conditions there was a 207% difference in the functional activity of the two promoter alleles, although this was not statistically significant. The difference in promoter activity after three, six and twenty-four hours was not significant, either, but ranged from a 100% to 140% increase in activity when the T allele was present. After forty-eight hours, the difference between the promoters was 115%, a statistically significant result.

As with the Hep G2 and T98G cells, there was a definite response to OSM (see table 3.1.6). There was a mean rise in functional response of 2-fold when the hepatocytes were stimulated with OSM whichever promoter allele was present. The OSM response in astrocytes differed to that seen in hepatocytes in a number of ways. Firstly, there seems to be a delay in the response. After three hours there was a decrease in activity. This decrease is not statistically significant, and it is more likely that there is no change in promoter activity. After 6 hours there is a statistically significant increase in functional activity when the T allele is present, then a small, non-significant rise after 12 hours, before activity rises to a peak after 24 hours. When the cells were stimulated with OSM for 48 hours the functional activity had dropped to approximately basal levels. This is

in contrast to the hepatocytes, which maintained a response to OSM for only 12 hours. The mean response to OSM was over 8 times greater in hepatocytes than seen in astrocytes. The hepatocytes showed a much greater mean increase to OSM, and at each time interval the activity in response to stimulation was much higher in these cells compared to astrocytes. In hepatocytes the mean increase in functional activity with OSM stimulation over the pGL3 enhancer alone was 18.9 fold with the T allele present, and 16.5 fold when the G allele was present. In astrocytes the values were 1.6 and 2.0 fold respectively.

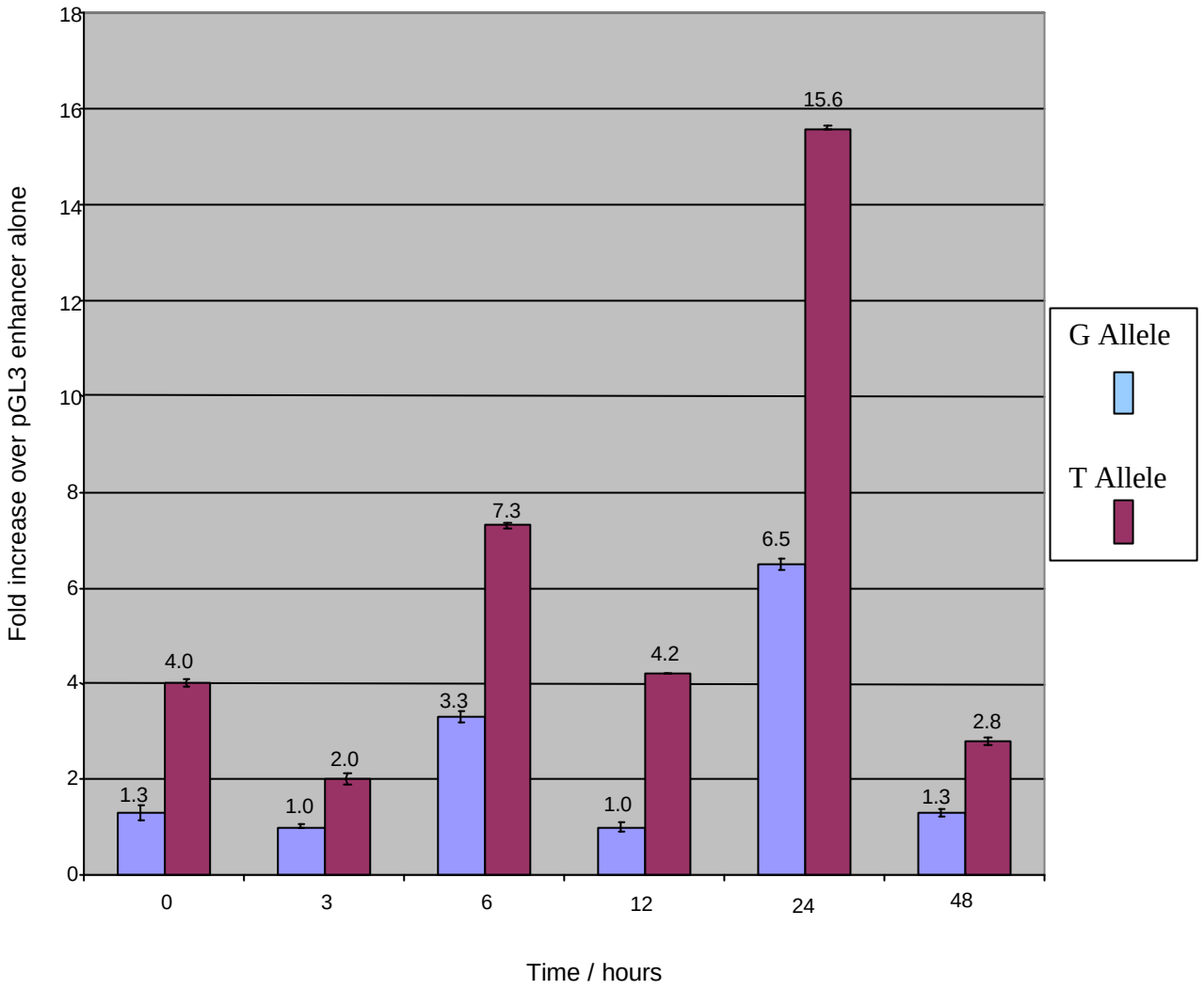

Figure 3.1.3: Functional response of the *ACT* promoter polymorphism to OSM stimulation in U373 MG (astrocyte) cells. Results of three experiments are shown as mean ± standard error of the mean (error bars).

	Exp1	Exp2	Exp3	Mean	Std. Deviation	% Change	p value
T Basal	4.6	4.7	2.8	4.0	1.1		
						207.7	0.054
G Basal	1.5	0.6	1.8	1.3	0.6		
T 3 hrs	1.6	3.2	1.2	2.0	1.0		
						100.0	0.156
G 3 hrs	1.2	1.0	0.8	1.0	0.2		
T 6 hrs	8.5	7.5	6.0	7.3	1.3		
						121.2	0.053
G 6 hrs	2.2	2.7	4.9	3.3	1.4		
T 12 hrs	4.1	4.2	4.3	4.2	0.1		
						320.0	0.014
G 12 hrs	1.3	1.0	0.7	1.0	0.3		
T 24 hrs	19.4	13.7	13.8	15.6	3.2		
						140.0	0.054
G 24 hrs	3.6	7.8	8.0	6.5	2.5		
T 48 hrs	2.5	3.8	2.1	2.8	0.9		
						115.4	0.040
G 48 hrs	1.0	1.8	1.2	1.3	0.4		

Table 3.1.5: Difference between activity of the pGL3E 'G' and 'T' constructs, shown as % change in U373 MG cells basally and following OSM treatment. The fold increases of luciferase activity, over the pGL3 enhancer vector alone are given as mean ± standard deviation. p values are derived using the Student's t-test on the log of the fold increases.

	T Allele			G Alleles		
Time	Mean increase over pGL3 enhancer alone	Fold increase over basal	p value	Mean increase over pGL3E enhancer alone	Fold increase over basal	p value
Basal	4.0±1.1	-	-	1.3±0.6	-	-
3 hrs	2.0±1.0	0.5x	0.060	1.0±0.2	0.8x	0.724
6 hrs	7.3±1.3	1.8x	0.019	3.3±1.4	2.5x	0.099
12 hrs	4.2±0.1	1.1x	0.728	1.0±0.3	0.8x	0.697
24 hrs	15.6±3.2	3.9x	0.013	6.5±2.5	5.0x	0.083
48 hrs	2.8±0.9	0.7x	0.105	1.3±0.4	1.0x	0.865

Table 3.1.6: OSM Response of *ACT* promoter alleles. Response of the pGL3E 'T' and 'G' constructs in U373 MG cells, comparing basal response to different OSM exposure times. Results (mean ± SD) are the mean of three experiments; % change is the percentage difference between activity of the pGL3E 'G' and 'T' constructs. Univariate analysis of variation of both variation over each time point provided a p value of <0.001. p values shown in table are calculated by performing post hoc Student's t-test on the log of the fold increase over the pGL3 enhancer alone.

3.1.4 *Effect of the* ACT *Promoter Polymorphism on Reporter Gene Activity in SH-SY5Y Cells*

Hepatocytes and astrocytes are known to synthesise ACT, and for that reason, the response to OSM of the *ACT* promoter was tested in these cells, but neuronal cells are not known to synthesise ACT. SH-SY5Y cells are neuronal cells, and the response of the *ACT* promoter to OSM was studied in this cell line to provide a contrast with cells involved in the acute phase response with cells that do not synthesise acute phase proteins.

Once more, the functional response of the T allele of the *ACT* promoter polymorphism is higher than the G allele (see figure 3.1.4 and table 3.1.7). The mean difference in functional response between the two promoters is a 44% increase in activity when the T allele is present. The greatest difference between the activity of the two alleles is at six hours, a difference of 112%. Statistically significant differences were seen until twelve hours of stimulation with OSM, when the difference is 21%. Before this time, the difference, which was 36% in unstimulated cells, had risen at each time interval. The smallest difference in functional activity was seen after twenty-four hours, when there was only a 5% difference in response between the two alleles.

There was no trend in increasing functional activity over time when exposed to OSM (see table 3.1.8). The mean response to OSM over each time interval was no different to the basal level of activity whichever promoter allele was present. In general, there was no significant difference between the functional activity of stimulated and unstimulated SH-SY5Y cells.

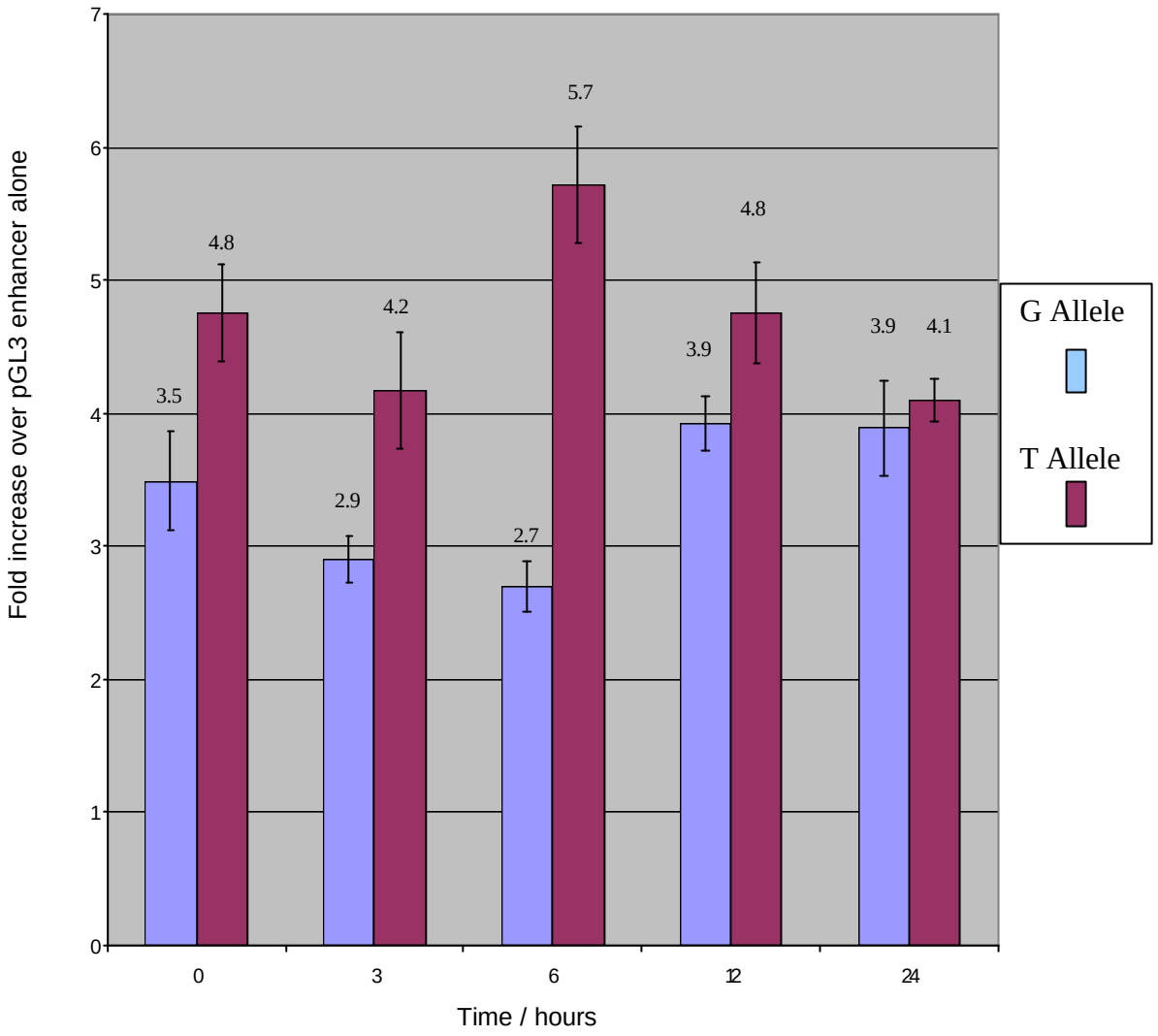

Figure 3.1.4: Functional response of the *ACT* promoter polymorphism to OSM stimulation in SH-SY5Y (neuronal) cells. Results of three experiments are shown as mean ± standard error of the mean (error bars).

	Exp1	Exp2	Exp3	Mean	Std deviation	% Change	p value
T basal	4.7	5.2	4.3	4.8	0.5	36.2	0.015
G Basal	3.7	3.5	3.3	3.5	0.2		
T 3 hrs	4.1	4.2	4.2	4.2	0.1	43.5	0.024
G 3 hrs	2.9	3.2	2.6	2.9	0.3		
T 6 hrs	6.1	6.4	4.6	5.7	1.0	111.9	0.006
G 6 hrs	3.0	2.5	2.5	2.7	0.3		
T 12 hrs	4.0	4.7	5.6	4.7	0.8	21.2	0.199
G 12 hrs	3.7	4.5	3.6	3.9	0.5		
T 24 hrs	4.4	3.8	4.1	4.1	0.3	5.3	0.609
G 24 hrs	3.7	4.7	3.2	3.9	0.8		

Table 3.1.7: Difference between activity of the pGL3E 'G' and 'T' constructs, shown as % change in SH-SY5Y cells basally and following OSM treatment. The fold increases of luciferase activity, over the pGL3 enhancer vector alone are given as mean ± standard deviation. p values are derived using the Student's t-test on the log of the fold increases.

	T Allele			G Allele		
Time	Mean increase over pGL3 enhancer alone	Fold increase over basal	p value	Mean increase over pGL3 enhancer alone	Fold increase over basal	p value
Basal	4.8±0.5	-	-	3.5±0.2	-	-
3 hrs	4.2±0.1	0.9x	0.152	2.9±0.3	0.8x	0.088
6 hrs	5.7±1.0	1.2x	0.102	2.7±0.3	0.8x	0.020
12 hrs	4.7±0.8	1.0x	0.957	3.9±0.5	1.1x	0.271
24 hrs	4.1±0.3	0.9x	0.243	3.9±0.8	1.1x	0.447

Table 3.1.8: OSM Response of *ACT* promoter alleles. Response of the pGL3E 'T' and 'G' constructs in SH-SY5Y cells, comparing basal response to different OSM exposure times. Results (mean ± SD) are the mean of three experiments; % change is the percentage difference between activity of the pGL3E 'G' and 'T' constructs. Univariate analysis of variation of both variation over each time point provided a p value of 0.138. p values shown in table are calculated by performing post hoc Student's t-test on the log of the fold increase over the pGL3 enhancer alone.

3.1.5 Summary of the ACT Promoter Responses

It is apparent, even looking at these data in isolation, that the response of the *ACT* promoter to OSM stimulation, and the responses of the two alleles of the polymorphism differ between cell lines. Table 3.1.9 is a summary of the results described in section 3.1.

The most striking observation was that in every cell line, the T allele had a higher functional effect than the G allele. In the Hep G2 cells, the functional activity of the T allele was higher than that of the G allele under basal conditions and following OSM stimulation. The difference between the functional activities of the alleles was most pronounced after 24 hours of OSM stimulation, with the T allele of the promoter showing 427% more activity than the G allele. The difference in functional activity between the two alleles was similar when the reporter gene constructs were transfected into T98G cells. As with the Hep G2 cells, the greatest difference between the alleles was after 24 hours of OSM stimulation (115%). The U373 MG cells again showed a higher functional effect when the T allele was present than the G allele. In contrast to the previous two cell lines, U373 MG cells showed the greatest difference between alleles after 12 hours of OSM stimulation, with the T allele showing a 320% greater functional effect than the G allele. When the SH-SY5Y cells were investigated the greatest difference between functional activity of the two promoter polymorphism alleles was seen after 6 hours of OSM stimulation.

The results of OSM stimulation were not as clear-cut. In all but the SH-SY5Y cells there was, on average, an increase in activity when the cells were stimulated compared to basal. However, the time course of this effect was not consistent between cell lines. In some cases, such as the T98G cells, the effect of OSM stimulation was apparent after 3 hours of OSM stimulation, and had diminished after 24 hours, whereas U373 MG cells did not show a definite response until they had been stimulated for 6 hours. The difference in activity seen between the OSM responses of the *ACT* promoter polymorphism alleles was not uniform either.

Although there are some differences in the effect of OSM on the different promoter polymorphism alleles in different cell lines, some general points can be made. With the exception of the SH-SY5Y cells, OSM caused an increase in functional activity of the *ACT* promoter, regardless of which allele was present. When the T allele was present, the increased functional activity of the *ACT* promoter caused by OSM stimulation was of a longer duration, with the response to OSM falling to near-basal levels first when the G allele was present.

Cell line	Allele Response						OSM Response	
	Basal		OSM		% Difference			
	T	G	T	G	Basal	OSM	T	G
Hep G2	2.9	1.5	54.8	25.3	90.3	116.6	18.9x	16.5x
T98G	33.6	25.3	118.4	83.3	32.8	42.1	3.5	3.3x
U373 MG	4.0	1.3	6.4	2.6	207.7	146.2	1.6x	2.0x
SH-SY5Y	4.8	3.5	4.7	3.3	37.1	45.5	1.0x	1.1x

Table 3.1.9: Summary of the functional assay data. Allele response under basal condition is the mean fold increase of both alleles of the *ACT* promoter over the SV40 enhancer alone. The values for OSM response are the mean of every OSM at each time interval combined over the SV40 enhancer alone. % Differences are the percent differences between alleles under basal and mean stimulated conditions. The OSM response is the mean OSM response over basal response.

3.2 Reverse-Transcriptase PCR

To confirm that the cell lines under investigation synthesise ACT mRNA, RT-PCR was performed on mRNA extracted from the four cell lines used in the functional assays. Of these cell lines, Hep G2, U373 MG and T98 G cells are known to synthesise ACT (Kalsheker, 1996; Kordula et al., 1998). The SH-SY5Y cells are neuronal cells, and it is not known whether they synthesise ACT.

3.2.1 RT-PCR in Cultured Cells

Two RT-PCR reactions were carried out for each cell line being studied. One was a positive control, amplifying cDNA from the housekeeping gene *HPRT*, and the other reaction was for the *ACT* gene. The RT-PCRs demonstrate that mRNA was being synthesised for both genes in Hep G2, U373 MG and T98G cells (figure 3.2.1). There is one strong band in both lanes, in every cell line, except SH-SY5Y. The presence of a band in the *HPRT* lane of SH-SY5Y demonstrates that mRNA was isolated from these cells, and cDNA was succesfully produced from this mRNA. The amplification of ACT cDNA from the other cell lines demonstrates that the reaction to amplify ACT cDNA was working in three of the four reactions. These results suggest that SH-SY5Y cells do not synthesise ACT mRNA.

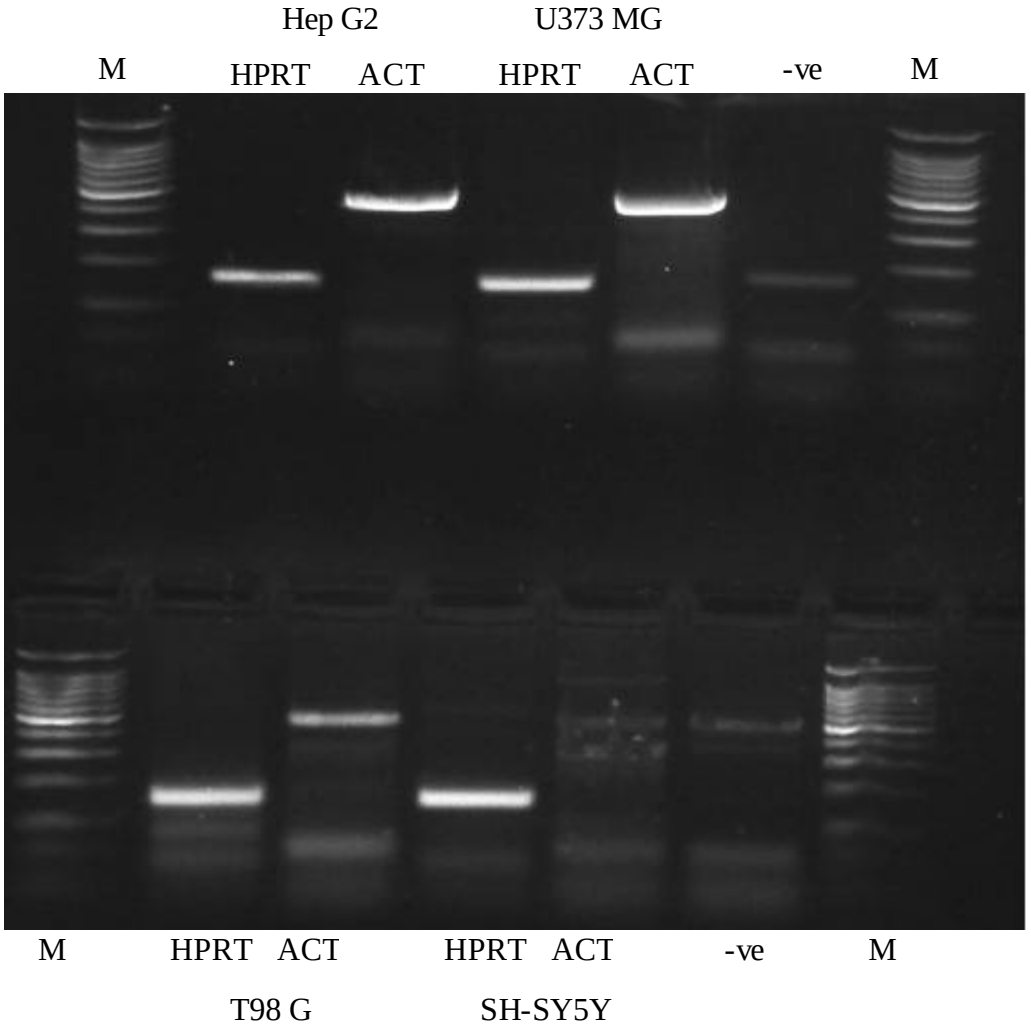

Figure 3.2.1: RT-PCR on cDNA from cultured cell lines. Bands show the products of quantitative RT-PCR on cDNA synthesise from mRNA from two genes, *HPRT* (180bp) and *ACT* (470bp). Other lanes show 100bp ladder (M) and negative controls, containing no cDNA (-ve).

3.3 Electrophoretic Mobility Shift Assays

Having obtained functional data that demonstrates a statistical difference in activity between alleles, the EMSAs were used in order to understand the underlying mechanism behind these differences. These assays rely on radiolabelled DNA, spanning the region of interest, to bind to nuclear proteins extracted from cultured cells. The cell lines chosen for this work were the Hep G2, U373 MG and T98G lines. The SH-SY5Y cells were not included, as RT-PCR showed they do not synthesise *ACT* and DLR assays showed no functional response to OSM.

3.3.1 Electrophoretic Mobility Shift Assay with Nuclear Proteins Extracted from Hep G2 Cells

The signal obtained with nuclear extract from stimulated Hep G2 cells is greater than that from unstimulated Hep G2 cells (figure 3.3.1). This is in agreement with the functional data, and demonstrates that more nuclear proteins are binding to this region of the *ACT* promoter when the cells are stimulated. This is to be expected, as OSM is known to upregulate ACT in hepatocytes. It is also apparent that more protein is binding to the probe that contains the T allele of the *ACT* promoter. Again, this agrees with the functional data, which showed that this form of the *ACT* promoter is more active, both under basal conditions and when stimulated with OSM.

There are at least three bands present in the lanes containing nuclear extract. The lowest band, which is also present in lanes that do not contain extract, is unbound probe. It is important to note that the amount of unlabelled probe is roughly equal in each lane. The probe is added in excess during the binding stage of the assay, and an equal amount of probe at the bottom of the gel suggests that the differences in the amount of protein bound to the probe are genuine, and not due to differences in loading.

The other bands (*) are the result of probe binding to proteins of different sizes. This is due, either to the ACT probe being able to bind to more than one protein,

or, the protein to which the probe binds is able to form a complex. The band closest to the unbound probe (†) is likely to be the result of degraded protein. This is possible, as the proteins that initiate transcription not only bind to DNA, but also form complexes due to protein/protein interactions.

Whatever the identity of the protein, or proteins, binding to the ACT probe, the signal in each lane is not present in equivalent amounts. As mentioned before, it seems to bind preferentially to the T allele of the *ACT* promoter, and when cells have been stimulated with OSM.

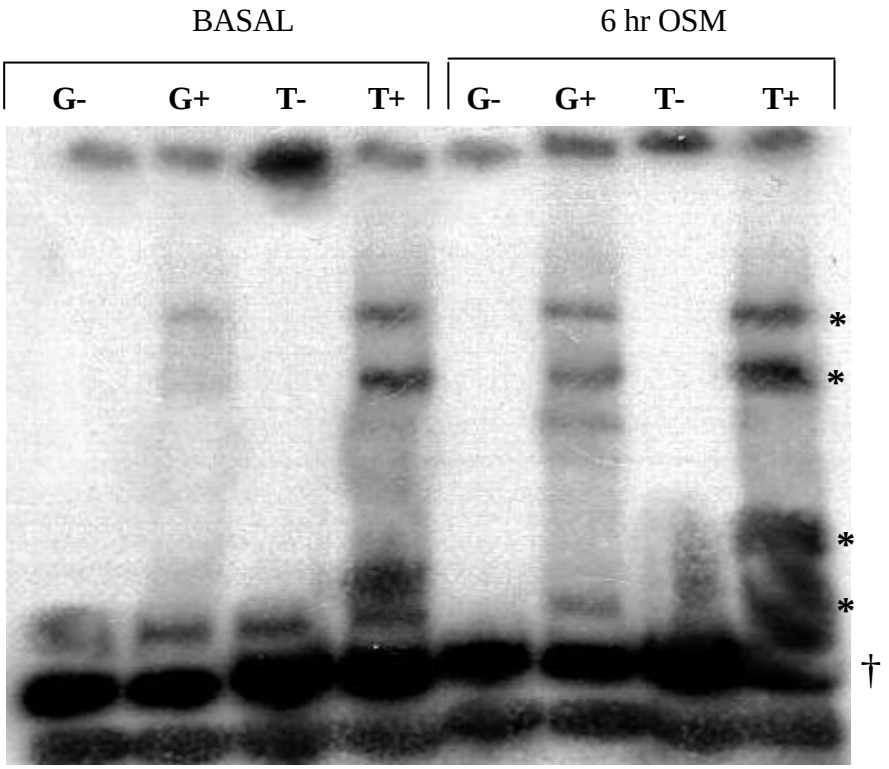

Figure 3.3.1: Electrophoretic mobility assay with nuclear proteins isolated from Hep G2 cells. Radiolabelled probes spanning the *ACT* promoter polymorphism were allowed to interact with nuclear proteins and then separated by size. '+' Represents lanes containing nuclear extract, '-' represents lanes containing radiolabelled probe only. '*' indicates position of DNA/protein complexes and '†' indicates unbound probe.

3.3.2 Electrophoretic Mobility Shift Assay with Nuclear Proteins Extracted from U373 MG Cells

When nuclear proteins extracted from astrocytes were allowed to interact with the radiolabelled ACT promoter probe a marked difference was seen between the two alleles (figure 3.3.2). Under both basal and OSM stimulated conditions the strength of the signal coming from the *ACT* T promoter probe was stronger than that obtained with the G allele probe. There was a large increase in the amount of nuclear protein binding to the T probe when the U373 MG cells were stimulated with OSM.

Although it is difficult to resolve the apparent differences between basal and stimulated binding patterns it is clear that the ACT promoter probe is interacting with protein. When nuclear protein from stimulated U373 MG cells interacts with the T probe under basal conditions, at least three bands are present (*), aside from the unlabelled probe band (†). The band nearest the unlabelled probe is most likely to be degraded protein. When the nuclear proteins were extracted from OSM stimulated cells, there are at least 5, possibly 6 bands present, including what is probably degraded protein. There was more protein binding to the probe in the stimulated extracts, and also a number of larger proteins or protein complexes present. When the probe to the G allele interacted to nuclear proteins, the proteins from the unstimulated cells bound less to the G allele probe than proteins obtained from stimulated cells. As with the T allele, a number of higher molecular weight proteins or protein complexes were present.

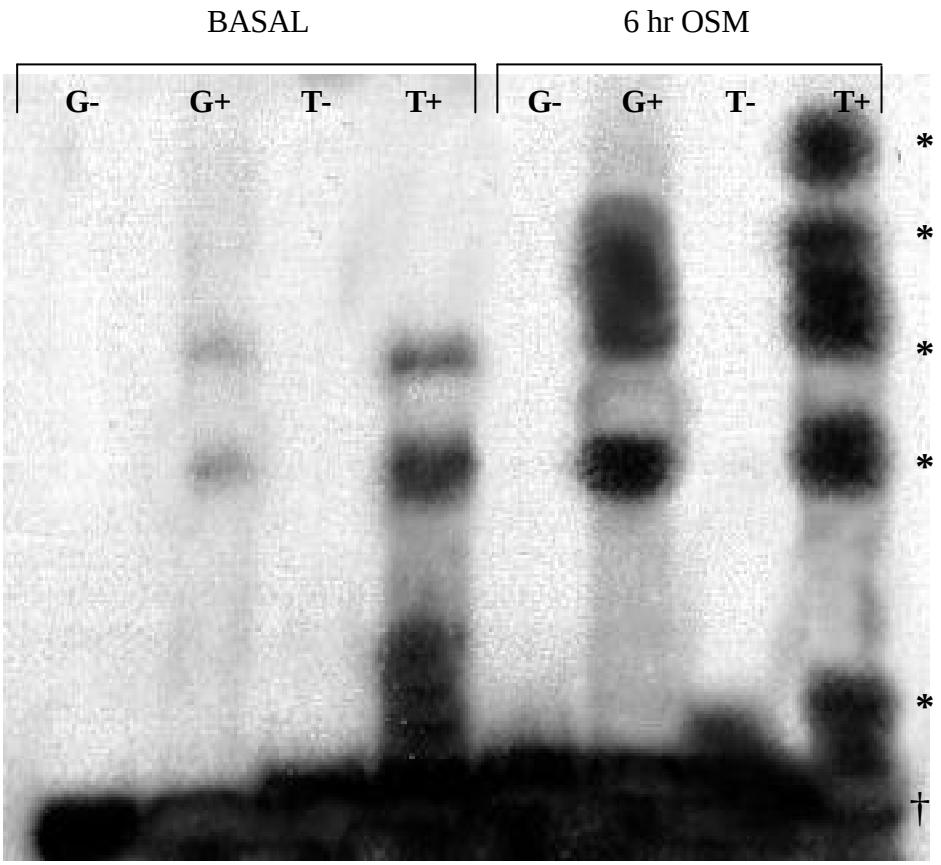

Figure 3.3.2: Electrophoretic mobility assay with nuclear proteins isolated from U373 MG cells. Radiolabelled probes spanning the *ACT* promoter polymorphism were allowed to interact with nuclear proteins and then separated by size. '+' Represents lanes containing nuclear, '-' represents lanes containing radiolabelled probe only. '*' indicates position of DNA/protein complexes and '†' indicates unbound probe.

3.3.3 Electrophoretic Mobility Shift Assay with Nuclear Proteins Extracted from T98G Cells

When the T98G cells were stimulated with OSM, there appeared to be similar amounts of protein binding to the *ACT* promoter as seen in the basal state (see figure 3.3.3). This was true for both alleles of the *ACT* promoter polymorphism. When the T allele was used there was more DNA-protein binding than when the G allele was used. The proteins that bind to the T allele could, on first examination, appear to be different than those that bind to the G allele. These appear as faint bands (marked '?'), and seem to bind only to the T allele. What is more likely is that the same proteins are binding to both alleles, however, only the proteins that bound most strongly to the T allele can be seen. There are at least three bands, representing different sized proteins, or protein complexes binding to the *ACT* promoter DNA, although the band closest to the unbound probe (†) is likely to be the result of degraded protein.

When anti-TFIIB antibody was added to the nuclear proteins from cells stimulated with OSM, the banding pattern changed. The strongest band seen when no antibody was present decreased in intensity, and a new band appeared near the top of the gel (arrowed). This indicates a larger protein complex due to the increased size of the protein originally present bound with the antibody (induced supershift). This suggests that the strong band is, at least in part, TFIIB. It is difficult to determine whether the other bands present were shifted by the addition of anti-TFIIB, so it cannot be said with certainty that each band represents a complex of protein that contains TFIIB, but it is clear that TFIIB can bind to the *ACT* promoter.

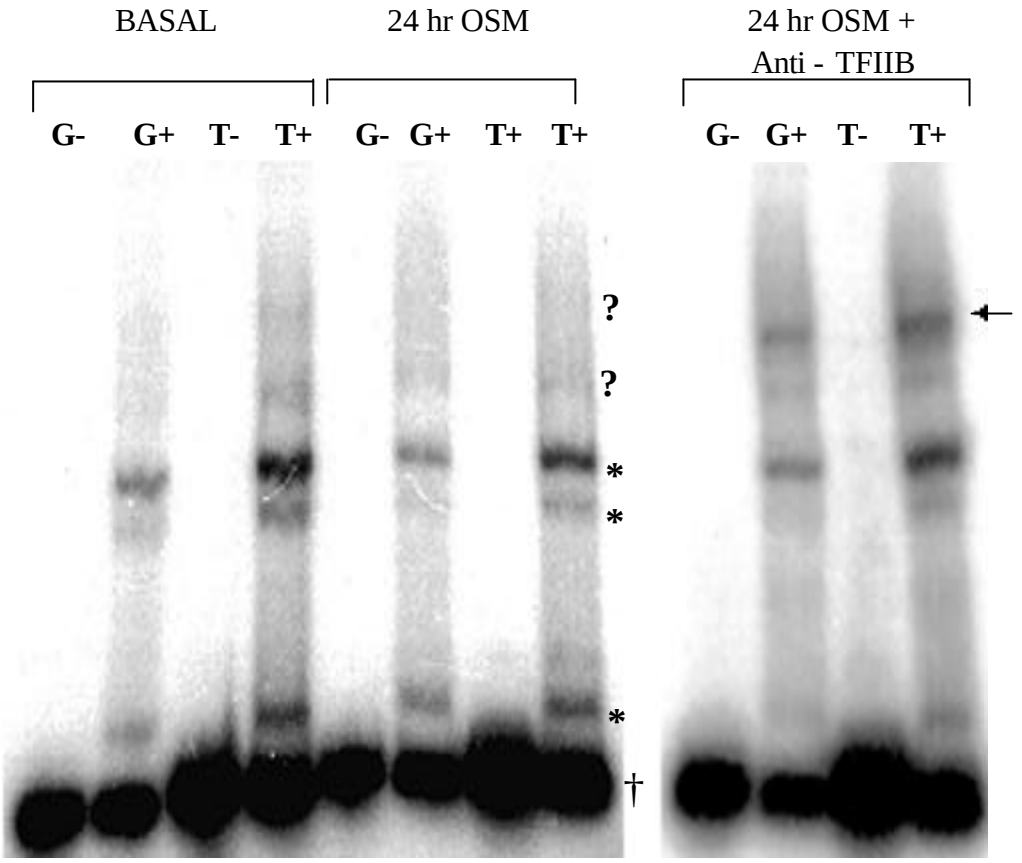

Figure 3.3.3: Electrophoretic mobility assay with nuclear proteins isolated from T98 G cells. Radiolabelled probes spanning the *ACT* promoter polymorphism were allowed to bind to nuclear proteins and then separated by size. In the lanes on the left, '+' represents lanes containing nuclear extract, '-' represents lanes containing radiolabelled probe only. '*' indicates position of DNA/protein complex, '?' indicates position of spurious DNA/protein complexes and '†' indicates unbound probe. In the lanes on the right, '+' represents lanes containing nuclear extract, radiolabelled probe and anti-TFIIB, '-' represents lanes containing radiolabelled probe alone. Arrow indicates position of supershifted DNA/protein complex.

3.4 Alpha-1-*Antichymotrypsin* Promoter Genotyping in AD Cases and Controls

Samples from two centres, Oxford and Nottingham, were used in this study (table 3.4.1). All samples were genotyped at the *ACT* –51 promoter and had previously been screened at the *APOE* polymorphism locus. The *ACT* genotypes were generated by MRC Geneservices (Hinxton, Cambridge) genotyping facility, and one third of samples had their genotype verified by RFLP (see figure 2.4.1, p93).

The numbers used in this study (335 controls, 394 AD) have over 99% power to detect an OR of 2 or more when the allele under investigation has a frequency of 49%. The OR of 2 was chosen arbitarily. The sample sizes are also large enough to have a greater than 99% power to detect an OR of 4 or more for the *APOE* e4 allele association. Previous studies have shown an OR for this association to be 4.5 (Lambert et al., 2002).

Centre	AD Status	n	Mean age±SD	Male	Female	AD Status	n	Mean age	Male	Female
Nottingham	All AD	191	83.3±8.7	69	118	Control	123	72.8±8.9[c]	78	38
Oxford	All AD	198	70.5±9.1	89	115	Control	212	76.3±9.5[d]	93	119
Total	All AD	389	75.6±10.4	158	233	Control	335	75.0±9.4	171	157
Nottingham	Confirmed AD	191	83.3±8.7[a]	69	118					
Oxford	Confirmed AD	112	71.1±9.0	51	67					
Total	Confirmed AD	303	77.3±10.1	120	185					
Oxford	Probable AD	86	69.5±9.2[b]	38	48					

Table 3.4.1: Demographic information for the two populations examined in the genotyping study. Each population was divided into groups based on AD status. Note some samples did not posses age or sex data; [a]7 samples, [b]3 samples, [c]2 samples, [d]5 samples.

3.4.1 ACT-*51bp Promoter Polymorphism and Alzheimer´s Disease*

Samples from both populations were classified according to disease status, confirmed AD, probable AD and control. Genotype and allele frequencies in each group, confirmed AD, probable AD and control, are shown in table 3.4.2. In the control groups from Nottingham and Oxford, the distribution of heterozygotes were as predicted by Hardy-Weinberg equilibrium. There was no significant difference between confirmed AD cases and controls in either population (Nottingham, $?^2$= 1.808, 1df, p=0.99 OR= 1.15, 95% CI 0.66 to 1.99; Oxford, $?^2$= 0.864, 1df, p=0.649, OR=0.98, 95% CI 0.59 to 1.63). Also, there was no significant difference when the Oxford probable AD group was compared with the Oxford control group ($?^2$= 1.848, 1df, p=0.39, OR=1.53, 95% CI 0.82 to 2.89). When both populations were combined, there was still no significant difference between genotype frequencies in confirmed AD cases and controls ($?^2$= 2.76, 1df, p=0.25, OR=1.12, 95% CI 0.79 to 1.58). The low odds ratios derived from these tests meant that the studies were underpowered. To gain 80% power with an odds ratio of 1.19 in a population with a mean age of 75, 2077 cases would be needed, with the same number of controls, assuming a 5% two-way error rate (false negatives and positives). This is assuming a 49% frequency of the T allele.

Centre	AD Status	Genotype Frequency			Allele Frequency		AD Status	Genotype Frequency			Allele Frequency	
		GG	GT	TT	G	T		GG	GT	TT	G	T
Nottingham	All AD	39 (20.5%)	103 (54.2%)	46 (24.2%)	181 (48.1%)	195 (50.8%)	Control	28 (22.8%)	57 (46.3%)	36 (29.3%)	113 (46.7%)	129 (53.3%)
Oxford	All AD	47 (23.0%)	105 (51.5%)	52 (25.5%)	199 (48.8%)	209 (51.2%)	Control	55 (25.9%)	99 (46.7%)	58 (27.4%)	209 (49.3%)	215 (50.7%)
Total	All AD	86 (21.9%)	208 (53.1%)	98 (25.0%)	380 (48.5%)	404 (51.5%)	Control	83 (24.9%)	156 (46.8%)	94 (28.2%)	322 (48.3%)	344 (51.7%)
Nottingham	Confirmed AD	39 (20.5%)	103 (54.2%)	46 (24.2%)	181 (48.1%)	195 (50.8%)						
Oxford	Confirmed AD	31 (26.3%)	60 (50.8%)	27 (22.9%)	122 (51.7%)	114 (48.3%)						
Total	Confirmed AD	70 (22.9%)	163 (53.2%)	73 (23.9%)	303 (49.5%)	309 (50.5%)						
Oxford	Probable AD	16 (18.6%)	45 (52.3%)	25 (29.1%)	77 (44.8%)	95 (55.2%)						

Table 3.4.2: *ACT* –51bp promoter polymorphism genotype and allele frequencies in different AD groups in two British populations. c^2 **tests demonstrated no association between *ACT* genotype or allele and AD risk, either when samples were combined or in separate populations.**

It has been reported that the *ACT* signal sequence polymorphism, which is in linkage disequilibrium with the promoter polymorphism, has a modifying effect on age of onset of AD. For this reason, the confirmed and probable AD groups from both populations were combined and the effect of *ACT*-51 genotype on mean age of onset was determined (table 3.4.3). When age of onset was unobtainable, age of death, less 10 years was used to estimate age of onset. No association was observed between *ACT* promoter genotype and age of onset.

ACT Genotype	n	Mean age	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
GG	85	75.5	11.5	1.3	73.1	78.0	50.5	95.0
GT	201	75.6	10.0	0.7	74.2	77.0	51.1	101.0
TT	96	75.6	10.4	1.1	73.5	77.7	49.3	97.0
Total	382	75.6	10.4	0.5	74.6	76.7	49.3	101.0

Table 3.4.3: Mean age of AD samples against *ACT* genotype. AD samples were from the confirmed and probable AD subgroups of both the Nottingham and Oxford populations. ANOVA test gave a p value of 0.997.

Although the *ACT* promoter polymorphism had no apparent effect on mean age of onset in the AD groups, there may still be an age-effect of the *ACT* polymorphism. When the AD cases, both confirmed and probable, are divided by age into early onset (defined as AD diagnosis made before the age of 65) and late onset (diagnosis of AD after the age of 65), the *ACT*-51 polymorphism genotype frequencies did not show a significant difference (figure 3.4.1).

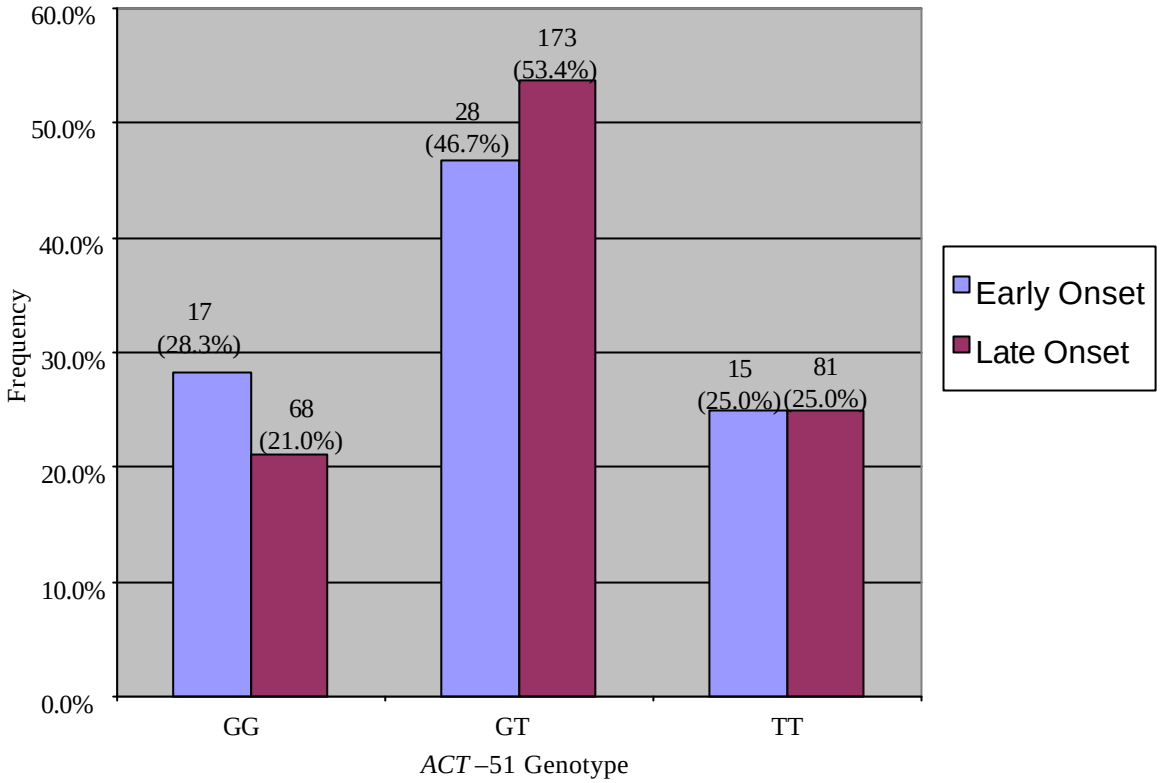

Figure 3.4.1: *ACT* –51bp promoter polymorphism genotype frequencies in early and late onset AD subgroups. Data labels show sample size and frequencies. Pearson $?^2$ returns a p value of 0.435; there is no significant difference in genotype frequencies between subgroups of AD in the two British populations.

3.4.2 Apolipoprotein E *Genotype and AD Status*

Previous studies have suggested that there is an association between *ACT* and *Apolipoprotein E* in AD. Although no mechanism has been suggested for this possible interaction, it has been suggested that the e4 allele of *APOE* results in a lower age of onset in AD. A table detailing *ACT* –51 and *APOE* genotypes in AD cases and control can be found in Appendix 2. The probable and confirmed AD groups were combined into an AD group, as there were insufficient probable AD cases for analysis. Similarly, the *APOE* genotypes were combined into presence or lack of e4, as some genotypes, such as e2/e2 were too rare to provide enough numbers for analysis. It was seen that AD patients were likely to have one or more *APOE* ε4 alleles (table 3.4.4). The odds ratio of 4.5 (95% CI 3.3 to 6.2) was in close agreement with previous studies (Lambert et al., 2002).

AD Status	*APOE* Genotype		
	No e4	e4	Total
AD	125 (40.6%)	183 (59.4%)	308
Control	245 (75.9%)	78 (24.1%)	323

Table 3.4.4: Comparison of AD Status with *APOE* genotype groups. Samples from Nottingham and Oxford were combined, as were the confirmed AD and probable AD subgroups, in order to provide enough cases for comparison. Pearson $?^2$ test returns a p value of less than 0.001, d.f = 1, OR= 4.5 (95% CI 3.3 to 6.2). Note that not all samples were categorised, as *APOE* genotype data was not available for every sample.

As with *ACT*, *APOE* has been reported as having an effect on the age of onset of AD. If this was the case, it would be expected that the mean age of AD patients in this study would be lower when the ε4 allele is present. The mean age of AD patients with and without *APOE* ε4 was compared and e4 patients have a significantly lower age of onset (table 3.4.5).

APOE Genotype Group	n	Mean age	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
No e4	158	77.1	10.5	0.8	75.4	78.7	51.7	97.0
e4	224	74.7	10.3	0.7	73.3	76.0	49.3	101.0
Total	382	75.7	10.4	0.5	74.6	76.7	49.3	101.0

Table 3.4.5: Mean age of AD samples against presence of *APOE* e4 allele. To determine if *APOE* genotype influences mean age of AD onset the age of onset was compared between confirmed and probable AD cases from Nottingham and Oxford based on presence of *APOE* e4. ANOVA test gives a p value of 0.026.

It is possible that the lack of association between the *ACT* –51 polymorphism and AD was masked by the strong association between the *APOE* ε4 allele and AD. When the *ACT* promoter polymorphism genotype frequencies were compared in subjects lacking an *APOE* ε4 allele no significant difference was seen (table 3.4.6).

AD Status	*ACT* Genotype Frequency			*ACT* Allele Frequency	
	GG	GT	TT	G	T
AD	37 (23.1%)	78 (48.8%)	45 (28.1%)	152 (47.5%)	168 (52.5%)
Control	67 (27.5%)	115 (47.1%)	62 (25.4%)	249 (51.0%)	239 (49.0%)
Total	104 (25.7%)	193 (47.8%)	107 (26.5%)	401 (49.6%)	407 (50.4%)

Table 3.4.6: Comparison of *ACT* genotype between AD cases and controls from Nottingham and Oxford in the absence of *APOE* e4. Pearson $?^2$ generates p value of 0.598, the *ACT* –51 polymorphism had no effect on AD risk in the absence of the *APOE* e4 allele.

Since there have been studies showing that ACT may be a risk modifier for AD in combination with the e4 isoform of ApoE, genotype frequencies of the *ACT* promoter polymorphism were compared in subjects, excluding those with no *APOE* e4 allele. No significant difference was seen in this comparison (table 3.4.7).

AD Status	*ACT* Genotype Frequency			*ACT* Allele Frequency	
	GG	GT	TT	G	T
AD	32 (18.8%)	93 (54.7%)	45 (26.5%)	157 (46.2%)	183 (53.8%)
Control	10 (15.2%)	30 (45.5%)	26 (39.4%)	50 (37.9%)	82 (62.1%)
Total	42 (17.8%)	123 (52.1%)	71 (30.1%)	207 (43.9%)	265 (56.1%)

Table 3.4.7: Comparison of *ACT* genotype between AD cases and controls from Nottingham and Oxford in the presence of *APOE* e4. Pearson $?^2$ generates p value of 0.215, the *ACT* –51 polymorphism had no effect on AD risk in the presence of the *APOE* e4 allele.

To determine if the *ACT* –51 polymorphism alters the mean age of AD patients in the absence of the *APOE* ε4 allele, the mean age of patients with each genotype of the *ACT* promoter polymorphism, lacking *APOE* ε4 allele were compared. There was no association with *ACT* promoter genotype and age at onset of AD in patients lacking *APOE* e4 (table 3.4.8).

	n	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
GG	37	76.8	11.9	2.0	72.8	80.7	54.8	94.0
GT	76	76.8	9.7	1.1	74.6	79.1	52.6	97.0
TT	45	77.7	10.8	1.6	74.4	80.9	51.7	97.0
Total	158	77.1	10.6	0.8	75.4	78.7	51.7	97.0

Table 3.4.8: Mean age of AD samples against *ACT* genotype in the absence of *APOE* e4 allele. AD samples are the confirmed and probable AD patients from Nottingham and Oxford, without an *APOE* e4 allele. ANOVA test gives a p value of 0.902.

To determine if *APOE* e4 is a necessary co-factor with *ACT* -51T in the pathology of AD, the mean age of patients with each genotype of the *ACT*

promoter polymorphism, with at least one *APOE* ε4 allele were compared. There was no association with *ACT* promoter genotype and age at onset of AD in patients with the *APOE* e4 allele (table 3.4.9).

	n	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
GG	48	74.6	11.2	1.6	71.3	77.8	50.5	95.0
GT	123	74.9	10.2	0.9	73.1	76.7	51.1	101.0
TT	51	73.8	9.8	1.4	71.1	76.6	49.3	93.0
Total	222	74.6	10.3	0.7	73.3	76.0	49.3	101.0

Table 3.4.9: Mean age of AD samples against *ACT* genotype in the presence of *APOE* e4 allele. AD samples are the confirmed and probable AD patients from Nottingham and Oxford, with at least one *APOE* e4 allele. ANOVA test gives a p value of 0.827.

4 DISCUSSION

4.1 Functional Activity of the *ACT* –51 Promoter Alleles

As described previously, the *ACT* promoter responds to OSM stimulation (Kordula et al., 1998). These experiments have extended previous work by demonstrating that the alleles of the –51bp polymorphism modify the functional activity of the *ACT* promoter, both basally and with OSM stimulation. Specifically, the T allele of this polymorphism has a higher activity, under basal conditions and when stimulated with OSM.

The acute phase response is the body's immediate reaction to a range of insults, including trauma, and results in a wide-ranging physiological reaction. This includes the generation of acute phase proteins that fulfil a number of requirements to co-ordinate a response to the insult, to reduce and repair any damage caused. The acute phase response sees an increase in the concentration of acute phase proteins in the circulation, co-ordinated by a range of cytokines (Roberts et al., 1995). These cytokines, which can influence local and distal reactions on cells, are tightly regulated, and the responses they provoke are strictly controlled (McGeer and McGeer, 1995). Cytokines often trigger cascade responses, whereby the genes they activate synthesise proteins that activate, directly or indirectly, other genes, and these cascades can also produce a feedback response. In these situations the product of a gene downstream can activate a gene upstream; in effect the system is self-maintaining. Polymorphisms in the regulatory regions of acute phase protein genes could therefore have a morbid effect, if they alter the cytokine response of the genes small effects could be amplified (Akiyama et al., 2000; McGeer and McGeer, 2001b).

Since AD pathogenesis is believed by many researchers to be a product of chronic neuroinflammation, the response of the two alleles of the *ACT* promoter

to an inflammatory cytokine was also investigated. The functional assays were chosen as the most appropriate method of testing the hypothesis that the base substitution in the *ACT* regulatory region alters the functional response of the *ACT* promoter.

The functional assays were used to demonstrate two different, but linked, effects. The difference in functional activity of the two alleles of the *ACT* promoter polymorphism was measured in different cell lines, and also, the effect of oncostatin M (OSM) exposure on functional effect was investigated. As the *ACT* promoter was known to respond to OSM (Kordula et al., 1998), the effect of OSM on the two alleles was also shown.

These effects were demonstrated in four different cell lines. Hepatocytes are the main source of ACT synthesis (Kalsheker, 1996), and the concentration of ACT in the circulation is dependent on ACT secretion from these cells. Hep G2 cells represented hepatocytes in this study. The origin of ACT in the brain is dependent on production by astrocytes, as this serpin is too large to pass through the blood brain barrier. Astrocytes were represented in this study by two cell lines, U373 MG, which is a pure astrocytic population, and T98G, a mixed population of astrocytes and neuronal cells. For purposes of comparison, the pure neuronal cell line, SH-SY5Y, was also included.

The most active allele in terms of functional activity did not vary according to the cell line under investigation. The T allele demonstrated higher activity, regardless of cytokine stimulation or cell line. There was also a cell-specific effect of the *ACT* promoter to OSM stimulation. The hepatocytes showed the largest response to OSM stimulation compared to the other cell lines, while the neuronal cells did not respond to OSM. The response to OSM in each individual cell line was similar whether the T allele or the G allele of the *ACT* promoter polymorphism was present overall.

Although these are *in vitro* data, if these trends reflect what occurs *in vivo*, the T allele of the *ACT* promoter polymorphism may result in a higher expression of ACT and this change will vary between cell types and tissues. This result is interesting as in AD amyloid plaques are localised to certain regions of the brain. It may be that the difference in *ACT* promoter activity leads to localised

differences in ACT concentration, causing Aß fibrilisation to increase in these regions.

4.2 Protein Interactions with the *ACT* –51 Promoter Alleles

The functional assays, described above, demonstrated that the different *ACT* promoter alleles have cell-specific functional effects and confirmed that the *ACT* promoter responds to stimulation by OSM. The mechanism by which these effects are mediated is unknown, although one possibility is that the promoter polymorphism alters the binding affinity of nuclear proteins responsible for initiating transcription. The electrophoretic mobility assay provides a qualitative demonstration of the differences in DNA-protein binding activity of the two promoter alleles. In this assay, DNA probes covering the polymorphic site of the *ACT* promoter interact with nuclear proteins extracted from cultured cells. The greater the affinities to the nuclear proteins, the more the probes will bind to them. Since the probes are labelled with a radioisotope the more probe that binds to the proteins, the more radioactive the DNA-protein complexes will be. The assumption being tested is that the polymorphism alters the binding affinity to nuclear proteins, and this will be revealed by differences in signal strength on the autoradiograph. If the probes for the different alleles bind to different proteins then the size of the DNA-protein complex will differ, and this will be revealed by the position of the complexes on the EMSA gels.

In general, all the EMSA patterns show a similarity in that the T allele probe of the promoter polymorphism binds nuclear proteins more avidly than the G allele probe. There does not appear to be a correlation between the increase in functional activity after OSM stimulation and increased DNA-protein binding. There are a number of possible reasons why this may be the case, for example the different complexes binding to the EMSA probes may be responsible for the magnitude of the OSM response.

The EMSA gels all show that the radiolabelled probes have bound protein complexes. One of the DNA-protein complexes appears to be common to all

cell lines. When nuclear extract obtained from T98G cells was incubated with radiolabelled probe and anti-TFIIB antibody the predominant band shifted, towards the top of the gel as a result of its increased size due to recognition by the antibody. This is evidence that the protein present in this band was at least in part TFIIB, which forms a core component of the RNA polymerase II transcription complex. By extension, it is possible that TFIIB is binding to all of the probes shown. Unfortunately, due to the presence of higher molecular weight complexes in the Hep G2 and U373 MG cells lines, it was not possible to test with the anti-TFIIB antibody, as the band would be shifted into the position of these bigger complexes. These higher order complexes either did not exist, or were present at levels difficult to detect in nuclear extracts from T98G cells.

Other complexes were also present, although different complexes were present in each cell line. These additional complexes appeared to bind less strongly, as their intensities varied. Without further study, isolating and identifying these proteins, it is difficult to state with any certainty what these bands could be, and what relevance, if any, they could have to the regulation of the *ACT* gene. It is known that a large number of proteins complex with TFIIB in the initiation of transcription. These additional bands could be the transcription initiation complex in different stages of assembly, either missing factors, or containing additional proteins. They may be the result of a non-specific binding, proteins that bind normally to a similar, perhaps related, DNA sequence. However, this is unlikely as all reactions were conducted using an optimal concentration of poly dI-dC, which minimises non-specific binding. What is interesting is the fact that there appear to be different proteins binding to the *ACT* promoter. This raises the possibility that this region could bind to different proteins depending on the cell line thereby modulating cell-specific expression and contain, as yet unidentified tissue-specific factors. Further work is required to determine if this is the case, or whether the bands represent the transcription initiation complex in different stages of assembly.

The EMSA assays add some detail to the results of the functional assays. It can be seen in all cases that the T allele appears to have a higher affinity than the G

allele for proteins isolated from cell nuclei. Although it is not possible to say definitely that the *ACT* promoter is binding TFIIB several results suggest that this is the case. The *ACT* promoter region contains sequence which matches the predicted sequence of a TFIIB-binding domain; binding is a function of the DNA sequence, which is changed by the polymorphic site. The evidence to suggest that TFIIB is involved comes from the antibody shift experiment. Knowing that the promoter potentially binds TFIIB in T98G nuclear extract suggests a possible mechanism for the difference in *ACT* promoter activity, and makes it more likely that the functional data is a true reflection of what occurs *in vivo.* TFIIB is more likely to bind to the T allele than the G, such that a transcription initiation complex forms more readily when the T allele is available. This implies that ACT mRNA will be present at higher levels when the T allele is found in the *ACT* promoter than the G allele. There is no data on mRNA splicing, or on translation, but if the level of ACT protein is dependent on the concentration of mRNA then differences in the *ACT* promoter polymorphism sequence will result in a greater transcription of the *ACT* gene, which will result in more ACT protein.

4.3 *ACT* –51 Promoter Polymorphism and AD

When the results of the binding assay are considered in the light of the functional data, bearing in mind the caveats mentioned above, it could be said that hepatocytes and astrocytes could produce a higher level of ACT depending on which form of the polymorphism is present. This would mean that individuals that have the T allele of the promoter polymorphism would produce more ACT, during an inflammatory response, compared to individuals without the T allele. Since hepatocytes appear to produce more ACT when the T allele is present, whether or not there is OSM stimulation, this would show up as an increase in plasma ACT compared to CSF ACT when there is no inflammation. This is precisely what has been shown in some studies to determine the viability of ACT as a biomarker in AD (Licastro et al., 2000a; Sun et al., 2003).

The functional and DNA-protein binding data suggest that the *ACT* –51 promoter polymorphism alters the functional activity of the *ACT* gene. Since the T allele of this promoter has a higher affinity for TFIIB, it is possible that this effect is mediated through the transcription initiation complex. If this has a role in the pathology of AD, it could be expected that the T allele might segregate with AD. To demonstrate this, DNA was genotyped from over 700 subjects with confirmed and probable AD as well as controls. In the control groups, both from Nottingham and Oxford as well as the combined populations, the genotypes were found to be in Hardy-Weinberg equilibrium, meaning that alleles are being distributed at random, not as the result of some selective pressure. This is an important point, as it suggests that any differences found when comparing cases with controls are due to alleles segregating with AD. When comparing the frequencies of the *ACT* promoter alleles between AD cases and controls no significant differences were found. This was true both of genotype frequency and allele frequency, in both populations separately and combined, and when considering confirmed AD, probable AD, or both.

That the TT genotype does not segregate with AD does not mean that the *ACT* promoter polymorphism has no effect on AD. It is possible that this polymorphism alters the pathology of AD, rather than the risk. One way the pathology could be altered is increasing the aggressiveness of the disease. Should this hypothesis be correct, there need not be a change in *ACT* promoter genotype frequencies in AD cases, but such a change would increase the severity of AD once it develops. However, the *ACT* promoter genotype frequencies did not show a significant change with age of onset in AD cases. When the AD cases were split into early onset and late onset, according to age at diagnosis, there was again no significant difference in the *ACT* genotype distribution. However, the numbers for this sub-analysis were very small and must be treated with caution in the light of other studies that show a potential involvement in early onset AD, for example, as shown in an Italian population (Licastro et al., 1999b).

The absence of an association between *ACT* genotype and AD is perhaps unsurprising. This study was designed to detect associations between alleles

and disease with an odds ratio of 2 or more, but on calculating the odds ratio for the association of the *ACT* T allele with AD, this was found to be less than 2, therefore not enough patients were included in the study. As the *ACT* promoter polymorphism is a recent discovery, no data was available for the risk associated with the T allele of this gene with AD. Overall in this study the odds ratio was 1.19 (95% CI 0.77 to 1.62), when all cases of AD were compared in both populations, without accounting for the *APOE* e4 allele. Using the Quanto 0.5 software, the number of cases required to gain a power of 80% for such a small effect can be computed. The number of cases needed would be 2800, with an equal number of controls for a population of 75-year olds.

4.4 *Apolipoprotein E*, Interactions with *ACT* and AD

Previous studies have demonstrated that *apolipoprotein E* is a major risk factor for AD. In particular, the e4 allele of *APOE* is associated with an increased risk of AD and e2 appears to have a protective effect. It has also been reported that ACT may have an effect on AD risk in association with *APOE* e4. The work of Licastro, *et al*. demonstrated that in both a mouse model, and in a human AD population, *APOE* e4 and the T allele of the *ACT* signal sequence polymorphism were associated with an increased risk of AD (Licastro et al., 1999a; Licastro et al., 1999b). A positive correlation was found between brain ACT levels and the *APOE* e4 allele in AD patients (Licastro et al., 1998). Other studies have either failed to show this effect (Durany et al., 1999; Helisalmi et al., 1997; Muller et al., 1996; Nacmias et al., 1996), or failed to prove or disprove an association (Axelman et al., 1999; Durany et al., 1999; Fallin et al., 1997; Kamboh et al., 1997; Nacmias et al., 1998; Scacchi et al., 1999; Scacchi et al., 2001).

The *apolipoprotein E* genotypes of the 405 AD patients and 307 controls were available. These were divided into e4 allele carriers and non-e4 carriers. The frequency of these allele groups in cases and controls were compared, and it was shown, in agreement with previous studies, that there was a greater number of e4 carriers in the AD groups (confirmed AD and probable AD) than in controls.

It has been reported that the *apolipoprotein E* e4 allele is associated with lowering the age of onset of AD. To demonstrate this effect the 382 AD patients for whom age was known were divided into e4 carriers and non-e4 carriers and the mean age was compared. It was shown that the e4 allele was associated with a lower mean age of onset.

In contrast to the *ACT* promoter polymorphism AD risk associated with the *APOE* e4 allele is very much higher. This was observed in this study, and agrees with data from previous studies. Since the association of *APOE* with AD is so large, the number of samples needed to effectively demonstrate an association between allele and disease is much lower. Due to this large effect on AD risk, the *APOE* genotyping had a very high power (greater than 99%), and the effect could have been reliably demonstrated with far fewer samples.

4.5 *ACT* –51 Promoter Polymorphism in AD Patients Lacking *APOE* e4

To demonstrate that the *ACT* –51 promoter polymorphism has an effect on the risk of AD, the *ACT* genotype frequencies were compared in 244 controls and 160 AD patients lacking the *apolipoprotein E* e4 allele. By excluding subjects with the *APOE* e4 allele, which has been shown to alter risk of AD, it is possible to look at the effect of the *ACT* polymorphism in isolation. The *ACT* –51 polymorphism genotype frequencies were not significantly different between cases and controls.

No association with AD and *ACT* genotype in the absence of *APOE* e4 was demonstrated, so the hypothesis that the *ACT* -51 promoter polymorphism may have an effect on the age of onset of AD was tested. The supposition was made that a lower age of onset would result in a mean lower age of diagnosis or death. This was again assuming that while the *ACT* promoter polymorphism does not alter the risk of developing AD it may alter the pathology of pre-existing AD. The 158 AD cases with data for *APOE* and *ACT* genotype together with age

were analysed. There was no significant difference between the mean ages of AD patients with any *ACT* –51 genotype.

The association of the *ACT* promoter polymorphism with AD in a population without the *APOE* e4 allele could not be demonstrated, but the number of samples used in the study was too low. To conclusively demonstrate a small effect such as this, 1590 cases and an equal number of controls would have to be included to gain 80% power.

4.6 General Discussion

Previous studies looking at association of the *ACT* gene with AD have not conclusively demonstrated a link (Axelman et al., 1999; Bass et al., 1998; Didierjean et al., 1997; Durany et al., 1999; Durany et al., 1998; Egensperger et al., 1998; Ezquerra et al., 1998; Fallin et al., 1997; Haines et al., 1996; Helisalmi et al., 1997; Itabashi et al., 1998; Kamboh et al., 1998; Kamboh et al., 1997; Kamboh et al., 1995; Kim et al., 2000; Licastro et al., 2000b; Licastro et al., 1999b; McGeer and McGeer, 2001b; McIlroy et al., 2000; Meng et al., 2000; Morgan et al., 1997; Muller et al., 1996; Muramatsu et al., 1996; Murphy et al., 1997; Nacmias et al., 1998; Nacmias et al., 1996; Rodriguez Martin et al., 2000; Scacchi et al., 2001; Talbot et al., 1996; Tang et al., 2000; Tysoe et al., 1997; Wang et al., 1998; Yamanaka et al., 1998; Yoshiiwa et al., 1997; Yoshizawa et al., 1997). The signal sequence polymorphism of *ACT*, believed to influence the secretion of ACT into the extra-cellular matrix, has been reported to modify the risk of AD, but there have been contradictory reports with some studies being unable to find such a link (Licastro et al., 1999b; Nilsson et al., 2001b; Wang et al., 2002). Using the *ACT* microsatellite there was some evidence for an increased risk of AD when the microsatellite allele A10 was present, but the presence of A*polipoprotein E* allele e4 was also required. This thesis investigates the recently discovered *ACT* promoter polymorphism, and attempts to demonstrate if there is a link between this polymorphism and AD. This work was designed to prove that the *ACT* promoter polymorphism does alter the

functional activity of the promoter in various cell lines, to give an insight into how this activity might be modified, and to determine if there is a relationship between the alleles of the polymorphism and AD risk.

The functional assays performed in the course of this study have not only confirmed results of previous work, that OSM increases the activity of the *ACT* promoter in astrocytes (Kordula et al., 2000; Kordula et al., 1998), but also, that the –51bp polymorphism in the promoter region of the *ACT* gene modifies the activity of the promoter. These effects were demonstrated in hepatocytes and astrocytes, both of which have previously been shown to produce ACT (Castell et al., 1989; Yoon et al., 2002). The main difference between the two cell lines is that Hep G2 cells containing either allele show an immediate response to OSM stimulation. In the liver cells, the response to OSM is rapid, but short-lived. In astrocytes, there is a stronger response to OSM when the T allele is present than when the G allele is present, it is slower to appear, but persists longer. This is interesting in the context of AD. If neuroinflammation is a feature of AD, as is a current belief amongst some researchers, the presence of the T allele might result in an elevated synthesis of ACT, which would not be the case if this allele were absent. This in turn would mean that the balance of Aß between the form of oligomers and polymers may possibly be altered, which could result in differences in the pathology of AD between individuals, such as earlier onset, or faster progression of the disease.

The response to OSM in the mixed population T98 G cells is broadly similar to that of astrocytes. This, perhaps, could be expected, as astrocytes are present in the T98 G population. During an inflammatory response a range of signalling molecules are produced from a range of different cells. These molecules, such as cytokines, influence other cells and modulate the inflammatory response. The difference in activity between a pure astrocyte population and a mixed population containing astrocytes is important when considering neuroinflammation. *In vivo* cells do not exist in isolation, and communicate in varied and often subtle ways. This well illustrates the problems of studying a model system and trying to draw conclusions about a more complex organism. At the very least, one can conclude that the choice of tissue used to study a

functional polymorphism is critical in generating meaningful results. The response of the promoter polymorphism is different when the reporter construct is transfected into Hep G2, U373 MG or T98 G cell lines, each of which could be chosen to study such a response. There is no known tissue specific element in the regulatory region of the *ACT* gene, but these results show that the promoter response varies in different tissues anyway.

The neuronal cells, SH-SY5Y, were included as a negative control. These cells are not involved in inflammation, and RT-PCR showed that these cells were producing no ACT mRNA. This is reflected in the comparison of reporter gene response to OSM, which is non-existent. There is, however, still a difference between the response shown by each allele. Although in previous cell types the basal activity was measured over different times, and showed no significant variation (data not shown), in these cells there is no response to OSM, so that at each time point the results are consistent with the basal activity. There is still a difference between the promoter activity of each allele, and that difference is consistent: the T allele is more active than the G allele. This is the case in every cell line, regardless of the effects of cytokine stimulation.

Having demonstrated that the polymorphism does have a functional effect, in an experimental model, there are legitimate grounds to question whether this polymorphism could have an effect on the risk and pathology of AD. The limitations of this model should be considered before going on to ask these questions. What is being measured is the effect of a cytokine on a reporter gene construct, not on chromosomal DNA. There are almost certainly other features of chromosomal DNA that are not present in these experimental vectors. The vectors used are modified plasmids, structures not found in mammalian cells. The regulatory elements of the *ACT* gene are found up to 13,000bp upstream of the transcription start site, while the DNA in the vector is only 345bp, or 2% of the regulatory elements (Kordula et al., 2000). While not every base of those 13kbp may be needed for the regulation of the *ACT* gene, there are other elements that do modify the activity of the *ACT* gene. These were not included in the reporter construct used in these experiments, and their effects were therefore ignored. This is not a fatal flaw, however, as the purpose of these

experiments was to look at the promoter polymorphism. Further polymorphisms can be examined in the future, and the combined effects can be investigated then. What is more important is that the chromosomal version of the *ACT* promoter does not exist, *in vitro*, as naked DNA. Genomic DNA cannot be considered as structurally uniform; it may be methylated, or wrapped around histones, or made inaccessible by its location on coiled DNA. The structure of genomic DNA is not static, as DNA can coil and uncoil, associate and dissociate with proteins that support, repair and replicate it. The experimental vectors are at best are a highly simplified model of the true picture of the mechanisms found in the nucleus.

In addition to this, the protein produced using the reporter gene constructs is not ACT, but luciferase. ACT, as it is found in the extracellular matrix, is the product of transcription, translation and post-translational modification. Although these events may take place to create a mature luciferase protein, they will not mimic exactly what occurs to ACT in the tissues. These experiments only show what effect the polymorphism has on promoter activity; they do not and cannot show what happens beyond this. Other changes that could occur at the level of transcription, translation, and secretion of ACT, all of which may have an effect on ACT levels in the brain, or CSF, or plasma, are not revealed. For example, it may be the case that an increase in promoter activity may result in a higher level of ACT mRNA being transcribed, but if there is no increase in translation, and this may be more dependent on ribosome availability, then the level of ACT protein might not vary. Whether this is the case or not is beyond the scope of this type of experiment to determine. What is shown is that there is a functional effect of the *ACT* promoter polymorphism, and that is worthy of further investigation.

It should be added that the OSM used in these assays is human recombinant OSM, synthesised in *E. coli.* While this form of OSM does produce a demonstrable response in functional activity, it was used at nanomolar concentrations. *In vivo,* the protein is matured in eukaryotic cells, and is present in picomolar concentrations (Slaviero et al., 2003). The differences between the

experimental model and the conditions found in living tissue could be significant.

The functional data appear to fit a model of AD rather well. There is a chronic inflammatory response in the brains of AD patients which results in cytokine signalling that, in turn, provokes the secretion of acute phase proteins. These result in alterations in the homeostasis of Aß, leading to neurodegeneration, and progression of AD. This is the basis on which the polymorphism might have an influence on the risk of AD, or the age of onset. According to the genotyping data, it would appear that the *ACT* promoter polymorphism alone is not sufficient to alter the risk of developing AD. It has been suggested that the polymorphisms in the *ACT* gene associate with polymorphisms in the *apolipoprotein E* gene and that together they modulate the risk of AD (Axelman et al., 1999; Kamboh et al., 1997; Kamboh et al., 1995; Lamb et al., 1998; Licastro et al., 1999b; Nacmias et al., 1996; Potter et al., 2001; Rodriguez Martin et al., 2000; Scacchi et al., 2001; Yoshiiwa et al., 1997). In the data presented in this thesis, a strong association was shown between *APOE* genotype and AD, and the e4 allele was associated with a lower age of onset. The effect of *APOE* e4 was so strong that it was difficult to determine if any allele of the *ACT* promoter polymorphism contributed to it. In the absence of the *APOE* e4 allele, however, there was still no association between *ACT* genotype and AD, nor was there a link between lower age of onset and *ACT* genotype. The number of subjects available to test this hypothesis was too low to make a definitive statement about this. A recent study has shown an increased risk of EOAD associated with the *ACT* TT genotype in an Italian and English population independently of the presence of the *APOE* e4 allele. In existing AD cases, the *ACT* TT genotype was also associated with faster cognitive decline in patients with the *APOE* e4 allele (Licastro, *et al*, submitted 2004).

It would seem that while there is a functional effect of the *ACT* promoter polymorphism, this does not correlate with an association in AD. There are several possible reasons for this. It could be that, as mentioned above, a change in promoter activity does not necessarily result in a change in protein level in

the extracellular matrix. However, patients with the TT genotype have been shown to have a higher serum ACT level compared to the GG genotype, so this is unlikely to be true (Licastro et al., 2000b). This could be due to events that occur after the initiation of transcription. The polymorphism may well influence the rate of transcription, and this effect could be exacerbated by cytokine stimulation, but if some other process alters the result of that transcription, this effect may be irrelevant to the level of protein found in the extracellular environment. One research group has suggested that another polymorphism, the *ACT* signal sequence polymorphism, influences the rate of protein secretion (Wang et al., 2002). If the rate of protein secretion were altered independently of the rate of transcription, the effect of the promoter polymorphism on extra-cellular ACT would be diminished. Linkage analysis has shown that the higher expressing T allele of the promoter polymorphism is tightly linked to the –17A signal sequence allele (Morgan et al., 2001).

Sequence analysis of the *ACT* regulatory regions has revealed more than one SNP, and at least one, in the enhancer region, may also have functional activity, although this is yet to be confirmed. If the –51bp polymorphism is one of a number of functionally active SNPs that alters the rate of *ACT* transcription then studying one SNP in isolation may not reveal an association between one genotype and AD. Haplotype analyses, looking at a number of SNPs in diseases clearly represents the way forward, as the technology for mass screening becomes more readily available (Dennis, 2003; Gabriel et al., 2002; Koch et al., 2002; Martin et al., 2000; Patil et al., 2001; Salisbury et al., 2003). A recent hypothesis states that several sporadic forms of neurodegenerative diseases may all be due to variation in the regulatory region haplotypes, and the difference in quantity of proteins produced by such variation is an important factor in the genetic risk of these diseases (Singleton et al., 2004). An investigation into haplotypes of the *ACT* regulatory regions in AD has already begun. It is possible that the –51bp polymorphism is part of a more complex picture. If the odds ratio associated with a haplotype is higher than that of a genotype of a single polymorphism then the number of samples required for 99% power in a study will be reduced. If there is a functional effect of each SNP in regulatory

region being studied, then the cumulative effect of all the SNPs in the haplotype will be considered.

Another layer of complexity is added when the interactions of cells under inflammatory conditions are considered. Unlike in the functional model used here, cells are not subject to the influence of one cytokine at a time during inflammation (McGeer and McGeer, 1995). A number of cytokines as well as other signalling molecules are involved. While the one-cytokine model used in the functional assays might produce a certain response in cultured tissues, the response of cells bathed in a cocktail of cytokines, in contact with different cell types, in a living organism is almost certain to be different. It has been shown that IL-1ß can inhibit activation of the a2-macroglobulin promoter by IL-6 (Bode et al., 2001). IL-1ß activates NF-?B, which can competitively bind to STAT responsive elements, preventing binding by STAT3. The activation of the *ACT* enhancer by IL-6 can also be inhibited by NF-?B, mediated by IL-1ß (Bode et al., 2001). An NF-?B consensus site is essential for IL-6-induced transcription (Bode et al., 2001) and it may be the case that the interplay between IL-1ß inhibition and IL-6 activation is more important than the effect of a single cytokine when considering the regulation of the *ACT* gene. If AD does result in a state of chronic neuroinflammation, it is possible that cells undergo changes to adapt to pathological conditions, resulting in a different response to stimulation in pathological tissues than healthy tissues (Black, 2002; Slaviero et al., 2003). Conversely, the effect of the *ACT* promoter polymorphism could influence other genes that might have an effect in AD. ACT is known to have DNA binding properties (Kalsheker, 1996), and while the ability of ACT to enter the nucleus and have a direct effect on DNA is unproven, ACT is known to have more properties than simply inhibiting serine proteases. Complexed ACT is removed from the circulation by receptor-mediated pathways, where it is presumably degraded. Is it possible that the DNA-binding domain once liberated from ACT-protein complexes could enter the nucleus? Preliminary data suggest that several genes in cells incubated with ACT and Aß are activated in the presence of these proteins together, but not separately (Baker and Morgan, unpublished data). This would again suggest

that the *ACT* promoter polymorphism is one SNP in a chain of genetic alterations needed to provoke AD. This situation is similar to that seen with the involvement of several proto-oncogenes in cancers. An environmental effect, coupled with several polymorphisms is required for the development of a disease. In the case of AD it could be that an initial trauma would trigger an inflammatory reaction, which, when certain alleles are present in different genes, would lead to the development of self-perpetuating neurodegenerative state. As mentioned in the introduction, a large number of genes have been investigated and shown to have an influence on the risk of AD. Some of these genes could very well be involved in the aetiology of AD, and although no single combination of polymorphisms in these genes may be needed to produce AD, a combination of some may be necessary, in some cases, to trigger this disease. The most obvious example of this is the *apolipoprotein E* polymorphism. It has been shown that while the e4 allele does influence the risk of AD, it is neither necessary nor sufficient to cause AD (Laws et al., 2003). This is compatible with a model where a number of different polymorphisms have a small effect on the likelihood of developing AD. The more "proto-AD genes" present, the greater the risk, with *APOE* e4 having the greatest effect of all these polymorphisms. This would also suggest the possibility that there is no one Alzheimer's disease, but a range of sub-types of Alzheimer's diseases, each having the same symptoms, but being caused by different polymorphisms. These subtypes could be more or less aggressive, depending on which genes were involved; the age of onset could vary, depending on when the disease is triggered and which genes are involved. The inheritance of AD would follow a non-Mendelian model, as several genes are necessary in this scenario. This is, of course, what is seen in AD, with sporadic and familial forms of the disease, as well as early- and late-onset subgroups. The familial form of AD is dependent on inheriting disease-associated forms of one of three genes: the *amyloid precursor protein* gene, *presenilin 1* and *presenilin 2*, while the genetic cause of the sporadic form, if any, is unknown (Rocchi et al., 2003).

If sporadic AD is a polygenic, multifactorial disease, it could alter the prospect for developing treatments. While there may be an underlying basis for the

aetiology, for instance, every polymorphism could alter the inflammatory response, then a treatment that targets the abnormal inflammatory response could be effective in every form of AD. If there is no commonality between subgroups, other than the end results, it may be difficult to design one treatment that is effective in each case. In this case, the pathway that leads to senile plaques might be different to that which causes tau pathologies, for instance. In some cases a treatment might prevent amyloidosis, but not avert development of tauopathies. Recent work on the aetiology of AD suggests this might not be the case, as it is starting to look as though there is a common pathway that produces both amyloid plaques and tau fibrilisation (Mudher and Lovestone, 2002). Determining which gene pathways are involved in AD, and to what extent, and how they influence the progression of AD is still likely to be extremely important to reach the goal of a cure, or even improved treatment of AD.

Whether or not these hypotheses are true, it is clear that further work is needed before a definitive conclusion can be made on the involvement of ACT in AD. Some of this work has already begun.

A search for new polymorphisms in the regulatory regions of the *ACT* gene has started, looking at both the 5' and 3' flanking regions of the gene. Once found, and some have already been discovered, they must be investigated to determine functional activity. Once this has been done, genotype and haplotype analyses can be made, to see if any are associated with AD. It is more likely that a group of functionally active SNPs will show an association with AD than one SNP considered alone (Martin et al., 2000). Even a SNP that has a large functional effect may be modulated by alleles at other loci, which means the overall functional effect of the haplotype is greater than the effect of the individual genotype. The "signal" of an association between alleles of any single SNP and AD may well be lost in the "background noise" of the effect of the other SNPs being investigated on an individual basis.

Following on from this, the post-transcriptional events will need investigation. It remains to be seen whether changes in DNA-protein binding results in changes in mRNA levels. The level of mRNA needs to be measured, for instance by quantitative RT-PCR and compared to either the *ACT* –51bp

polymorphism genotype or the haplotype of a range of *ACT* regulatory SNPs. If there is no difference between mRNA level, there could be differences in mRNA length, due to differences in splicing, which might alter the mRNA stability, although there is no evidence yet for alternative splicing in the *ACT* gene. If the mRNA half-life varies, this may have a greater effect on translation than the concentration of mRNA, for instance, if a particular form of mRNA is unlikely to survive long enough to reach a ribosome, or if it can be translated repeatedly. It is possible to determine mRNA stability and this might be an interesting line of research for the future.

As well as post-transcriptional events, there are post-translational events to be considered. Most proteins undergo modifications before becoming mature proteins. While it is implausible that a SNP in the non-coding, regulatory regions of the gene would influence post-translational modification, a regulatory SNP could be in linkage disequilibrium with a coding SNP. The –51bp polymorphism, for instance, is in almost complete linkage disequilibrium with the signal sequence polymorphism found in exon one of the *ACT* gene (Morgan et al., 2001). While the amino acids coded for by this exon are not present in the mature protein, it does influence the secretion of the protein. If there were other SNPs that do result in a modified form of the mature protein, which are in partial linkage disequilibrium with a functionally active regulatory SNP, the effects on AD would be worth investigating.

Changes in *ACT* activity and function alone do not cause AD. It would seem that ß-amyloid is a key protein in the development of AD, and there are many other proteins that are involved in the processing of amyloid precursor protein to Aß, and still more proteins that have an effect on the *APP* gene. In the case of *APOE*, there is a demonstrable effect on AD risk, but there are many other candidate proteins, and therefore genes, which may modify the risk of AD (Rocchi et al., 2003). With recent improvements in technology to allow rapid sequencing and comparison of sequences, it is easier than ever to search for SNPs in candidate genes for AD (Chandra et al., 1983; De Quervain et al., 2004; Sun et al., 2000). The growing availability and improvements of micro-arrays means that it is becoming easier to test for functional activity of such

polymorphisms in the presence of several cytokines. Similarly, the effects of novel molecules, such as Aß, on gene regulation can be investigated with greater ease than before. Where, only a few years ago, investigating the functional effects of one cytokine on one gene was a time consuming affair, studying the expression of an entire gene pathway can now be achieved in the fraction of the time (Lee et al., 2000). As more data are obtained, the computational models for predicting regulatory regions from sequence data will become more accurate and powerful (Rodriguez-Antona et al., 2003; Schlotterer, 2003; Segal et al., 2003). This will make it easier to find regulatory regions to test, and it may one day be possible to predict the functional activity of regulatory region of DNA by computer analysis alone, although it would be advisable to confirm this experimentally. Until that day arrives, new ways of studying the genetic involvement of AD are becoming available.

A transgenic strain of *Caenorhabdis elegans* has been developed which expresses human Aß (Link et al., 2003). The *C. elegans* model has several advantages over previous model organisms, such as rodents and primates. It is a simple, multi-cellular organism, with a short lifespan. The lifespan of *C. elegans* is considerably shorter than mice, which means that the effects of Aß over a lifetime can be studied in a shorter time than previously. It is also easier to produce and house large numbers of offspring, so that experimental groups can be larger, generating more reliable data, at a lower cost. Finally, while more complex than tissue culture, the *C. elegans* approach is far simpler than mouse models, so there are fewer interrelated genes and environmental factors to take into account with this model. This means that the effect of one gene on the organism may be demonstrated more clearly in the worm than the mouse. If a transgenic worm can be produced that expresses Aß, a worm that produces ACT is also possible, as has been achieved with mouse models (Nilsson et al., 2001a). Genes can then be studied in relation to each other, and their effects demonstrated more clearly than before, and more rapidly.

The results of these experiments have shown that a functional effect of a polymorphism in the regulatory region of the *ACT* gene might have a bearing on

AD. While they do not demonstrate that this polymorphism does influence the risk of AD, it is one small part of a much greater, complex web of cause and effect. Newer technology is emerging that will in future make it far easier to study more of these integrated networks. The results obtained in this study have not closed the door on the possibility that this polymorphism is a risk factor for AD, but neither has this been demonstrated. What has been shown indicates that there is still a need for further work that might demonstrate some role for *ACT* in AD, although this could be one factor amongst many that contribute to the disease process. A recent report has shown that, in transgenic mouse models, the process of Aß fibrilisation is more neurotoxic than monomeric, oligomeric or fibrillar Aß (Nilsson et al.), possibly due to the formation of a neurotoxic intermediary between the different forms of Aß. ACT and ApoE are pathological chaperones critical to the process of Aß fibrilisation.

4.7 Conclusions

These experiments have confirmed previous observations that the *ACT* promoter responds to oncostatin M stimulation. In addition, they have shown that the T/G polymorphism located in the promoter region at –51bp is also functional. The T allele of this polymorphism is responsible for increased activity of the promoter, both under basal conditions and when stimulated by OSM. The G allele is functionally less active, but responds to OSM in the same fashion as the T allele. The response of the promoter polymorphism varies between different cell lines.

The polymorphism appears to alter the binding afinity of TFIIB to the ACT promoter with the T allele having a greater afinity for this transcription initiation complex subunit than the G allele.

No genetic association between the *ACT* –51bp polymorphism and late onset AD was detected, and there was no apparent effect of this polymorphism on influencing age of onset of AD in the two UK populations studied. However, the small size of the study means that the question of any risk associated with

the *ACT* –51 promoter polymorphism and early onset AD remains to be established.

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6 APPENDIX ONE

6.1 The 5' Flanking Sequence of *ACT*

CTGAGGTGGGCAGATCACGAGGTCAGGAGGTCGAGACCAGTCCGGCCGACAAGGTGAAACCCTG

F1

TCTCTACGAAAAATACAAAAATTANCTGGGCGTGGTGGTGTGTGCCTGTAGTCCCAGCTACTCG

GGAGTCTGAGGCAGAAAGTTGCTTGAACCCGGGAGTCAGAGGTTGCAGTGAGCTGAGATCACGC

CACTGCACTCCAGCCTGGGCAACAAAGCAAGACTCTGTCTCAAAATAAAAATAATAAATAAAAA

F2

GAAATAAAAAAGAAATATACCCACATGTTAGCTGGGGTCTTCTCTGGGTAGTAAAGTGCTGGGG

GATATTTTCCAAAGTCCTTCTTTACATTCTCTGAGTTTTTCCATGTTCTTCAATGAGTATTTAA

TAAGCAGATAAAAACTAATACAACAAAGGATTTTTTCTGTGTGCTTTTTTGACCTTTGGAGGAA

GAGATTAGAGCTAGTCCCATAACCAGGTTATTTGAGTAGGTCTAATAAGCCCGTATTACCAGAA

F5

ATTATCATCTGGTCATTTCCAGTCCGAGAACAGAACACTTGGTTGTCCTGGCATTTCCCAAGCA

R2 R5 +1

GGGGGAGGAGTTCTCTGCAGGAATAAATAAGCCTCAGCATTCATGAAAATCCACTACTCCAGAC

TATA box

R1

AGACGGCTTTGGAATCCACCAGCTACATCCAGCTCCCTGAGGCAGGTAATCCATGATGTTTTAC

ATCCTGGGAGCGGAGGAATCTGTTTTTCCAGGAGAGTTTTAGGCAGCAGCCTGGAGTGTGTGGA

GTGTGAGGGGTAAGCAGAG

Figure 6.1.1: The 5' Region of the *a_1-antichymotrypsin* gene. Highlighted sequence shows primers and the TATA box, +1 is the transcription start site, G is the -51 polymorphism.

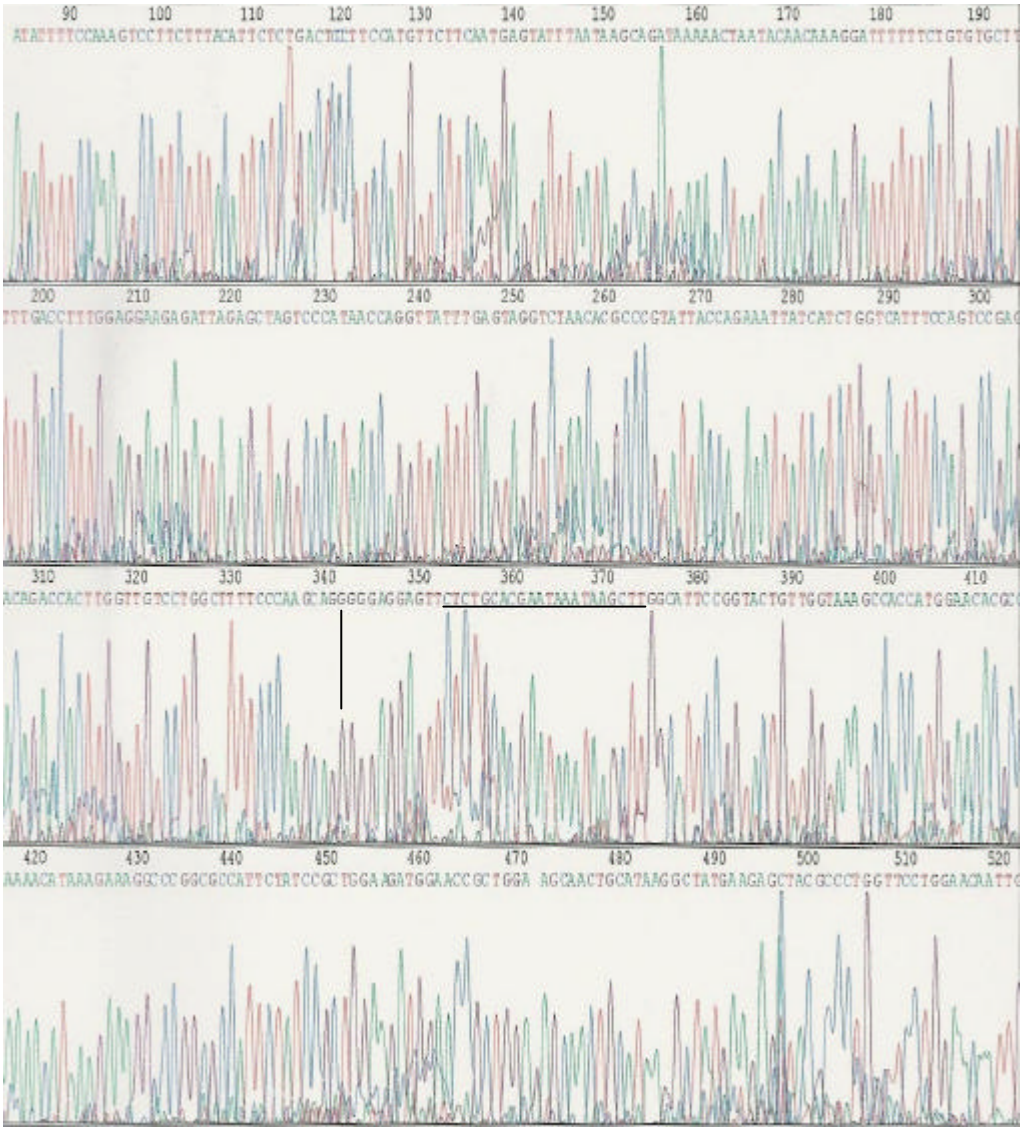

Figure 6.1.2: The 330bp *ACT* promoter region insert in pGL3E construct. Arrow shows the position of the G allele, and underlined bases shows the position of the R2 primer.

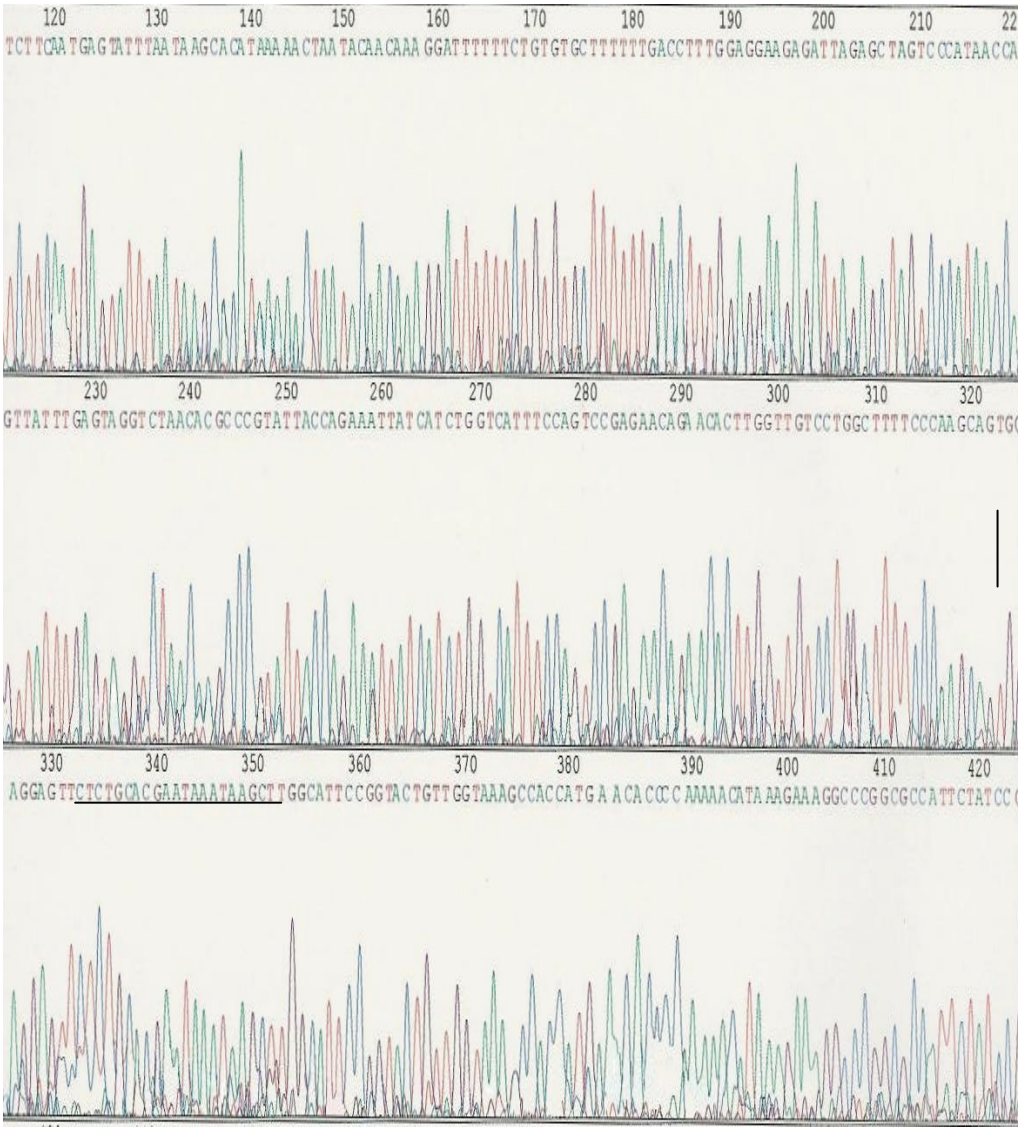

Figure 6.1.3 The 330bp ACT promoter region insert in pGL3E construct. Arrow shows the position of the T allele, and underlined bases shows the position of the R2 primer.

7 APPENDIX TWO

7.1 Apolipoprotein E and a_1-Antichymotrypsin Genotyping Data

APOE	*ACT*	Confirmed AD	Probable AD	Total AD	Control	Total
	GG	26	6	32	51	83
e3/e3	GT	54	13	67	96	163
	TT	31	8	39	49	88
	GG	0	0	0	0	0
e2/e2	GT	0	1	1	1	2
	TT	0	0	0	1	1
	GG	26	6	32	10	42
e3/e4	GT	78	15	93	30	123
	TT	33	12	45	26	71
	GG	4	1	5	16	21
e2/e3	GT	7	3	10	18	28
	TT	3	3	6	12	18
	GG	9	2	11	1	12
e4/e4	GT	16	7	23	2	25
	TT	4	2	6	0	6
	GG	5	1	6	1	7
e2/e4	GT	8	1	9	4	13
	TT	2	0	2	3	5

Table 7.1.1: Frequencies of *Apolipoprotein E* and *a_1-antichymotrypsin* genotypes in AD and control subjects.

APOE	*ACT*	Mean Age	n
	GG	78.83	32
e3/e3	GT	76.63	66
	TT	77.48	39
	GG	74.28	31
e3/e4	GT	75.06	91
	TT	74.26	43
	GG	76.37	5
e2/e3	GT	78.20	9
	TT	78.81	6
	GG	74.69	11
e4/e4	GT	70.37	23
	TT	67.99	6
	GG	75.92	6
e2/e4	GT	84.80	9
	TT	82.26	2
	GG	0	0
e2/e2	GT	78.57	1
	TT	0	0

Table 7.1.2: Mean Age of AD Onset in *Apolipoprotein E* and *a_1-Antichymotrypsin* Genotypes