Mechanisms Controlling the Unique Phenotype of Brain Endothelium

Thesis

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Mechanisms Controlling the Unique Phenotype of Brain Endothelium

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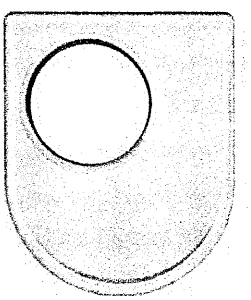
A thesis submitted for the degree of Doctor of Philosophy



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ABSTRACT

Endothelial cells from different tissues differ widely in the expression of junctional proteins like occludin and transporters like the transferrin receptor. The mechanism(s) responsible for the differential expression of these proteins is not known. In this project we have studied how the occludin promoter interacts with nuclear transcription factors (TFs) from brain and non-brain endothelium. EMSA data indicates the TFs Sp1, Sp3 and YY1 are responsible for the specific binding to the occludin promoter in hCMEC/D3 cells, a transformed brain endothelial cell line. Using ChIP assays, we confirmed the interaction between these three transcription factors and DNA as these complexes were active in live cultured cells from transformed and primary brain endothelium. We investigated the expression and localisation of Sp1, Sp3 and YY1 in these cells and compared with lung endothelial cells and report the specific association of the TFs Sp3 and YY1 in brain endothelium which is absent in non brain endothelium. In addition, we have compared the activity of the occludin promoter in hCMEC/D3 cell to that in primary human dermal and lung endothelial cells by transfection with reporter vectors under the control of the full length and fragments of the occludin promoter. Our work identifies a group of transcription factors present in brain endothelium which may regulate the expression of the tight junction protein occludin. We propose a model whereby the TF Sp3 is necessary for the transcription of the occludin promoter in brain endothelium and YY1 negatively regulates promoter activity in non-brain endothelium by controlling access of Sp3 to the initiation sites on the occludin promoter.

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Abbreviations

AA	Amino Acid
AP1	Activator Protein1
BBB	Blood Brain Barrier
FGF2	Basic Fibroblast Growth Factor
BMEC	Bone Marrow Endothelial Cells
BSA	Bovine Serum Albumin
CBP	CREB Binding Protein
c/EBP	CCAAT/Enhancer Binding Protein
CCAAT	Box at ~ -70 bp from transcription start site in eukaryotic promoters
ChIP	Chromatin Immunoprecipitation Assay
CTF/NF1	CCAAT box Binding Transcription Factor/Nuclear Factor1
CVOs	Circumventricular Organs
DARC	Duffy Receptor for Chemokines
DlgA	Drosophila disc large tumour suppressor
DMVEC	Dermal Microvascular Endothelial Cells
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ELL	RNA Polymerase II Elongation Factor
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Signal Regulated Kinase
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GATA	Globin Transcription Factor
Glut 1	Glucose 1 Transporter
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
G-protein	Guanine Nucleotide Binding Protein
GSK-3β	Glycogen Synthase Kinase 3-beta
GTPases	Guanine Triphosphate Hydrolase Enzymes
HAT	Histone Acetyl Transferases
HDAC	Histone Deacetylases
HBEC	Human Brain Endothelial Cells
HBSS	Hanks Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HUVEC	Human Umbilical Vein Endothelial Cells
IAEC	Iliac Artery Endothelial Cells
ICAM-2	Intercellular Adhesion Molecule 2
IFN-□	Interferon-Gamma
Ig	Immunoglobulin
IL	Interleukin
JNK	Jun N-terminal Kinase
LFA-1	Leukocyte Functional Antigen-1
LMVEC	Lung Microvascular Endothelial Cells

LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
MAdCAM	Mucosal Addressin cell adhesion molecule
MAGuK	Membrane Associated Guanylate Kinases
MHC	Major Histocompatibility Complex
MUPP1	Multiple PDZ Domain Containing Protein
NC2	Negative Cofactor 2
NF1	Nuclear Factor 1
NF-Y	Nuclear Factor-Y
PALS1	Protein Associated with Lin Seven 1
PATJ	PALS1-Associated TJ Protein
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDZ	PSD95 DlgA ZO1
PI3K	Phosphatidyl Inositol 3-Kinase
aPKC	Atypical protein Kinase C
PKC	Protein Kinase C
PMP22/EMP	Peripheral Myelin Protein-22/Epithelial Membrane Protein
PTF	Plasma Thromboplastin Factor
PSD95	Post Synaptic Density Protein
Ser	Serine
SH3	Src homology 3
TER	Transendothelial Electrical Resistance
TESS	Transcriptional Element Search Software
TGF	Transforming Growth Factor
TGFβR1	Transforming Growth Factor beta Receptor 1
Thr	Threonine
TNF	Tumour Necrosis Factor
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand factor (factor VIII)
ZnF	Zinc Finger
ZO1	Zona Occludens 1
ZONAB	ZO1 Associated Y-Box Factor

Chapter 1

Introduction

1 Introduction

1.1 Endothelium

Endothelial cells which line the inside layer of blood vessels and the lymphatic system form the endothelium. This layer of cells constitutes all physiological communication between the circulation and the proximal tissue. Functions of the endothelium include maintenance of homeostasis, exchange of nutrients, trafficking of cells during an inflammatory response, regulation of vessel permeability, control of vasomotor tone and angiogenesis (Jaffe 1985; Nawroth, Kisiel et al. 1985; Simionescu and Simionescu 1986).

Structural differences in endothelium are associated with corresponding variations in capillary permeability (Fig 1.1) (Risau 1995). Throughout much of the body, the endothelial lining is continuous and capillaries with continuous endothelium are found in the lungs, muscle and central nervous system. In the brain the specialised blood brain barrier is the most restrictive of all continuous endothelia in the body and limits paracellular and transcellular traffic to and from the brain. Nutrients and molecules either diffuse through plasma membranes or via specific membrane transporters (Rubin and Staddon 1999).

However, the blood brain barrier is absent in the choroid plexuses and in the following organs: the pineal gland, subfornical organ, organum vasculosum of the lamina terminalis, paraventricular organ, median eminence, neurohypophysis, subcommissural organ and the area postrema. These structures are located around the third and fourth cerebral ventricles and commonly referred to as the

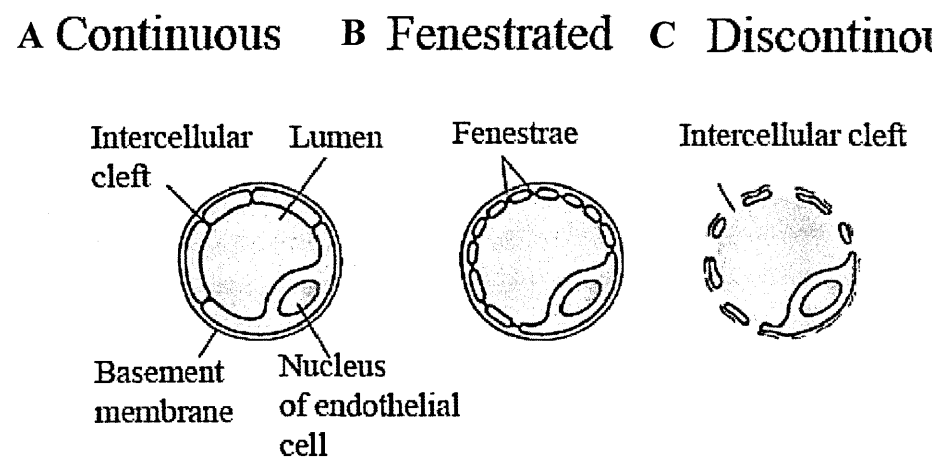
circumventricular organs (CVOs) (Joly, Osorio et al. 2007). CVOs possess a specialised type of endothelium that is continuous but is characterised by circular transcellular gaps referred to as fenestrae. Presence of fenestrae enables important communication between the brain and the blood because fenestrated capillaries are relatively more permeable to unrestricted passage of high-molecular weight and polar substances.

Fenestrated capillaries resemble continuous endothelium in having an uninterrupted basement membrane but are characterised by regional thinning leading to the presence of circular transcellular gaps referred to as fenestrations (Stan 2007). The diameter of the fenestrae ranges from 60-80 nm and are readily permeable to hydrophilic molecules of low molecular weight and hence their location in tissues with high rates of fluid exchange such as the sinusoids of the liver, small intestine, glomeruli of the kidney, and in most endocrine glands. In the kidney and choroid plexus, signalling from the epithelial layer which is always in close proximity to the endothelium is believed to be important in the differentiation and maintenance of the fenestrae. High constitutive expression of VEGF which is known to induce permeability is highly expressed in epithelial layers and therefore could induce the phenotype of the fenestrated endothelium (Risau 1998).

Discontinuous endothelium found in liver, spleen and bone marrow has wide intercellular spaces, approximately 80-200 nm in width, and with no basement membrane. This allows for almost unrestricted transport of molecules from interstitium to the capillary lumen (Aird 2007).

Freeze-fracture electron microscopy analyses have elucidated the differences between continuous and discontinuous endothelia. Tight junctional structures in the latter do not form a continuous barrier but as the term suggests a discontinuous series of stranded clusters. In addition, the spatial distribution of tight junction components within the endothelial cell membrane is different. Components of the tight junction are associated with either the internal (P-face) or external (E-face) membrane leaflets (Lane, Reese et al. 1992). Tight and leaky junctions are characterised by components with associations at the P-face and E-face respectively (Schneeberger and Lynch 2004). In the endothelial cells of the blood brain barrier, it has been found using immunogold labelling and freeze fracture technique that 57% of the particles are within the internal P-face and about 44% of the particles associate with the external leaflet (Kniesel, Risau et al. 1996) . This distribution contributes to the integrity of the blood brain barrier. In pathology, increased permeability is associated with a shift of components and enzymatic activities from the P-face to the E-face (Wolburg, Neuhaus et al. 1994). The molecular mechanisms involved in the gain and loss of P-face association of tight junctions are unknown.

Figure 1-1 Structural Features of Adult Microvascular Endothelial Phenotypes



(Cleaver and Melton 2003)

(a) Continuous capillaries have no openings in their walls and are lined continuously with the endothelial cell body.

(b) Fenestrated capillaries have small openings, called fenestrae, which are covered by a small, nonmembranous, permeable diaphragm, and allow the rapid passage of macromolecules. The basement membrane of endothelial cells is continuous over the fenestrae.

(c) Discontinuous capillaries, also called sinusoids, have a large lumen, many fenestrations with no diaphragm and a discontinuous or absent basal lamina (Cleaver and Melton 2003).

1.2 Blood Brain Barrier (BBB)

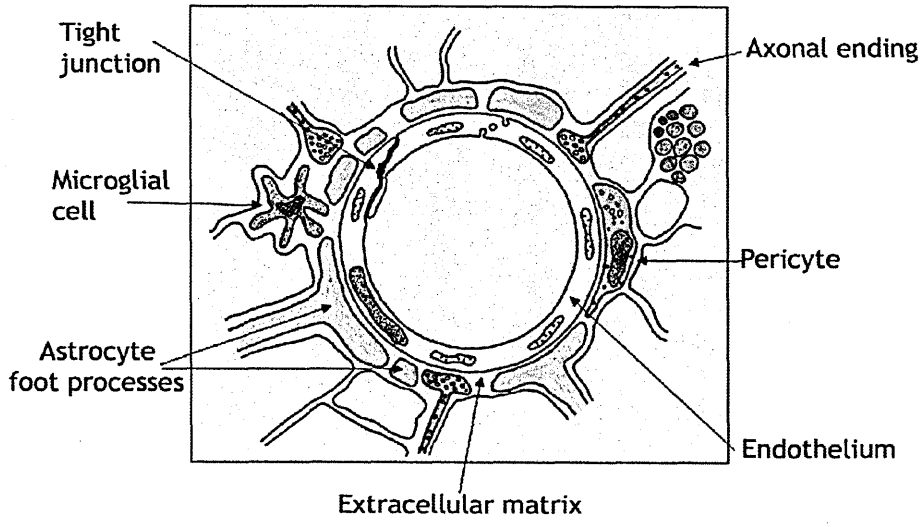
The blood-brain barrier (BBB) is formed by a complex network of endothelial cells, astroglia, pericytes, perivascular macrophages, and a basal lamina (Fig 1.3). It was first reported in 1885 by the German pathologist Ehrlich (Ehrlich 1885) and subsequent electron microscopy studies showed that intravenous administered electron dense particles, such as horseradish peroxidase, are prevented from entering the brain by the capillary endothelial cells (Reese and Karnovsky 1967).

Brain endothelial cells differ from endothelial cells in the periphery by forming continuous tight junctions (TJ) and lack fenestrations except in the circumventricular organs. TJs severely restrict passage of water-soluble compounds, including polar drugs and are the structural basis for the paracellular impermeability and high electrical resistance (Reese and Karnovsky 1967; Rubin and Staddon 1999). In comparison with transendothelial electrical resistance (TEER) values of 2–20 $\Omega.cm^2$ in peripheral capillaries, brain capillaries exhibit TEER values exceeding 1,000 $\Omega.cm^2$. In addition, the presence of very few endocytic vesicles (Sage, Wilson et al. 1998) limits transcellular transport and expression of specific transport systems and carrier molecules restrict entry to only required nutrients (Wolburg and Lippoldt 2002; Begley 2004). CNS endothelium is also characterised by the presence of increased mitochondrial content which contributes to about 8-11% of the endothelial cell volume in contrast to 2-5% in non-CNS endothelial cells (Oldendorf, Cornford et al. 1977).

Structurally, the basic unit of the blood brain barrier is composed of endothelial cells which completely surround the lumen of a capillary. Pericytes associate with

the endothelial cells at the abluminal surface and the cells are embedded in a common basement membrane which is about 30 to 40 nm thick and composed of extracellular matrix proteins including laminins (8 and 10), collagen type IV, heparan sulphate proteoglycans and fibronectin (Farkas and Luiten 2001). The basement membrane provides both mechanical support and a barrier function. The astrocyte end feet ensheathe the capillary and the plasma membranes of the foot processes are closely connected to the basement membrane.

Figure 1-2 Representation of the Blood Brain Barrier Unit



The cerebral endothelial cells form tight junctions at their margins where they meet. Pericytes are distributed discontinuously along the length of the cerebral capillaries and partially surround the endothelium. Foot processes from astrocytes form a network fully surrounding the capillaries. Axons from neurons are also present close to the endothelial cells and contain vasoactive neurotransmitters and peptides. Microglia and perivascular macrophages are derived from systemic circulating monocytes and form the resident immuno competent cells of the brain (Begley 2004).

1.2.1 Development of the Blood Brain Barrier

In rodents, brain capillaries differentiate to blood brain barrier endothelium during late embryonic and early postnatal development. The increase in vessel impermeability to protein correlates with a conformational change of the TJs. There is an increasing association of tight junctional particles with the P-face membrane leaflet from embryonic day 13 to postnatal day 125 (Robertson, Du Bois et al. 1985). It should be remembered that tight junction components in the periphery are usually associated with the E-face *in vivo*.

1.2.1.1 Role of Astrocytes

Astrocytes, whose processes form end-feet that surround brain microvessels (Janzer and Raff 1987; Abbott, Ronnback et al. 2006) have been shown to play important roles in conferring BBB properties to the endothelial cells. The development of complex tight junctions, specialized enzyme systems, expression and asymmetrical localization of transporters in the endothelial cells has been attributed to astrocytic signalling (DeBault and Cancilla 1980; Boado, Wang et al. 1994; Dehouck, Dehouck et al. 1994; Roux, Durieu-Trautmann et al. 1994; Wolburg, Neuhaus et al. 1994; Hayashi, Nomura et al. 1997; Rist, Romero et al. 1997). The astrocytic end feet display a high density of purinergic P2Y receptors, K+ channels and the water-channel protein aquaporin-4, indicating a possible role in the regulation of water permeability in the brain (Simard, Arcuino et al. 2003).

Transplanted astrocytes *in vivo* (Janzer and Raff 1987), and astrocytes *in vitro* (Hayashi, Nomura et al. 1997) induce BBB properties in adjacent non-neural endothelial cells from different species. Evidence supports the importance of

astrocytes in mediating expression and localisation of markers including p-glycoprotein, GLUT1 and γ-glutamyl transpeptidase (γ-GTP) (Abbott, Ronnback et al. 2006). The parenchymal basement membrane, one of the two basement membranes associated with the blood brain barrier, is produced by astrocytes. Two components namely agrin (Barber and Lieth 1997) and β2 laminin (Hunter, Llinas et al. 1992) in this membrane are important in the terminal development of the blood brain barrier.

In addition, astrocytes secrete growth factors like transforming growth factor-β (TGF-β), angiopoetin 1 (ANG1), glial derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) which influence development of the barrier phenotype. In 2003, Lee and colleagues identified a single gene SSeCKS which is upregulated in astrocytes in response to oxygen tension. Ectopic overexpression in rat cerebral cortex astrocytes led to downregulation of VEGF and increased expression of ANG1. VEGF is a known mediator of increased permeability and ANG1 mediates angiogenesis and vessel maturation (Ferrara, Gerber et al. 2003). Supernatants from SSeCKS expressing cells also upregulated the expression of ZO1 and claudin 1, constituents of the TJs of the blood brain barrier. Hence it is clear that the gene product of SSeCKS is instrumental in the development and maturation of the blood brain barrier (Lee, Kim et al. 2003).

Bidirectional astrocyte–endothelial calcium signalling has been demonstrated in co-cultures employing astrocytes and endothelial cells (Braet, Paemeleire et al. 2001) and in brain slices (Zonta, Angulo et al. 2003). The calcium oscillations seen in astrocytes have been shown to be important for vasodilation induced by

neuronal activity (Zonta, Angulo et al. 2003). Hence it appears that the complex interactions between cerebral endothelial cells, astrocytes and neurons regulate circulation in the CNS.

However the first known markers of brain endothelial cells and the accumulation of p-glycoprotein appear even before astrocytes is present (Qin and Sato 1995). So it seems unlikely that all the properties are acquired simultaneously. It also appears that astrocytes are important in maintaining the barrier properties in the adult although short term loss of astrocytic end feet does not affect the function of the blood brain barrier (Krum and Rosenstein 1993). Some of the BBB-inducing properties of astrocytes are mimicked by corticosteroids (Romero, Radewicz et al. 2003).

1.2.1.2 Role of Pericytes

Pericytes are present in the basement membrane formed by the capillary endothelial cells and astrocytes (Fig. 1.3) and play important role in conferring BBB properties to the cerebral endothelial cells (Lai and Kuo 2005). Pericytes possess long cytoplasmic extensions and along with gap junctions are involved in maintaining contact with endothelial cells. During embryonic development, the ligand PDGF-B required for angiogenesis is expressed by capillary endothelial cells, but its receptor PDGFRβ is expressed on pericytes (Betsholtz, Lindblom et al. 2004). Pericytes induce endothelial differentiation and growth arrest (Sims 2000; Gerhardt and Betsholtz 2003). Pericytes in the brain can function as macrophages and also exhibit phagocytic activity (Thomas 1999). They have also been shown to protect the integrity of the blood brain barrier in hypoxia *in vitro*

(Hayashi, Nakao et al. 2004).

1.2.1.3 Role of Neurons

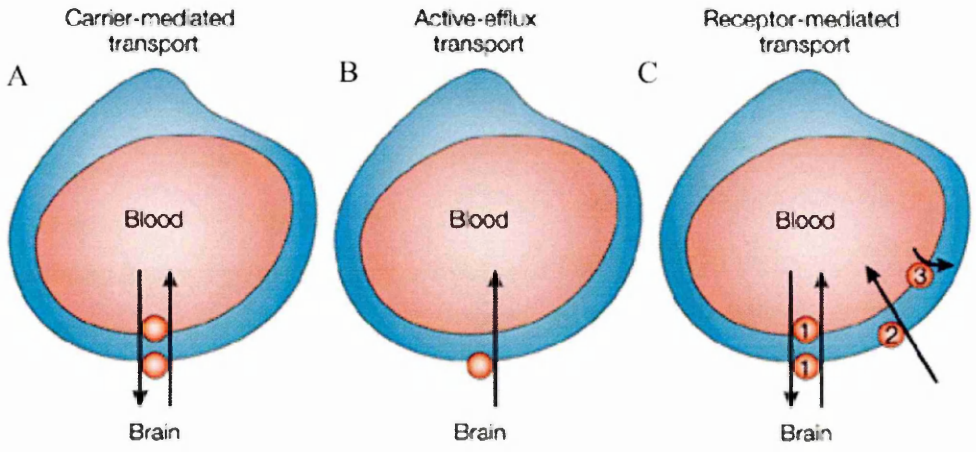
It is possible that non-astrocytic factors are also important as different kinds of nerve endings are also associated with brain capillaries (Kobayashi, Magnoni et al. 1985; Petty and Lo 2002). In the developed brain, groups have demonstrated roles for noradrenergic (Cohen, Molinatti et al. 1997), serotonergic (Cohen, Bonvento et al. 1996) and GABAnergic (Vaucher, Tong et al. 2000) neurons in the activation of endothelial cells. Inhibition of noradrenergic contacts to the CNS vasculature increases susceptibility of the BBB to hypertension (Ben-Menachem, Johansson et al. 1982). Neurons have been shown to be as effective as astrocytes in inducing barrier properties *in vitro.* In 2000, Savettieri and colleagues investigated the role of neurons in the induction of occludin, a TJ protein and showed the expression and localisation of occludin is dependent on presence of neurons in the culture (Savettieri, Di Liegro et al. 2000). It can be said that neurons are critical in maintaining the phenotype of the established blood brain barrier but their importance in its formation is yet to be clearly demonstrated. The same group also has also shown extracellular matrix protein collagen IV influences occludin mRNA expression but not the protein levels (Savettieri, Di Liegro et al. 2000).

1.2.1.4 Molecular Characteristics of the Blood Brain Barrier

1.2.1.5 Transport Systems

The continuous tight junctions between the cerebral endothelial cells abrogate access to polar substances unless they are transferred by specific transport pathways. At the BBB, various membrane transporters on the luminal and abluminal membranes of the capillary endothelium function to regulate the entry of essential molecules into the brain, as well as effluxing harmful substances and waste products from the brain into the blood (Fig. 1-4) (Abbott and Romero 1996). Transport systems (Fig.1-4) at the BBB include carrier-mediated transport (CMT, Table 1.1), active efflux transport (AET, Table 1.2) and receptor-mediated transport (RMT) (Begley 2004).

Figure 1-3 Three Classes of BBB Transport Systems



(Pardridge 2002)

CMT systems (Table 1.1, Fig. 1-4) CMT systems are expressed on both the

luminal and abluminal membranes of the capillary endothelium, so that transport in either the blood-to-brain or brain-to-blood direction can be mediated (e.g. amino acid transporters).

AET systems (Table 1.2, Fig. 1-4) are responsible for the transport of substrates usually from the brain to blood (e.g. P-glycoprotein).

The RMT systems (Fig. 1-4) are responsible for the transport of endogenous molecules. The transferrin receptor depicted as system 1 in panel C, is a bidirectional system that transports transferrin in either the blood-to-brain or brain-to-blood direction. The blood–brain barrier Fc receptor which is depicted as system 2 in panel C selectively transports immunoglobulin G molecules in the brain-to-blood direction only. The type I scavenger receptor which is depicted as system 3 in panel C, can only mediate the uptake of circulating ligand, such as acetylated low-density lipoprotein, into the brain capillary endothelial compartment, without transcytosis or release into brain. (Pardridge 2002)

Table 1-1 CMT at the Blood Brain Barrier

Molecule	Transport
Nucleosides/nucleic acid transport	Nucleosides/nucleic acid
transport	
System ASC/B^{0+}	L-Ala/L-Ser/L-Cys & others
System B^{o+}	Basic AAs
Choline transport	Choline
GLUT1	D-Glucose, Ascorbic acid
LAT/4F2hc (system L)	Large neutral AA's
CAT1 (system y^+)	Cationic AA's
EAAT1, 2, 3	Anionic AA's
System A	Small neutral AA's
MCT1	L-Lactate/monocarboxylates

Table 1-2 AET at the Blood Brain Barrier

Gene	Protein	Function
ABCB1 (MDR1)	P-glycoprotein	multidrug resistance
ABCC1 (MRP1)	MDR1-related protein	drug resistance
ABCC2 (MRP2)	cMOAT	organic anion efflux
ABCC3 (MRP3)	MRP3	drug resistance
ABCC4 (MRP4)	MRP4	nucleoside transport
ABCC5 (MRP5)	MRP5	nucleoside transport
ABCG2 (BCRP)	breast cancer resistance	drug resistance

1.2.2 The Blood Brain Barrier in Disease

The blood brain barrier is metabolically active and hence it is possible to modulate the gene expression of endothelial cells and subsequent changes in function and permeability across the barrier. Transient opening of the barrier occurs at times to allow for passage of other blood borne substances into the brain. Cytokines such as tumour necrosis factor-α (TNF-α), interleukins IL-1α and IL-6, histamine, glutamate, free radicals, nitric oxide, purine nucleotides are known to increase the permeability of the barrier.

Breakdown of the BBB is associated with a variety of CNS disorders and results in aggravation of the condition. In multiple sclerosis, the barrier function is compromised along with the loss of laminin (Oki, Takahashi et al. 2004) in the basement membrane and the tight junction proteins claudin -1 and -3 (Minagar, Ostanin et al. 2003). Post mortem CNS tissue from HIV-1 patients with encephalitis displayed decreased immune reactivity for occludin (Dallasta, Pisarov et al. 1999).

Endothelial cells comprising blood vessels in malignant gliomas, metastatic and benign tumours fail to form TJs or form leaky TJs. Occludin and claudin-5 are down regulated in human gliomas and are absent in metastatic tumour vessel TJs (Liebner, Fischmann et al. 2000; Papadopoulos, Saadoun et al. 2001; Rascher, Fischmann et al. 2002). Two reasons for the poor quality or absence of TJs can be attributed to decreased astrocyte numbers in the tumours and increased secretion of angiogenic factors (Janzer and Raff 1987; Bates, Lodwick et al. 1999).

In bacterial meningitis, free radicals and presence of interleukins IL-6 and IL-1β generated in response to lipopolysaccharide increase permeability (Gaillard, de Boer et al. 2003). Exposure to stress or hypoxia results in the increased levels of cAMP and Glut1 and a subsequent increase in terminal resistance and upregulation of Pgp activity (Kis, Deli et al. 2001).

BBB in Alzheimer's disease is characterised by increase in GLUT1 expression (Kalaria 1999), altered agrin levels (Berzin, Zipser et al. 2000) and accumulation of amyloid-β due to decreased clearance (Lee and Bendayan 2004). Microglia and astrocytes are closely associated with neuronal plaques and activated by the accumulated β-amyloid protein to release cytokines, reactive oxygen species etc (Giulian, Haverkamp et al. 1995). β-amyloid stimulates NFκB a transcription factor that has been implicated in altering the barrier function. The transcription factor activates transcription of TNF-α, IL-1, IL-6 and monocyte chemoattractant protein (MCP)-1 all known potentiators of increased permeability of the blood brain barrier (Akama, Albanese et al. 1998; Brown and Davis 2005).

Restoration of the BBB is thus one strategy during therapy of CNS diseases. One of the agents used to reduce inflammation is dexamethasone, a corticosteroid that is commonly used to treat oedema (Kaal and Vecht 2004). Some of the potential ideas for therapy include employing modulators that can increase activity of the enzymatic machinery and/or affect gene expression in the endothelial or surrounding cells resulting in restoration of barrier function and hence integrity. Their success depends on the knowledge of the structural and functional aspects of the blood brain barrier.

1.2.3 *In vitro* Models of the Blood Brain Barrier

Over the past two decades, many groups have devised *in vitro* models of the blood brain barrier in order to facilitate understanding of the unique properties and regulation of endothelial cells. Some of the *in vitro* models are discussed below.

1.2.3.1 Isolated Brain Capillaries

(Siakotos and Rouser 1969) described the isolation of capillaries from homogenised post-mortem human brain tissue by filtration. This model was the first system used to demonstrate receptor mediated transport of insulin and transferrin in capillary endothelial cells. However, isolated capillaries cannot be used to measure transendothelial transport across the BBB.

1.2.3.2 Primary Cultures of Endothelial Cells

Primary endothelial cells maintain morphology, biochemistry and function in *in vitro* growth conditions. Detailed methodology of obtaining viable endothelial cells from donor tissue has been described in the General Materials and Methods section. However, there are several constraints including availability and quality of human post mortem donor tissue; slight variation in phenotypic properties between different donor tissue; mouse, bovine or porcine based primary endothelial cell cultures cannot be compared with human cell cultures because of well recognised differences in gene expression (e.g. p-glycoprotein). Establishment of immortalised cell cultures has answered some of the problems of primary cultures. Endothelial cell lines used in the field are tabled below.

Table 1-3 Immortalized Brain Capillary Endothelial Cell Lines Reported in the Literature

Cell line	Species	Nature of transformation	Reference
SV-HCEC	Human	SV40 T antigen	(Muruganandam, Herx et al. 1997)
hCMEC/D3	Human	SV40 T antigen + hTERT	(Weksler, Subileau et al. 2005)
HBEC-51	Human	SV40 T antigen	(Xiao, Yang et al. 1996)
BB19	Human	E6E7 gene	(Prudhomme, Sherman et al. 1996)
MBEC	Mouse	E6E7 gene	(Tatsuta, Naito et al. 1992)
S5C	Mouse	Adenovirus E1A	(Sobue, Yamamoto et al. 1999)
TM-BBB4	Mouse	SV40 T antigen	(Asaba, Hosoya et al. 2000)
RBE4	Rat	Adenovirus E1A	(Couraud, Greenwood et al. 2003)
GPNT	Rat	SV40 T antigen	(Regina, Romero et al. 1999)
CR3	Rat	SV40 T antigen	(Lechardeur, Schwartz et al. 1995)
GP8.3	Rat	SV40 T antigen	(Greenwood, Pryce et al. 1996)
RBEC1	Rat	SV40 T antigen	(Kido, Tamai et al. 2000)
RCE-T1	Rat	Rous Sarcoma virus	(Mooradian and Diglio 1991)
t-BBEC-117	Bovine	SV40 T antigen	(Sobue, Yamamoto et al. 1999)
SV-BEC	Bovine	SV40 T antigen	(Durieu-Trautmann, Foignant-Chaverot et al. 1991)
BBEC-SV	Bovine	SV40 T antigen	(Stins, Prasadarao et al. 1997)
PBMEC	Porcine	SV40 T antigen	(Teifel and Friedl 1996)

Adapted from (Gumbleton and Audus 2001)

1.3 Tight Junctions

Cell junctions can be classified into occluding, anchoring and communicating junctions. The occluding junctions commonly referred to as the tight junctions (TJs) represent the connections between adjacent cells and represent the apical most type of junctional complexes in vertebrate epithelium (Wolburg and Lippoldt 2002). They are also observed in endothelial cells and mesothelial cells, as well as several other types of cells including Schwann cells, Sertoli cells and oligodendrocytes. By transmission electron microscopy, tight junctions appear as a series of very close membrane appositions of adjacent cells. On freeze–fracture, these contact sites principally correspond to continuous networks of tight junction strands and complementary grooves in the protoplasmic and extracellular faces, respectively (Vorbrodt and Dobrogowska 2003).

TJs are crucial in limiting paracellular transport and in maintaining polarity of cells. In addition TJs function as specialized micro domains in the plasma membrane and coordinate vesicle protein docking and actin organization. Components of the TJs especially the cytoplasmic components are important signalling proteins regulating differentiation and proliferation. These multiple functions of TJs are important in segregating the epithelial and endothelial cellular layers and in establishing distinct tissue compartments within the body to ultimately maintain homeostasis.

Disturbance of the functions of TJs is considered to cause or contribute to the pathology of diseases, such as inflammatory bowel disease (IBD), infections and

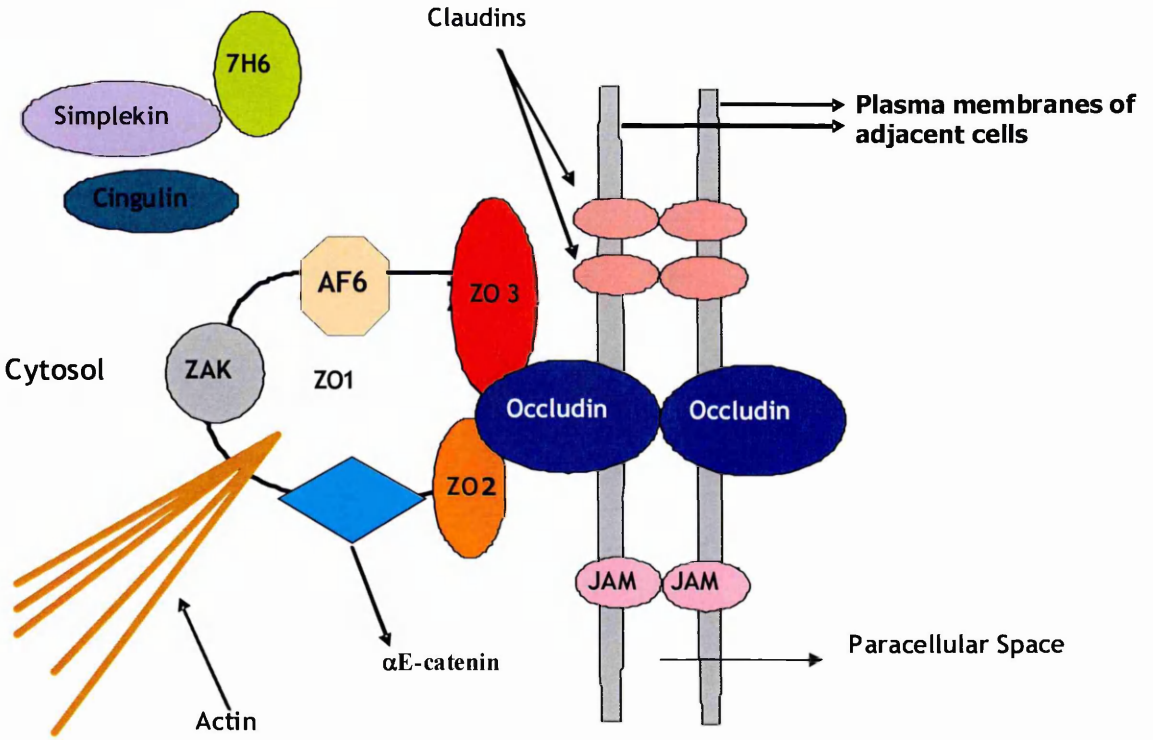
cancers, as well as vasogenic oedema and blood-borne metastases. In turn, the barrier function of tight junctions also restricts drug delivery to underlying tissues and therefore how to overcome the paracellular barrier is critical for treatment of diseases.

Table 1-4 Recruitment of Proteins into TJs During Development

Protein	**Stage at which first detected**	**Assembly into TJ**	**Model**	**Reference**
Claudin 1	Not determined	32-cell stage	Mouse (Epithelium)	Fleming *et al.* 2001
Claudin 5	Embryonic Day 5	Embryonic day 10	Chick (Retinal pigment epithelium)	Kojima *et al.* 2002
Occludin	Throughout all embryonic stages	Early 32-cell stage	Mouse (Epithelium)	Sheth *et al.* 2000
ZO-1 α^-	Throughout all embryonic stages	Punctate staining 8-cell embryos	Mouse (Epithelium)	Sheth *et al.* 1997
Z0-1 α^+	Beginning of the blastocyst stage	Early 32-cell stage	Mouse (Epithelium)	Sheth *et al.* 1997

(GONZALEZ-MARISCAL, BETANZOS, NAVA, JARAMILLO, 2003)

Figure 1-4 Molecular Constituents of Tight Junctions



TJs are composed of a set of transmembrane proteins- occludin, claudin 5 and junctional adhesion molecule-JAM. Adaptor molecules, ZO-1, -2 and -3 bind the cytoplasmic tail of occludin and actin, AF-6, ZAK-(ZO1 associated kinase) and alpha-catenin. The function of simplekin, cingulin and 7H6 which also localise to the junctions is not yet known. Many of the cytoplasmic junctional components are signalling proteins or exhibit sequence similarities with tumour suppressors, and so might function in transducing signals to and from the cell interior.

1.3.1 Molecular Constituents of TJs

1.3.1.1 Occludin

Discovered by (Furuse, Hirase et al. 1993), occludin is an approximately 65-kD type II transmembrane protein composed of four transmembrane domains, two extracellular loops, and a large C-terminal cytosolic domain. When observed by freeze fracture electron microscopy, occludin is concentrated directly within the tight junction fibrils (Fujimoto 1995).

1.3.1.1.1 Structure and Interactions

Occludin protein can be subdivided into five domains based on its structure. Referred as domains A-E, these regions include a carboxy terminal, amino terminus, two extra cellular loops and a cytosolic loop (Fig 1.5)

Domain E (carboxy terminus) is localised to the cytoplasm and is composed of 225 residues which are enriched for charged amino acids. Occludin mutants with truncations in domain E localise to the TJs but the barriers generated are leaky indicating that the this domain is necessary for correct TJ assembly and function (Chen, Merzdorf et al. 1997). Protein interaction studies have indicated this domain binds the ZO protein family. Within the 150-amino acid region that interacts with the ZO proteins, is a 27-amino acid stretch with hydrophobic residues clustered in a pattern that has identified to be consistent with a coiled-coil structure. In an elegant study (Nusrat, Chen et al. 2000), the authors have shown that this region is responsible for most of the interactions of the full length occludin protein.

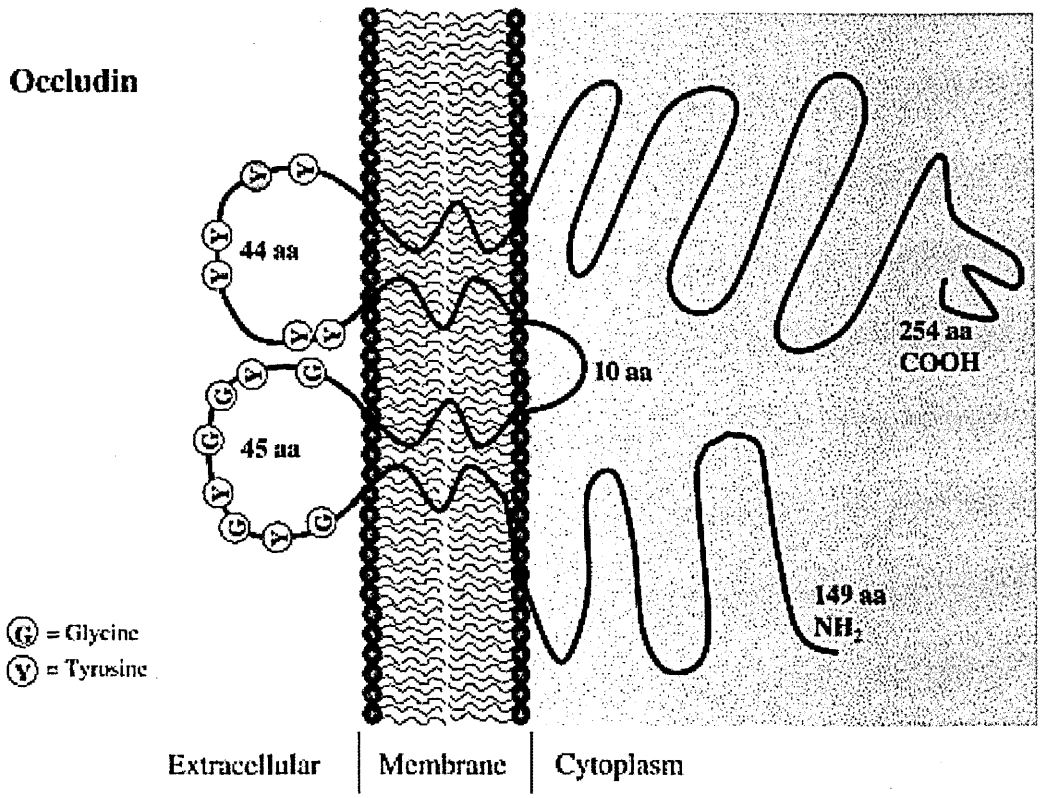
These include associations with members of the TJ and proteins associated with the plasma membrane. The following proteins c-Yes, the regulatory (p85) subunit of PI3K, PKC-ζ, and the gap junction component, connexin 26 have been shown to interact with this domain. There are however differences in the interactions within the proteins; PI3K interacts with both interfacial surfaces of the domain in contrast to PKC-ζ and c-Yes which interact solely with the hydrophobic surface. Connexin-26 interacts with the hydrophilic surface of the coiled-coil (Nusrat, Chen et al. 2000).

The N-terminus referred to as domain A is comprised of 65 amino acids and is directly involved in barrier function. Occludin constructs lacking this domain when over expressed localise to the TJ and also interact with ZO-1 but this localisation and subsequent interaction with other TJ proteins is not functional as the barriers formed are leaky (Bamforth, Kniesel et al. 1999). Domains A and E are enriched in serine and threonine residues. Since occludin at the TJ is hyper phosphorylated, it is likely these residues are potential targets for phosphorylation and hence are important in function (Feldman, Mullin et al. 2005).

The two extracellular domains, B and D, are rich in tyrosine and glycine residues (Feldman, Mullin et al. 2005) and are separated by a short (10 aa) cytosolic loop. Occludin constructs lacking domain D, or both the domains when ectopically expressed in epithelial cells, were restricted to the basolateral cell surface indicating the domains are important in the targeting of the protein to the TJ (Medina, Rahner et al. 2000). There are two conserved protein domains, the MARVEL and the Occludin-ELL domain. The MARVEL domain is a membrane-

associating domain often found in lipid-associating proteins and may contribute to the machinery of membrane apposition events. The occludin-ELL domain is a conserved region of approximately 100 residues between occludin and the RNA polymerase II elongation factor ELL.

Figure 1-5 Structural Representation of Occludin



(Feldman, Mullin et al. 2005)

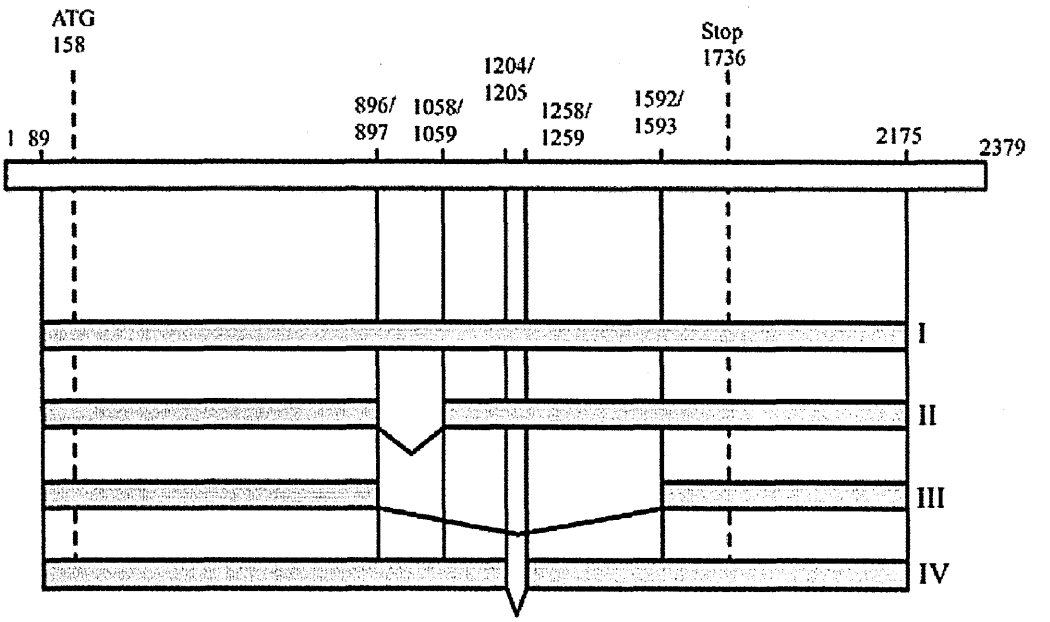
Occludin has four transmembrane regions; two extracellular domains and the amino and carboxyl terminal ends are oriented towards the intracellular region. Both extracellular loops of occludin are of approximately the same size, lack charged residues and are very rich in tyrosine (Y). More than half of the first loop residues are tyrosines and glycines (G). (Feldman, Mullin et al. 2005).

1.3.1.1.2 Splice Variants

In 2002 (Mankertz, Waller et al. 2002), identified four differentially spliced occludin-specific mRNA transcripts (Fig 1.6). Two splice variants, occludin types II and III, lack the fourth transmembrane domain and do not co-localize with ZO-1. An occludin isoform lacking the TM4 domain was discovered by RT-PCR analysis of human tissues, embryos and cells using primers spanning the TM4 and adjacent C-terminal region (Ghassemifar, Sheth et al. 2002). It is possible that this isoform might modulate intercellular adhesion.

Occludin 1B variant contains a 193 bp insertion that results in a longer form of the protein with a unique domain A comprising of 56 amino acids. The localization of occludin 1B on ectopic expression in MDCK cells is similar to that of the full length occludin (Muresan, Paul et al. 2000). Occludin 1B has been observed in T84 human colon carcinoma cells and in a range of epithelial tissues (Ghassemifar, Sheth et al. 2002). Co-expression of occludin and occludin 1B has also been seen in various murine tissues. It appears that occludin IB expressed in the gut is conserved between mouse and human. The presence of occludin 1B in epithelium and conservation across species implies that occludin 1B is potentially a significant player in the modulation of TJ barrier properties in vascular endothelium (Muresan, Paul et al. 2000).

Figure 1-6 Structural Comparison of the Occludin mRNA Variants



(Mankertz, Waller et al. 2002)

Primary nucleic acid sequences from occludin-specific cDNAs (types I, II, III, and IV) were compared to the published occludin sequence (GenBank accession number U49184). The sequence boundaries are determined by the location of the oligonucleotides used for amplification. Translation start and stop codons are marked by broken lines, regions of homology are indicated by filled boxes, and deletions are represented by thin lines (Mankertz, Waller et al. 2002).

1.3.1.1.3 Expression

Occludin is expressed in a broad range of epithelial cells in addition to the blood brain barrier and blood vessel endothelium. It is clear that expression of occludin correlates with barrier function.

Blood Brain Barrier: Occludin is localized at the TJs of cerebral endothelial cells. (Hirase, Staddon et al. 1997) first reported that occludin protein was strongly expressed and distributed continuously at the interface of brain endothelial cells. Occludin expression has been shown to be relatively lower in TJs of endothelial cells in non-neural tissue. Occludin levels at the TJs of brain endothelial cells increase with development. The levels of occludin protein in brain and non-neural tissue are mirrored at the mRNA level (reviewed in Wolburg and Lippoldt 2002).

Blood Retinal Barrier: Occludin is also highly expressed in the capillaries of the retina, which represents the blood–retinal barrier (BRB). Occludin is strongly expressed between adjacent endothelial cells in all retinal blood vessels. In contrast, low levels of occludin were detectable in choroid vessels, which do not exhibit blood–barrier properties (Morcos, Hosie et al. 2001).

Epithelium:

Occludin expression is seen in stratified (Langbein, Grund et al. 2002) as well as simple epithelium (Brandner, Kief et al. 2002) where it contributes to barrier function. Occludin has been found localised to focal strands between granular cells in the outer most zones of the epidermis in rodents (Moroi, Saitou et al. 1998). Occludin mRNA expression has also been reported in human neo-natal and adult keratinocytes (Tebbe, Mankertz et al. 2002). Immunostaining analyses for the protein shows its presence in the intercellular space of the granular layer. It appears that occludin contributes to formation of TJs between keratinocytes in the epidermis (Brandner, Kief et al. 2002). Increase in occludin protein expression in regenerating epidermis suggests that it may function during epidermal wound

healing (Malminen, Koivukangas et al. 2003).

Its expression has been reported in bladder epithelium of mouse, rat and rabbit where it localises to the TJ and contributes to the high resistance of the uroepithelial-associated TJ complex (Acharya, Beckel et al. 2004).

Studies investigating occludin expression in liver epithelium demonstarted that its localisation alters during cell division which differs from other peripheral proteins like ZO1 and ZO2 (Kojima, Kokai et al. 2001) (Fallon, Brecher et al. 1995). Occludin and another integral TJ protein claudin 1 are strongly expressed in the mid body between daughter cells. This was one of the first studies wherein a difference in the regulation of integral versus periperhal TJ proteins was seen in cell division. (Kojima, Kokai et al. 2001).

Occludin is expressed in the acinar cells of the salivary gland (Hashimoto, Ochiai et al. 2000). The protein localizes to the abluminal side in adjacent acinar cells. Since salivary fluid secretion is dependent on paracellular routes as well as transcellular routes, occludin regulates the salivary fluid secretion by means of its presence at the tight junction.

Occludin is a component of corneal epithelium (Ban, Dota et al. 2003). Studies on the canine kidney have shown weak and irregular to strong, continuous immunostaining for occludin. For e.g. the protein was highly expressed in the ascending and descending loop of Henle and in the cells of the distal tubule and was weak in the cells of the proximal tubule. There is a strong correlation between the TER and the level of occludin expression thereby implying this protein is

required for the function of the renal TJ (Kwon, Myers et al. 1998).

Occludin is concentrated at the TJs in the Sertoli cells of the murine testis (Moroi, Saitou et al. 1998) where it is highly phosphorylated and its concentration increases with maturity. (Kevil, Okayama et al. 1998) observed that occludin in endothelial monolayers was more concentrated in arterial junctions than in venous junctions both *in vivo* and *in vitro*.

1.3.1.1.4 Occludin Knock-out Mouse

The occludin knockout mouse showed growth retardation, brain calcification, thinning of bone, male sterility, chronic inflammation and hyperplasia of gastric epithelium (Saitou, Furuse et al. 2000). However, TJ formation in gut epithelial cells and function was not affected (Saitou, Fujimoto et al. 1998). Similarly, TJs of embryoid bodies originating from occludin-deficient embryonic stem cells were similar to their wild-type counterparts (Saitou, Fujimoto et al. 1998).

1.3.1.1.5 Functions

There is considerable evidence for the importance of occludin in TJs. Over expression of chicken occludin in cultured MDCK cells increases the number of TJ strands, and in increase in transepithelial resistance (TER). Introduction of C-terminally truncated occludin into MDCK cells or Xenopus embryo cells resulted in the increased paracellular leakage of tracers of low molecular mass. The TER of cultured Xenopus epithelial cells is reduced by addition into the culture medium of a synthetic peptide corresponding to the second extracellular loop of occludin. Occludin confers adhesive property to transfected fibroblasts which can

be blocked by peptides to any of the two extracellular loops (Anderson and Van Itallie 1995; McCarthy, Skare et al. 1996). But nevertheless, occludin is not absolutely required for the formation of TJs. Well-developed TJ structures between adjacent epithelial cells developed from embryonic stem cells lacking occludin.

1.3.1.1.6 Regulation of Occludin Protein

Cytokines

Interferon-γ (IFN-γ) an inflammatory cytokine released by activated T cells and natural killer cells leads to decreased expression of ZO1, redistribution of occludin and ZO2 and also the disruption and disorganization of actin *in vitro*. In combination with TNF-α, it induces a loss of claudin 5 and occludin. Both cytokines act to increase paracellular permeability and have been shown to regulate the barrier in gut epithelium (Schmitz, Fromm et al. 1999).

Treatment with TGF-β1 results in loss of cell–cell contact, rearrangement in actin cytoskeleton and impairment of endothelial permeability (Barrios-Rodiles, Brown et al. 2005). Other cytokines such as interleukin (IL)-1, IL-4, IL-13, TGF-β, insulin-like growth factor (IGF)-I and -II, and vascular endothelial growth factor (VEGF) have been documented to decrease the barrier properties of endothelial cells (Walsh, Hopkins et al. 2000).

MAP and PI3 Kinase Signalling

MAP signalling pathway modulates blood brain barrier function by regulating the expression or phosphorylation status of TJ proteins (Yuan 2002). Activation of the

PI3 kinase, and PKB pathway by reactive oxygen species leads to the down regulation of occludin in brain endothelial cells (Schreibelt, Kooij et al. 2007). In human umbilical vein endothelial cell, alcohol induced toxicity, activation of ERK1/2, JNK and p38 signalling results in phosphorylation of Ser residues in occludin resulting in its degradation (Kevil, Oshima et al. 2000). HIV-1 Tat protein activates ERK1/2 leading to relocalisation of occludin from cell surface to the cytosol in brain endothelial cells (Andras, Pu et al. 2003).

The cytokine, TGFβ1 activates the PI3K pathway and independently upregulates the expression of the transcriptional repressor snail (Ozdamar, Bose et al. 2005). During epithelial to mesenchyme transitions (EMT), expression of TJ and adherens junction proteins is down regulated. The ZnF transcription repressor, Snail has recently been implicated in EMT. Snail acts as a transcriptional repressor and binds DNA with the carboxy terminal zinc fingers. Snail binds the E-box motif, 5'-CA (G/C) (G/C) TG, which is found in the E-cadherin promoter (Cano, Perez-Moreno et al. 2000) and in the occludin and claudin-3, -4, and -7 promoters which contain one, six and eight, E-boxes, respectively. In a human epithelial carcinoma cell line, Snail over expression in epithelial cells lead to the complete repression of occludin, claudin-3, -4, and -7, and E-cadherin expression at both mRNA and protein levels, and this was accompanied by the disruption of both TJs and adherens junctions with subsequent induction of EMT (Ikenouchi, Matsuda et al. 2003).

The effects of PI3K signalling on TJ physiology depends on the stimulus as activation of the signalling cascade by different agents can induce opposite effects

on the TJ proteins. In Caco2 cells, activation of the PI3K pathway by oxidative stress mediates the loss of occludin (Sheth, Basuroy et al. 2003). Glucocorticoids lead to the recruitment of PI3K subunit p85 and its association with ZO1 and the C-terminal domain of occludin to positively affect the TJ barrier in brain endothelial cells (Forster, Silwedel et al. 2005). PI3K signalling can also act to inhibit GSK-3β mediated blocking of β-catenin and snail degradation. In addition, it positively upregulates Smad signalling which promotes the transcription of the transcription factor LEF1, which promotes EMT and down regulation of TJ proteins in epithelial cells (Bachelder, Yoon et al. 2005).

Phosphatases PP1, PP2A and PP2B

Ser/Thr phosphorylation of several TJ proteins promotes the barrier function of TJs and it is expected that Ser/Thr phosphatases exert opposite effects on the barrier function. Protein phosphatases PP2A and PP1 interact with the carboxyl terminal tail of occludin and dephosphorylate occludin on Ser residues leading to decrease in TER and increased paracellular permeability. PP2A also associates with aPKCζ, leading to the inhibition of its activity and promoting its relocalisation to the cytosol. This subsequently leads to the disappearance of TJ proteins at cell-cell contacts. PP2B in turn appears to blocks the phosphorylation and thus activation of cPKCα, the kinase that promotes TJ disassembly in epithelial cells (Seth, Sheth et al. 2007).

Rho Signalling

Rho family of small GTPases includes the proteins, RhoA, Rac and Cdc42 (Wennerberg and Der 2004). These proteins regulate apical and basal actin

structures in response to extracellular cues (Nusrat, Giry et al. 1995). Many of the integral proteins present at the TJ are stabilised by virtue of direct or indirect interactions with actin (e.g., occludin, ZO family AF-6 and cingulin). Investigations into the effect of dominant active and negative mutants of the Rho family indicate all the members are involved in conserving the integrity of the barrier. A dominant-negative mutant or an inhibitor of RhoA prevented occludin phosphorylation (Hirase, Kawashima et al. 2001). RhoA signalling pathway has also been shown to promote the migration of small lung cancer cells through brain endothelium (Li, Zhao et al. 2006).

Other Modulators

The E3 ubiquitin ligase Itch was identified to be a binding partner for the amino terminus of occludin in a study by Traweger et al. 2002. Itch is a member of the HECT domain-containing ubiquitin protein ligases and is responsible for the ubiquitination of occludin and subsequent degradation in epithelial cells (Traweger, Fang et al. 2002)

1.3.1.2 Claudins

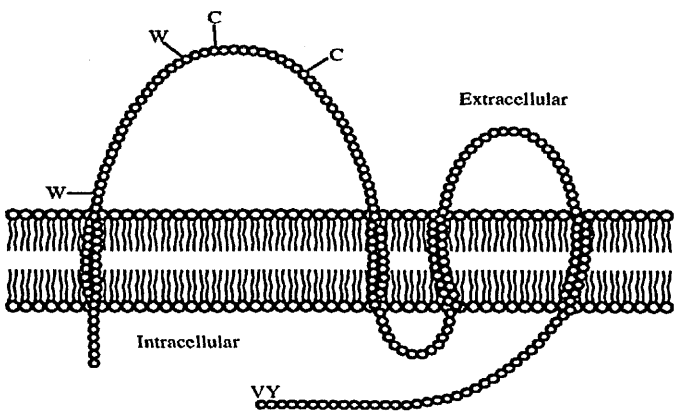
The association of tight junctional particles with the P or the E membrane leaflet is dependent on various claudins (Tsukita and Furuse 2000). Claudins were identified as major cell-adhesion molecules of TJs and belong to the PMP22/EMP/MP20/claudin mammalian superfamily. 20 different claudins have been identified in different tissues so far with molecular weights ranging from 20–27 kDa (Turksen and Troy 2004). Claudin 1 was the first member of the family to

be identified as a TJ component (Furuse, Fujita et al. 1998).

1.3.1.2.1 Structure

The claudin family is characterised by four transmembrane helices, two extracellular domains, a short N-terminal sequence (2–6 residues), and a long C terminus tail (Fig 1.7). The N- and C-termini are localised to the cytoplasm. The first extracellular domain ranges from 49–52 residues and includes the conserved motif of the claudin family, a set of highly conserved amino acids, W-GLW-C-C. The second extracellular domain is comprised of 16-33 amino acids and is a known receptor for bacterial toxins. The C-terminus binds cytoplasmic proteins through a PDZ (PSD95 DlgA ZO1) motif. The cytoplasmic tails are the most diverse in sequence and vary in length from 21–63 residues. The membrane-proximal region of the C terminus is palmitoylated on conserved cysteines, and it is probable that most claudins can be phosphorylated on serines and/or threonines in the cytoplasmic tail. Claudins may form hexamers, as suggested by studies of claudin-4 and a distant relative, MP20. (Reviewed (Matter and Balda 2003).

Figure 1-7 Structural Representation of Claudins



(Turksen and Troy 2004)

1.3.1.2.2 Interactions

Multiple claudins interact in both homotypic and heterotypic fashion and confer selective permeability in different tissues (Fig 1.8). All claudins (except claudin-12) end in PDZ-binding motifs. This motif is responsible for associations with related PDZ domains in the cytoplasmic junction proteins ZO-1, -2, and -3; MUPP1, PATJ and possibly other proteins. However, blocking of the PDZ-binding sites does not restrict localisation. Claudins still localize to cell-cell contacts and form freeze-fracture strands, suggesting that they have an inherent ability to polymerize, independent of PDZ interactions. However, the strands formed by PDZ-blocked claudins are poorly organized and not restricted to the apical border (Tsukita and Furuse 2002).

1.3.1.2.3 Expression

Northern blotting analysis for claudin expression has shown that most tissues expressed more than two species of claudins. For example, kidney epithelium expresses claudins -4 and -8 (Acharya, Beckel et al. 2004); claudins -1, -2, and -3 are present in the liver epithelium (Morita, Furuse et al. 1999). Therefore, it is likely that most TJ strands are heteropolymers of claudins (Fig 1.8), although specialized TJ strands in the myelin sheaths of oligodendrocytes and in the Sertoli cells in the testis appeared to be mainly composed of a single species of claudin, claudin-11 (Morita, Sasaki et al. 1999). Claudin 5 was originally described to be restricted to endothelial cells. However, the protein was also found in epithelium of the stomach and of the large and small intestine (Morita, Furuse et al. 1999).

1.3.1.2.4 Claudins in the CNS

The only claudins detected in endothelial cells thus far are claudin-1 and claudin-5 (Morita, Sasaki et al. 1999; Lippoldt, Liebner et al. 2000). A novel anti-claudin-3 antibody recognized cerebral capillary endothelial cell TJs (Engelhardt and Wolburg). Additionally, claudin-11 localizes to TJs in myelin sheaths and claudins -1, -2 and -11 are present in the fenestrated endothelium of the choroid plexus (Wolburg, Wolburg-Buchholz et al. 2001). Claudins 2, 4, 5, 6, 8 and to a lesser extent -7 were observed in rat hippocampus and cortex (Lamas, Gonzalez-Mariscal et al. 2002).

1.3.1.2.5 Function

The claudin family members are essential for the formation of TJs in epithelial and endothelial cells and hence have important roles in the control of paracellular transport and in the maintenance of cell polarity. Multiple claudins interact in a homotypic as well as in a heterotypic fashion to confer selective permeability in different tissues.

In $Cld5^{-/-}$ mice, the blood vessels showed normal morphology and distinctly formed TJs comprising claudin-12. However, the TJs were not fully functional as the BBB was severely affected against molecules less than ~800 D, but not larger molecules. Nevertheless, the mice died within 10 hours of birth (Nitta, Hata et al. 2003).

In addition to barrier functions, claudins are involved in development. Mutations in claudin genes lead to various familial diseases such as neonatal sclerosing cholangitis (Claudin 1) (Hadj-Rabia, Baala et al. 2004), nonsyndromic

recessive deafness (Claudin 14) (Wilcox, Burton et al. 2001), and familial hypomagnesaemia (Claudin 16) (Lai-Cheong, Arita et al. 2007).

Recent analyses also show the role of the claudin family in the progression of various cancers (Morin 2005). One of the hallmarks of metastasis is loss of tight junctions and hence loss of expression in TJ proteins is expected. However, expression of claudins -1, -3, -4, -5, -7, -10, -16 are not lost but altered in various cancers; claudins 3 and -4 are increased in ovarian, breast, prostate and pancreatic tumours (Morin 2005). Claudin 17 is elevated in stomach cancer but is decreased in breast cancer (Kramer, White et al. 2000).

Claudins -3 and -4 are being used as targets for therapy in malignancies over expressing these proteins. Clostridium perfringens enterotoxin (CPE) a natural ligand for Claudins-3 and -4 (Van Itallie, Betts et al. 2008) binds to the proteins resulting in rapid cytolysis of the cells (Katahira, Sugiyama et al. 1997).

1.3.1.3 Submembranous TJ Associated Proteins

The transmembrane components are tethered to the actin cytoskeleton and properly positioned at the tight junction by a group of cytoplasmic proteins including ZO1, -2, -3, AF6, MUPP1, PAR3, Cingulin, Simplekin and 7H6.

1.3.1.3.1 ZO (Zona Occludens) Proteins

ZO proteins are members of the Membrane Associated Guanylate Kinase (MAGuK) homologue family and are structurally composed of three PDZ domains, an SH3 domain and a non-catalytic guanylate kinase homology domain. In addition, they have an acidic and a basic domain, a proline rich C-terminus and

a leucine zipper dimerization motif (Gonzalez-Mariscal, Betanzos et al. 2000). ZO family members interact with each other to form heterodimers via their PDZ domains. The PDZ domain of the ZO family mediates binding to the C-terminal regions of claudins 1-8 (Itoh, Furuse et al. 1999; Umeda, Ikenouchi et al. 2006). The GuK domain and the acidic domain mediate binding to occludin in vitro. Recombinant ZO-2 and -3 also interact with occludin *in vitro* (Wittchen, Haskins et al. 1999) (Fig. 1.10).

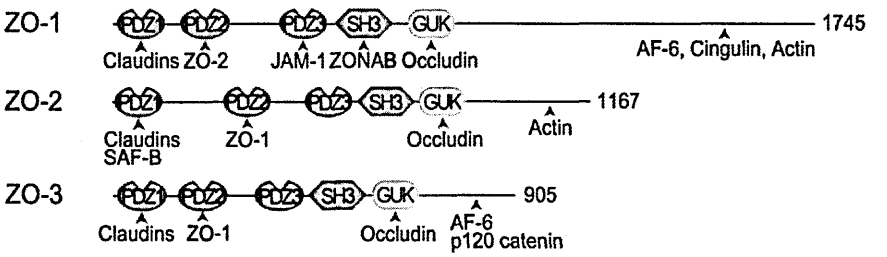
ZO proteins are anchored to the cytoskeleton by association of the proline rich C-terminus with F-Actin. The SH3 domain mediates binding to G proteins such as Gα12. ZO1 also interacts with the Ras target, AF-6 (Yamamoto, Harada et al. 1997) and this binding can be disrupted by the over expression of activated Ras. ZO-1 binds to Cingulin (D'Atri, Nadalutti et al. 2002), JAM A (Ebnet, Suzuki et al. 2004) and the gap junction protein, Connexin 43 (Toyofuku, Yabuki et al. 1998) and also the adherens junction protein α-catenin (Gonzalez-Mariscal, Betanzos et al. 2000; Salama, Eddington et al. 2006).

ZO-1 expression is regulated at the post-transcriptional level by alternate splicing leading to the generation of two isoforms that can be differentiated by the presence or absence of the α motif. The α motif is an 80 amino acid domain within the C-terminus. The α^+ isoform is present in epithelial cells and the α^- isoform in cells of endothelial origin (Willott, Balda et al. 1992). Additional splice variants in the C-terminus gives rise to the β and γ isoforms (Gonzalez-Mariscal, Islas et al. 1999).

ZO1 has been shown to interact with ZONAB, a TF homologous to Y-box

proteins (Balda and Matter 2000). The two proteins functionally interact in the nucleus to modulate the expression of the *erbB-2* proto-oncogene, indicating a role for regulation of gene expression for TJ proteins (Balda, Garrett et al. 2003). Data supports the view that the two proteins also interact to promote paracellular impermeability either through occludin or affecting the gene expression of TJ proteins.

Figure 1-8 Structure of ZO Family Members



(Gonzalez-Mariscal, Betanzos et al. 2003)

Domains are represented by labelled boxes. Intermolecular associations with other TJ and cytoskeletal proteins are indicated underneath the domains. (Gonzalez-Mariscal, Betanzos et al. 2003).

1.4 Transcriptional control in eukaryotes

RNA polymerases I, II, and III allow for the transcription of rRNA, mRNA, and small RNAs in eukaryotes. Much of the regulation of transcription occurs at the promoter region where specific DNA sequences effect the recruitment of the polymerases and regulatory proteins (transcription factors) (Kornberg 2007). However, gene expression can be controlled at the processing of the RNA transcript, transport of RNA to the cytoplasm, translation of mRNA, and mRNA and protein stability. General transcriptional machinery includes:

1. Basal transcription machinery: RNA polymerases and promoter region.
2. Long range regulatory elements and transcription factors (TFs)
3. Co activators and co repressors.

Table 1-5 Regulatory Proteins in Transcription

	Factor	Function
General transcription machinery	RNA Pol II	Catalysis of RNA synthesis
	TFIIB: TF for RNA Pol II B	Stabilization of TBP–DNA interactions, recruitment of RNA Pol II–TFIIF
	TBP: TATA-binding protein	Core promoter recognition, TFIIB recruitment
	TAF: TBP-associated factor	Core promoter recognition
	TFIIE: TF for RNA Pol II E	TFIIH recruitment
	TFIIF: TF for RNA Pol II F	Recruitment of RNA Pol II to promoter DNA–TBP–TFIIB complex
	TFIIH: TF for RNA Pol II H	Promoter melting, helicase, RNA Pol II CTD kinase
	Mediator	Transduces regulatory information from activator and repressor proteins to RNA Pol II
Co activators and co repressors	Chromatin modification complexes:	
	HATs: Histone acetyltransferases	Acetylate histones
	HDACs: Histone deacetylases	Deacetylate histones
	CBP: CREB-binding protein	HAT activity
	HMTs: Histone methyltransferase	Methylate histones
	LSD1: Lysine-specific demethylase1	Demethylates histones
Elongation factors	FACT: Facilitates chromatin transcription	Transcription-dependent nucleosome alterations
	Elongator	Exact function unknown
	TFIIS: TF for RNA Pol II S	Facilitates RNA pol II passage through regions that cause transcriptional arrest

1.4.1 Eukaryotic Promoters

The promoter of a gene is the cis-regulatory DNA region that can drive the transcription of its target gene in response to environmental signals. Eukaryotic promoters can be defined into three regions:

1. Core-promoter situated ~80–100 bp surrounding the transcription start site.
2. Proximal-promoter present at ~250–1000 bp upstream of the core-promoter. DNA motifs in this region can predict tissue-specific gene expression.
3. Distal-promoter located further upstream, normally excluding enhancer or other regulatory regions whose influences are position/orientation independent.

1.4.1.1 Upstream and Downstream Core Promoter Elements

Known core promoter elements include the TATA box, the initiator element (Inr), the TFIIB recognition element (BRE), the downstream promoter element (DPE), and the motif ten element (MTE). TATA box, Inr and BRE form the upstream promoter elements; DPE and MTE, the downstream promoter elements.

1.4.1.1.1 TATA Box

The TATA Box is a T/A-rich sequence that is usually located 25–35 base pairs upstream of the transcription start site(s) and is essential for transcription initiation. However, TATA boxes are present in only 32% of 1031 potential core promoters and transcription from promoters lacking TATA boxes takes place

through enhancer elements and or GC boxes. The consensus sequence of TATA Box is TATAWAWN (where N is any base and W denotes A-T base pair without regard to end-for-end orientation) and recruitment of the TFIID complex which includes the TBP and TBP associated factors, directs assembly of the pre-initiation complex (PIC). TATATAAG has been identified as the optimal TBP recognition sequence.

One factor that is present at promoters used by all three RNA polymerases is the TATA-binding protein (TBP). TBP along with factors such as the TAF_Is, TAF_{II}s, and TAF_{III}s that bind to class I, II, and III promoters determine general and gene-specific regulation of gene expression. Among the eight factors that interact with TBP, TAF_I, TAF_{II}s, TAF_{III}s, and PTF/SNAPc function in promoter selection and the other four factors namely, SAGA, Mot1, NC2, and Nots function together with TAF_{II}s to regulate expression of protein-coding genes. All but one of these factors (PTF/SNAPc) is highly conserved among eukaryotes. In the case of genes transcribed by RNA polymerase II, TBP is responsible for promoter recognition and binds directly to DNA in the minor groove of an AT rich sequence (consensus TATA T/A A T/A X) (Matangkasombut, Auty et al. 2004; Sandelin, Carninci et al. 2007).

1.4.1.1.2 Initiator (Inr) Element

Functionally similar to the TATA box, the Inr element whose sequence is PyPyA+1N(T/A)PyPy is recognized by TAF1 and TAF2 and can function independently of the TATA box. However, in TATA-containing promoters, it acts synergistically to increase the efficiency of transcription initiation (Gross and

Oelgeschlager 2006).

1.4.1.1.3 Downstream Promoter Element (DPE)

The DPE is a distinct seven nucleotide element conserved from the fly to humans and is bound by the subunits TAF6 and TAF9 of the TFIID complex. The DPE consensus sequence is (A/G)G(A/T)(C/T)(G/A/C) and is located about +30 bp relative to the transcription start site. It functions in TATA-less promoters and requires the presence of an Inr element for activity (Kadonaga 2002)

1.4.1.1.4 Motif Ten Element (MTE)

MTE whose consensus sequence is C(G/A)A(A/G)C(G/C)(C/A/G)AACG(G/C) is located at positions +18 to +27 relative to the transcription start site and promotes transcriptional activity and binding of TFIID along with the Inr element. Although it can function independently of the TATA box or DPE, it exhibits strong synergism with both of these elements (Lim, Santoso et al. 2004; Juven-Gershon, Hsu et al. 2006).

1.4.1.2 Proximal Promoter Elements

1.4.1.2.1 CCAAT box

The CCAAT box was one of the first elements identified in eukaryotic promoters. The pentanucleotide sequence has been described in different, unrelated promoters in vertebrates, plant and yeast and the various transcription factors that interact with the sequence have also been described. In higher eukaryotes, the CCAAT box is present in reverse ATTGG orientation in 60% of the promoters

and usually, genes lacking TATA boxes show a preference for the ATTGG box (Ramji and Foka 2002). The transcription factors, c/EBP, CTF/NF1, CDP, NF-Y (also known as CBF, CP1) have been described extensively to regulate transcription from this element. TFs NF1 and c/EBP are discussed in the next section.

The CCAAT displacement protein (CDP) is a homolog of the Drosophila homeodomain protein Cut (Neufeld, Skalnik et al. 1992). CDP/Cut proteins were found to function primarily as transcriptional repressors and some examples of the genes targets are H2B, NCAM, c-Myc, etc. Repression can occur through competition with activators for a binding site or through the two active repression domains identified within the C terminal domain (Nepveu 2001).

The NF-Y is a heteromeric protein that is composed of three subunits NF-YA, –YB and –YC and requires all the CCAAT nucleotides for binding and interaction. NF-Y sites are predominantly found in proximal promoters and in the absence of TATA-TBP interactions, NF-Y has been known to function in connecting the upstream activators with the general transcription machinery allowing for RNA polymerase to mediate its effects from the start site (Mantovani 1999).

GC box is discussed in detail in the Sp transcription factor section.

1.4.2 Long Range Regulatory Elements

Transcriptional control also involves additional regulatory DNA sequences namely enhancers and silencers, insulators, locus control regions (LCRs), and matrix attachment regions (MARs).

1.4.2.1 Enhancers and Silencers

Usually situated 700–1000 bp or more from the transcription start site, these elements can be found downstream, upstream, or within an intron, and can function in either orientation relative to the promoter (Maston, Evans et al. 2006). A typical enhancer is around 500 bp in length and exhibits binding sites for several different transcription factors. Enhancer activation could result in chromatin reorganisation and access of the promoter to transcription factors (Bulger and Groudine 1999; Engel and Tanimoto 2000). Activation of the enhancer element could also lead to interaction of proteins separately bound at the promoter and enhancer regions resulting in the looping out of DNA and the formation of a promoter/enhancer holocomplex (Hatzis and Talianidis 2002). Similar elements that repress gene activity are called silencers (Pozzoli and Sironi 2005).

1.4.2.2 Insulators

An insulator is a DNA sequence element, typically 300 bp to 2 kb in length that functions as a chromatin boundary marker, marking the border between regions of heterochromatin and euchromatin (Geyer and Clark 2002; Brasset and Vaury 2005). Another important function is to block enhancer blocking activity to prevent inappropriate cross-activation or repression of neighboring genes by blocking the action of enhancers and silencers (Brasset and Vaury 2005). It is believed that insulators tether the DNA to subnuclear sites, forming loops that separate the promoter of one gene from the enhancer of another.

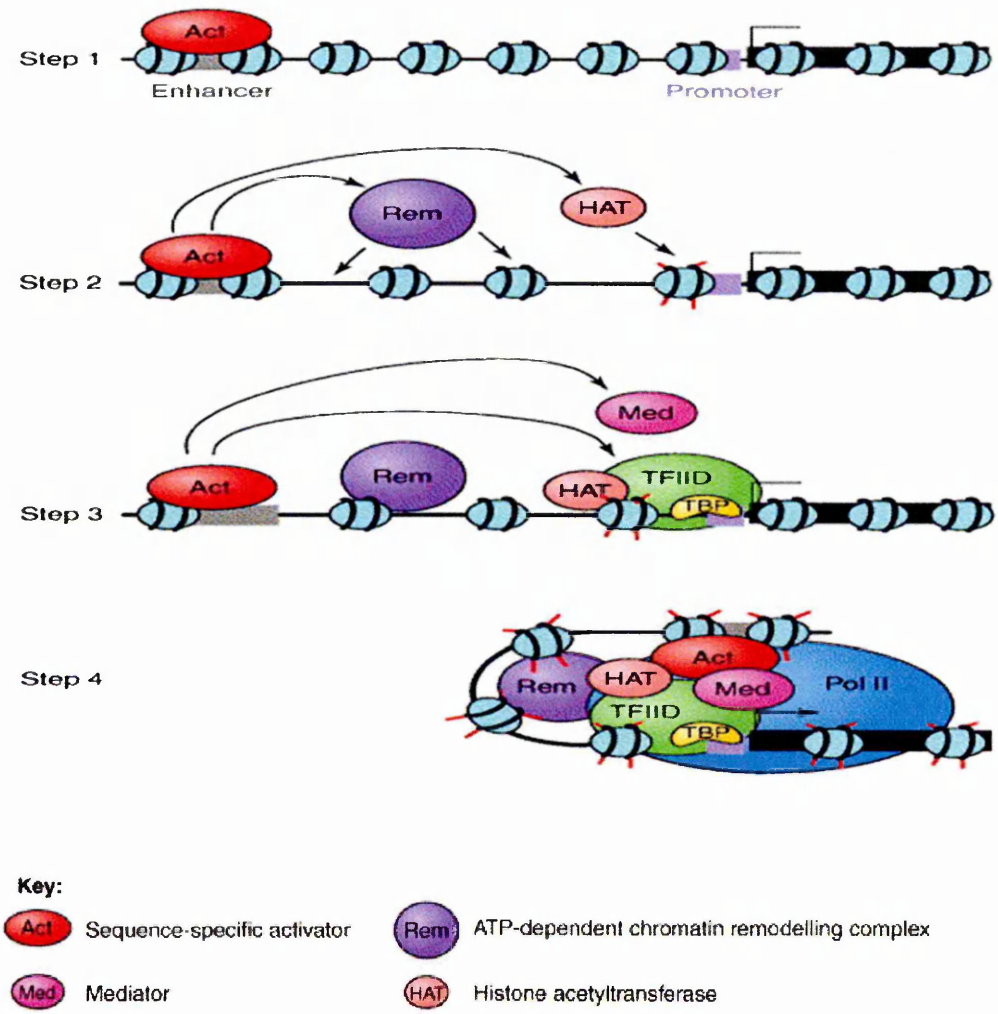
1.4.2.3 Locus Control Regions (LCRs)

LCRs are DNA sequences that organize and maintain a functional domain of active chromatin to enhance the transcription of downstream genes and operate in an orientation-dependent manner (Dean 2006). LCRs have been shown to be important in the transcription of the β-globins (Sawado, Halow et al. 2003), MHC II proteins (Masternak, Peyraud et al. 2003), human growth hormone (Horan, Millar et al. 2003), serpins (Zhao, Friedman et al. 2007) and cytokines (Dean 2006).

1.4.2.4 Matrix Attachment Regions (MARs)

MARs are genomic DNA fragments that have the ability to bind to isolated nuclear matrices *in vitro* (Cockerill and Garrard 1986). Located near enhancers in 5′ and 3′ flanking sequences, MARs sequences are scattered throughout the genome at 5-200 kb intervals and play important roles in modulating chromatin structure in interphase and metaphase (Laemmli, Kas et al. 1992; Bode, Stengert-Iber et al. 1996). The MARs recognition signature (MRS) comprises two individual sequence elements AATAAYAA (where Y = C or T) and AWWRTAANNWWGNNNC (where W = A or T, R = A or G, N = A, C, G or T) that are <200 bp apart (van Drunen, Sewalt et al. 1999). MARs recruit TFs and chromatin-remodelling enzymes resulting in the formation of chromatin loops (Mirkovitch, Mirault et al. 1984). Associations of MARs with other factors can be altered by interactions with components of enhancers and LCRs.

Figure 1-9 Model Describing the Activation Process of a Eukaryotic Gene in Chromatin



(Szutorisz and Dillon, et al 2005)

Step 1: The gene (black box) has a core promoter element (purple box) and is regulated by a distal enhancer (grey box). Step 2: The enhancer-bound activator recruits ATP-dependent chromatin-remodelling complexes (Rem) and histone-modifying factors (HATs) to the core promoter. Step 3: Mediator complexes (Med) establish the link between the enhancer-bound activator and the basal transcription machinery at the promoter. Step 4: The PIC remains inactive until a

direct contact (looping) occurs between the distal enhancer and the promoter resulting in Pol II recruitment and initiation of transcription (Szutorisz and Dillon, et al 2005)

1.4.3 Transcription Factors

Transcription factors are proteins that bind to the promoter or enhancer elements upstream of genes and function to either initiate or inhibit transcription. Transcription factors are composed of two essential functional regions: a DNA-binding domain and an activator domain. The DNA-binding domain consists of amino acids that recognize specific DNA bases near the start of transcription and the structure of the DNA-binding domain has been used to classify transcription factors. The activator domains are responsible for interacting with RNA polymerase and other regulatory proteins. This interaction often enhances the efficiency with which the basal transcriptional complex can be built and bind RNA polymerase II. Other mechanisms by which TFs can activate transcription are:

1. Induction of a conformational change or post-translational modification (such as phosphorylation) that stimulates the activity of the general transcription machinery.

2. Interaction with proteins that are involved in chromatin modifications so as to permit enhanced accessibility of the promoter region to transcription factors or coactivators.

There are several families of transcription factors and those that are relevant in the

project are discussed below:

1.4.3.1 Zinc Finger Proteins (ZnFs)

Zinc finger proteins include the ubiquitous transcription factor Sp1, Krüppel, GR, GATA family, YY1 etc. The proteins have two or more α-helical domains which are linked by a centrally located zinc ion. In addition to their role in binding DNA, roles for ZnFs have been implicated in protein: protein (RING family) and protein: lipid interactions.

1.4.3.2 The Sp Family

G-rich elements such as GC (GGGGCGGGG) and GT/CACC boxes (GGTGTGGGG) are widely distributed cis-acting elements required for the appropriate expression of genes important in development as well as house-keeping and viral genes. Sp1 was the first transcription factor that was shown to bind GC elements. However, other proteins related to Sp1 namely Sp2, Sp3, Sp4 and members of the KLF family have been characterised to act through the GC elements (Philipsen and Suske 1999). Sp family members bind not only GC-box motifs but also GT (GGGTGTGC) and CTC (CTCCTCCTC) motifs although with different affinities. Sp/KLF family contains at least twenty identified members. The Sp proteins are identical in their zinc finger region and N-terminal motifs. The KLF family is more heterogeneous in comparison and includes factors, BTEB1, TIEG1 and TIEG2 which are closely related to the Sp family and the subfamily, XKLFs which includes the factors, BTEB2, GBF/ZF9, ZNF741 and AP-2rep (Suske, Bruford et al. 2005).

Family members bind the GC boxes with different affinities and hence have different transcriptional properties. Since cells can express multiple family members, there is a possibility of synergistic activation and repression within a network of Sp/KLF factors. The prototype family member, Sp1 can activate transcription by functioning as a basal promoter element and as an upstream activator simultaneously. In many genes lacking TATA boxes, a proximally positioned Sp1 site determines promoter activity and the start site of transcription (Emami, Burke et al. 1998).

1.4.3.2.1 Expression

Except for Sp4 whose expression is restricted to the brain, Sp1, -2 and -3 are ubiquitously expressed (Table 1.4). Individual cells hence can express multiple members of the family (for e.g. Sp1, Sp3 have been detected in vascular endothelial cells (Yet, McA'Nulty et al. 1998), which leads to the possibility that the members can modulate each others activity at the level of expression or at promoters or via direct interactions at the level of protein.

Table 1-6 Expression of Sp Family Members

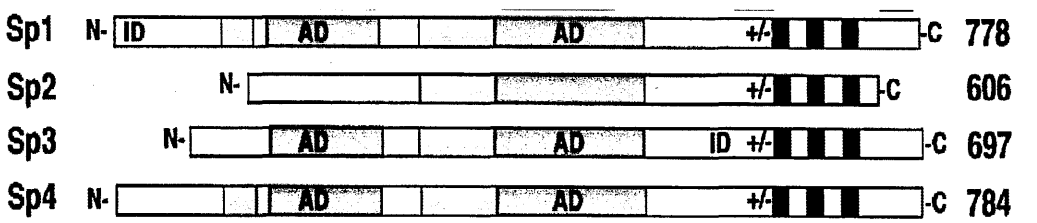
TF	Expression	Function
Sp1	Ubiquitous	Activator
Sp2	Absent in Brain endothelium	Activator
Sp3	Ubiquitous	Activator/Repressor
Sp4	Brain, Epithelial tissues, Testis	Activator/Repressor

1.4.3.2.2 Structure

Sp family members have similar domains and are characterized by a highly

conserved C-terminal DNA-binding domain containing three zinc fingers (Fig 1.11). Critical amino acids in the zinc fingers are conserved in Sp1, Sp3 and Sp4 but not in Sp2 and hence the former are more closely related to each other than to Sp2. Despite conservation in the DNA binding domain, the family members bind target motifs with different affinities (Suske 1999).

Figure 1-10 Structural Features of Sp Family TFs



(Suske 1999)

Their length in amino acids is indicated on the right. Sp3 full length isoform is represented here. Red boxes indicate regions rich in glutamine and yellow boxes indicate serine/threonine rich regions. The black boxes represent the ZnFs and the region preceding the first zinc finger (+/−) is rich in charged amino acids. Known activation (AD) and inhibitory (ID) domains are indicated. (Suske 1999).

1.4.3.2.3 Structural Variants of Sp3

There are three distinct Sp3 isoforms: a full length 110-115 kDa Sp3 protein and two shorter isoforms approximately 80 and 78 kDa species. The lower weight species are generated by internal translational initiation within Sp3 mRNA and possess functional DNA binding domains and are nuclear localised. They bind the Sp target motif with similar affinities (Suske 1999) and function as potent

inhibitors of Sp3 mediated transcription (Kennett, Udvadia et al. 1997).

1.4.3.2.4 Regulation of Gene Expression by Sp1 and Sp3

Sp1 functions primarily as an activator. Sp3 can function either as an activator or a repressor depending on the promoter context and ratio of Sp1:Sp3 in a cell (Sjottem, Anderssen et al. 1996) . Furthermore, functions of Sp3 may depend on the number of functional Sp1 binding sites. Promoters containing a single binding site are activated, whereas those containing multiple Sp1 sites are not activated or respond weakly to Sp3 (Dennig, Beato et al. 1996). In addition, Sp3 protein is a target for phosphorylation. In the regulation of the VEGF promoter, ERK phosphorylates a key serine residue in the Sp3 protein and enhancing its activity *in vitro* (Pages 2007). Sp3 is also modified by acetylation (Braun, Koop et al. 2001) and sumoylation (Ross, Best et al. 2002). These modifications are likely to determine the role of Sp3 in regulating transcriptional activity. Furthermore, unlike Sp1, binding of Sp3 to GC- and GT-boxes is influenced by flanking nucleotides. Amino acid triplet KEE in the inhibitory domain of Sp3 is responsible for the poor activity (Hata, Duh et al. 1998) and mutation of these amino acids to alanine residues converted Sp3 to a strong activator.

1.4.3.3 YY (Yin Yang) 1

YY1 (NF-E1, UCRBP, and CF1) is multifunctional ZnF transcription factor belonging to the GLI-Krüppel class of ZnFs and can act as a transcriptional repressor, activator, or initiator element binding protein (Thomas and Seto 1999) YY1 plays an important role in embryonic development, cell cycle, oncogenesis, X-chromosome inactivation in differentiating and non-dividing cells of all types,

chromatin remodelling, etc (Sui, Affar el et al. 2004; Gordon, Akopyan et al. 2006; Kim, Hinz et al. 2006).

This transcription factor has been implicated in the regulation of a very large number of genes involved in many metabolic processes in the cell. YY1 may target histone deacetylases and histone acetyltransferases to a promoter thereby implicating histone modification as one of the functions. Chronic activation of YY1 is associated with increased cellular proliferation, resistance to apoptotic insults and metastatic potential. The number of promoters that have been reported to be regulated by YY1 is increasing and include human transferrin receptor, p53, viral LTRs, c-Myc, α-actin, IgH enhancer, β-casein, IFN-γ, c-Fos, E6 and E7 of HPV, P6 of B19-parvovirus etc (reviewed in Shi, Lee et al. 1997).

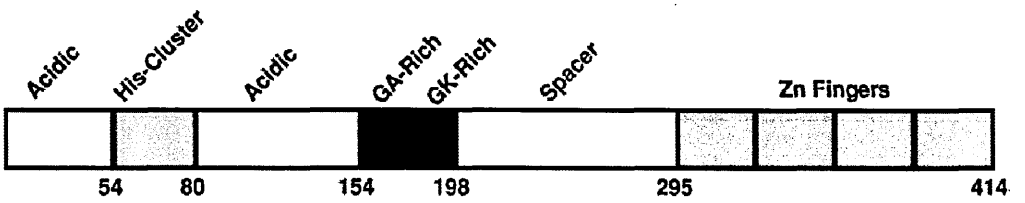
1.4.3.3.1 Related Proteins

In 2004, (Nguyen, Zhang et al. 2004) described the identification of a protein YY2 with significant structural similarity to the transcription factor YY1. YY2 is a transcriptional activator and it appears that it binds only a few YY1 target sites on promoters leading to the suggestion that accessory DNA motifs are needed to recruit YY2 to the DNA. Further investigations are needed to ascertain the function of YY2. (Wang, Liang et al. 2004) reported the identification of an YY1 associated protein (YY1AP). YY1AP is ubiquitously expressed in human tissues and has been shown to enhance activation of YY1 and hence regulate expression from promoters responsive to YY1. The mechanism by which YY1AP synergises YY1 activity is not clear but it is possible the factor is recruited by YY1 itself and tethered to the DNA.

1.4.3.3.2 Structure

The YY1 protein is characterised by four C2H2-type zinc-finger motifs. Regions comprising amino acids 298-397 in the c-terminus serves as an inhibitory domain. Sequences within the zinc finger motifs and a glycine rich region between amino acids 157 and 201 promote the activity of the inhibitory domain. The N-terminus region (AA 43–53) acts as a potent activation domain (Shi, Lee et al. 1997). The role of the 11 consecutive histidine residues (AA 70–80) is not known (Fig.1.13)

Figure 1-11 Domain Structure of YY1



(THOMAS AND SETO 1999)

1.4.4 Basic Leucine Zipper proteins

Basic leucine zipper (bZIP) family of proteins which includes c/EBP, AP1 and yeast GCN4 are a class of enhancer-type transcription factors that play important roles during embryonic development and in the adult organism (Sanyal, Sandstrom et al. 2002; Nerlov 2007) . The leucine zipper motif that characterizes these proteins is composed of leucine residues separated by 7 amino acid residues and repeating at least 3 times (LxxxxxxLxxxxxxL) (Landschulz, Johnson et al. 1988). On binding to a promoter, adjacent basic regions undergo conformational change and also interact with the promoter. This domain is followed by a regulatory domain that can interact with the promoter to stimulate or repress

transcription.

1.4.4.1 GATA1

GATA1, along with five other structurally related proteins, -2 to -6, regulates gene expression in many different cell types. The consensus DNA sequence is A/TGATAA/G which is bound through two highly conserved ZnF domains. The conserved ZnFs also function in interaction with other proteins such as PU.1 and CBP/p300 both of which function as important co-factors enhancing activity GATA-1 activity. The main function of GATA1 is the transcriptional control of genes that are important in hematopoietic lineage. Mutations in the zinc fingers lead to severe dysfunction in the hematopoietic system (Morceau, Schnekenburger et al. 2004).

1.4.5 Other TFs

1.4.5.1 GR (Glucocorticoid Receptor)

GR regulates the transcription of genes carrying the specific GR binding sequence, the glucocorticoid response element (GRE). Some of the BBB-inducing properties of astrocytes are mimicked by corticosteroids (Romero, Radewicz et al. 2003). GR has a single DNA-binding domain that and a ligand-binding domain which is receptive to ligands like cortisol. Inactive GR localises to the cytoplasm to a large multiprotein complex containing heat shock proteins (hsp90, hsp70). Activation by ligand induces conformational changes resulting in the exposure of the GR nuclear localization signal and subsequent nuclear translocation (Chandran and DeFranco 1999) where it interacts with specific DNA sequences, known as

glucocorticoid response elements (GREs), in target genes eliciting a specific transcriptional response. The GREs are normally palindromic, with two hexameric half sites separated by three nucleotides, the perfect palindromic GRE reading 5'-AGAACAnnnTGTTCT-3'. GR acts by transactivation or inhibition of the transactivating function of other transcription factors (transrepression) such as AP1 and NFκB (Schoneveld, Gaemers et al. 2004).

1.4.5.2 NF1

The CTF/NF-1(CCAAT/Nuclear Factor 1 TF) family was first described in the replication of Adenovirus DNA and it has been established that this family is important in the growth state of cells and oncogenesis. The binding motif is TTGGC (N5) GCCAA and the protein has been shown to bind to individual half sites-TTGGC and GCCAA though with reduced capacity. NF1 proteins have been identified as negative regulatory elements of a number of promoters including peripherin, eta-globin, Pit-1 and Glut4 genes (Dusserre and Mermod 1993).

1.4.5.3 c/EBP

The c/EBP (CCAAT/enhancer binding protein) family consisting of six members (c/EBP-α, –β, –γ, –δ, –ε, and CHOP-10/GADD153) is bZIP transcription factors which have been shown to bind to the dyad-symmetric sequence ATTGCGCAAT either as homodimers or heterodimers. Members of the C/EBP family have been implicated in regulating the differentiation of adipocytes, hepatocytes, and myelomonocytes (Nerlov 2007).

1.4.6 Co activators and Co repressors

The coactivators and co repressors proteins effect transcriptional activity through protein–protein interactions without binding DNA directly. Genomic DNA is packaged efficiently around protein complexes called histone octamers which serves the purpose of packing DNA in the nucleus but at the same time, this highly compact state limits access of TFs and other regulatory proteins to their target sites.

One mechanism by which chromatin is made more accessible to regulatory proteins is by histone acetylation. Histones are positively charged proteins that interact tightly with the negatively charged DNA. Acetylation of histones reduces charge and loosens their interaction with DNA leading to an increase in binding of transcription factors. Several transcription factors in a variety of organisms have now been found to be histone acetyltransferases (reviewed in (Kuo and Allis 1998; Roth, Denu et al. 2001). To counteract HATs are the histone deacetyltransferases (HDACs) which catalyse the removal of acetyl groups from lysine residues in histones leading to chromatin condensation and repression of gene transcription (Kuo and Allis 1998).

1.5 Previous work

Promoters of transferrin receptor, claudin 5, and p-glycoprotein-1 were investigated for common sequence motifs in the 2000 bp region 5' to the initiation codon. Expression of these proteins is seen in brain endothelium and not in other endothelia. However, these proteins are not restricted to brain endothelium and

found in other cell types such as the epithelial tissue.

Commonly expressed endothelial markers Duffy antigen receptor for chemokines, P-selectin and p-glycoprotein-3 were used as controls. None of these proteins overlap in function. Work by David Male identified repeated motifs - (GGGC; GCCCC) in these regions which are heavily over-represented in the genes restricted to brain endothelium, but were present at standard frequencies in a set of control genes. Presence of cluster of common motifs in the cis-5' regions of diverse brain endothelial genes suggests these are under common transcriptional control. The GC content in the control gene promoters and the CNS gene promoters is similar and hence the high incidence of the motif only in the CNS genes rules out non-specificity.

Sp1/3 (target site is –GGGCGG-) is a known transcription factor that could bind this motif partly due to the similarity to the –GGGGC- motif. Analysis of the claudin 5 gene yielded a second cluster of 8 –GGGGC- motifs at –750 to –1100 bp which were regularly spaced at every sixth turn of the DNA helix (69bp), shown below, which suggests a target site for a zinc-finger transcription factor. Table 1.7 shows the number of iterations of this motif in a segment of 500bp located between –150bp and –650bp of the first exon of the three proteins and in a previously identified enhancer region of p-glycoprotein-1

The sequence below shows an example of an identified motif in the 5' region of the human claudin 5 promoter.

caggcccagg**gcccc**agcctcacccccatgccactcactgcctctctggagcctgagtctctggcaaaaagcggt

ggcacaggggctttccccctgcctggcgacccccactctcccaagccgcaggggcttcccagacctctcaatcttca

cagggggctgctcctcttcctgcggtactgcccgcaccccatccttgggggccagttcaggtgacaccacttcagga

agttcccagtgacccagcacactgggtgtagcacccaggggcagtggtggccccaggcctagcagcctgctctggc

cttcagacaggagagacaaagggacacggagggggctgtgccctgccctcccaccagtggcgatggtgtccctggc

accccagccccccaggccaccctccggaagccaacttgga

Table 1-7 GC-rich Motif in Enhancers of Brain Endothelial Specific Genes.

Protein	Gene	Expression	Forward (cis)	Reverse
Claudin 5	CLD5	CNS endothelium	6	1
Transferrin receptor	TFR	CNS endothelium & dividing cells	9	2
Pgp-1	MDR1	CNS endothelium	2	-
occludin	OCCLD	CNS endothelium	3	5
p-glyc. enhancer	MDR1	CNS endothelium	3	-
Duffy ARC	FY	Endothelium & erythrocytes	1	2
P-selectin	PSL	Most endothelia & platelets	-	-
Pgp3	MDR3	Epithelia outside the CNS	-	-

1.6 Aims of the Project

Endothelial cells in different tissues vary widely in structural phenotypes and function and are highly specialised in order to meet the requirements of the local tissue environment. For example, some of the important characteristics that

distinguish brain- from non-brain endothelium include the presence of continuous tight junctions, expression of specific receptors and transporters, high expression of heparan sulphate glycoproteins and sialic acid on the cell surface, high expression of the chemokine receptor CXCR2. Tight junction proteins like occludin and claudin 5 determine the integrity of tight junctions and hence the function of the brain endothelium. Occludin is an integral membrane protein at the tight junction and is restricted to endothelium in the CNS. It is however, present in other cell types such as gut epithelium where continuous tight junctions are seen. The molecular mechanisms regulating differential gene expression in brain and non-brain endothelia are not known. The constituents of the tissue environment such as astrocytes, pericytes and soluble mediators have been known to contribute to the development of the barrier function in the brain. Studies have identified growth factors, transcription factors and signalling pathways that are responsible for the initiation of the endothelial phenotype in various tissues. It is possible these factors could be expressed in the endothelial cells and maintaining the expression of these factors can be responsible for the terminal phenotype. The general aim of this work is to identify transcriptional mechanisms involved in the regulation of brain endothelial specific proteins which could shed light on terminal differentiation of endothelium. We have analysed the regulation of occludin in this present study. Unlike other studies which have examined cues from cells in the native tissue, this study will aim to investigate the possibility that terminal differentiation in the brain is undertaken by transcription factors which are either activated only in brain endothelium or interact in a specific manner absent in non-brain endothelium.

The specific aims of the present study are:

1. Analyse activity of the occludin promoter in brain and non-brain endothelial cells

Full length and promoter fragments cloned upstream of GFP sequence in a promoter deficient vector on transient transfection in brain (hCMEC/D3) and non-brain (lung) cells will provide data on any differences in promoter activity.

2. Analyse transcription factor binding profiles *in vitro* and *in vivo*

Labelled promoter fragments will be analysed for binding of transcription factors present in nuclear preparations of brain and non-brain endothelial cells. Any difference in profiles of transcription factors leads to possible candidates. Immunoprecipitation of endogenous promoter in the different endothelial cells with antibodies to the possible transcription factors will provide information of transcription factors bound in a 'living' state.

3. Analyse expression and localisation of relevant transcription factors in brain and non-brain endothelium and investigate for any possible interactions between the target transcription factors.

4. Generation of reporter vectors containing occludin promoter fragments with deletions in specific transcription factor binding motifs and determine activity in brain endothelium.

5. Inhibition of target transcription factor(s) and investigate changes in expression of the occludin protein in brain endothelium.

Why is the study of transcriptional regulation in endothelia important?

1. Phenotype of endothelium is dictated in large part by space and time specific differences in gene expression

For e.g. glomerular endothelium in the kidney and endothelium of the blood brain barrier (BBB)

This provides important insights into the molecular basis of vascular diversity

2. Transcriptional networks act as signal transducers in the endothelium coupling input to output. For example, ECs in the blood brain barrier receive input from surrounding astrocytes

This helps understand the link between microenvironment and cellular phenotype

3. Each Transcription Factor (TF) is coupled to multiple upstream signals and downstream target genes

Characterisation of these small 'hubs' of activity in the context of network topology will provide:

I. Important mechanistic information

II. Foundation for targeted therapy

Chapter 2

General Materials and Methods

2 General Materials and Methods

2.1 Primary Antibodies

Table 2-1 Primary Antibodies

Antibody	Species of Ab	Immunogen	Clone /PAD*	Application	Dilution	Company
Claudin 5	rabbit	peptide from C-terminal region of claudin 5 protein	Z43.JK	WB, FACS, IHC, IF	1:500 1:50	Zymed/ Invitrogen
Claudin 5	rabbit	167-218 aa's of CLD5 protein	H-52	WB, FACS, IF	1:500 1:100	Santa Cruz
Occludin	rabbit	peptide from C-terminal region of occludin protein	ZMD.467	WB IF, IHC	1:500 1:100	Zymed/ Invitrogen
Occludin	mouse	fusion protein containing the C-terminal region of occludin	Z-T22	WB IF, IHC, FACS	1:500 1:50 1:100	Zymed/ Invitrogen
ZO1	rabbit	recombinant ZO1 containing a region that lies N-terminal to the α–motif	Z-R1	IF	1:100	Zymed/ Invitrogen
TR (Transferrin receptor)	mouse	Rec. human transferrin receptor	H68.4	WB FACS	1:500 1:100	Zymed
Von Willebrand factor (vWF)	rabbit	aa's 2514-281 mapping at the C-terminus of vWF	H-300	FACS	1:100	Santa Cruz
YY1	rabbit	aa's 1-414 representing full-length YY1	H-414	WB, IF, IHC, IP	1:500 1:100 2 µg	Santa Cruz

* PAD: Polyclonal antibody designation

2.2 Cell Culture

All cell lines were human in origin. hCMEC/D3 (D3) (Weksler, Subileau et al. 2005), a brain microvascular endothelial cell line, bone marrow endothelial cell line (BMEC), primary lung (LMVEC) and primary human brain endothelial cells (HBMEC) were used in all experiments.

hCMEC/D3 cells were grown on collagen (Sigma, Cat No: C8919) coated flasks or culture plates and maintained in EGM-2 MV medium (Cat No: CC-3202, Lonza Biologics, Cambridge, UK) supplemented with 2.5% foetal bovine serum, and a quarter of the supplied growth factors-vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), human fibroblastic growth factor (FGF) but fully complemented with the supplied quantities of hydrocortisone, ascorbic acid and gentamicin sulphate.

Lung (LMVEC) and dermal (DMVEC) endothelial cells were purchased from Clonetics/Biowhittaker (Wokingham, UK) and grown in fully supplemented EGM-2 MV medium according to the manufacturer's recommendations.

The transformed human BMEC line was donated by Babette Weksler. Cells were maintained in DMEM (Cat No: 31885-023, Invitrogen) with low glucose (1mg/ml), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and 2.5 μg/ml amphotericin-B and with 10% FCS.

hCMEC/D3 cells were used between passages 21 and 30; lung and dermal

endothelial cells were used at passages between 3 and 7; bone marrow endothelial cells at passages 22-28 and primary human brain endothelial cells at passages 1-4. The different endothelial cells were passaged when cultures reached 60-70% confluency. The monolayers were washed with HBSS without Ca^{2+} and Mg^{2+} (Cat No: H6648, Sigma Aldrich) and subjected to 0.25% trypsin-EDTA (Cat No: 25200-072, Invitrogen) solution for 5 min at 37°C after which the cells were centrifuged in excess medium and plated as required. For all the analyses described in the methods, confluent monolayers were rested in EGM-2 MV medium without growth factors but with serum, antibiotics and hydrocortisone for 48 hours before assay.

2.3 Isolation of Primary Brain Endothelial Cells (HBEC) from Human Donor Tissue

Buffers

Buffer A: HBSS without Ca^{2+} and Mg^{2+} supplemented with 10 mM Hepes (Sigma Aldrich, Cat No: 4034)

Collagenase/Dispase (C/D) solution: lyophilised powder (obtained from Roche Diagnostics Ltd, Cat No: 10269638001) was reconstituted in HBSS without Ca^{2+} and Mg^{2+} to a final concentration of 1mg/ml and filter sterilised through a 0.22 μm (Schleicher & Schuell) membrane filter and 20 ml aliquots were stored at -20°C. The final concentration of the enzymes was, collagenase: 0.1 U/ml and dispase: 0.8 U/ml.

DNase I solution: 2000 U of the lyophilised enzyme (obtained from Roche Diagnostics Ltd, Cat no: 11284932001) was dissolved in 1 ml of sterile HBSS without Ca^{2+} and Mg^{2+} to achieve a final concentration of 20 U/ml. The solution was aliquoted into equal volumes of 200 µl and stored at -20°C. One aliquot was used to reconstitute 20 ml of collagenase/dispase solution.

TLCK (Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride) solution: (Sigma Aldrich, Cat No: T7254). Final concentration of 0.147 mg/ml was achieved by dissolving 2.94 mg of TLCK in 20 ml of sterile HBSS without Ca^{2+} and Mg^{2+} and filter sterilised through a 0.22 µm membrane filter and 1 ml aliquots were stored at -20°C. 20 µl was added to 20 ml of collagenase/dispase solution just before use.

24% BSA: 5 gm of bovine serum albumin powder (Sigma Aldrich, Cat no: A4161) was dissolved in 20 ml of buffer A and filter sterilised serially using a 0.8 µm and then a 0.22 µm membrane filter and stored at 4°C.

Percoll® gradient: 4.5 ml of Percoll® (Sigma Aldrich, Cat no: P4937) was added to 500 µl of 10x HBSS without Ca^{2+} and Mg^{2+} and 5 ml of 1x HBSS without Ca^{2+} and Mg^{2+} in DuPont tubes and centrifuged at 16500 rpm for 1 hour at 4°C.

Post mortem tissue obtained from MS patients (MS society, Charing Cross Hospital, London, UK) or from tissue obtained from temporal lobectomy resections for epilepsy at the Kings College hospital (London, UK) was used for the isolation of primary brain endothelial cells. The work was approved by the ethics committee at KCH.

Primary HBEC were isolated according to the method developed by Hughes and

Lantos in 1986 with slight modifications. After removal of the meninges, the tissue was cut into smaller pieces with a scalpel and centrifuged at 1500 rpm for 5 min in buffer A. The tissue was resuspended in 15 ml of the collagenase/dispase solution and incubated for 1 hour at 37°C with occasional shaking. The suspension was then triturated with a Pasteur pipette until all the grey matter was dispersed and centrifuged at 1500 rpm for 5 min and the supernatant discarded. The digested tissue was resuspended in 17 ml of 25% BSA, mixed gently and centrifuged at 2900 rpm for 15 min. After removal of the myelin and the top layers, the capillary pellet was washed in buffer A and incubated with 5 ml of the enzyme digest solution for 2 hours at 37°C with occasional shaking.

The digested capillary pellet was washed and resuspended in 2 ml of buffer A and gently layered over a pre-spun 50% isotonic percoll gradient and subjected to density gradient centrifugation at 2900 rpm for 10 min to separate capillary fragments from single cells surrounding vessels and erythrocytes. The middle fraction of the gradient containing endothelial cells was gently aspirated and added to excess of buffer A. After centrifugation to wash off the percoll, microvessel fragments were plated on collagen coated flasks or cover slips with EGM-2 MV medium supplemented with 2 μg/ml puromycin (Sigma, Cat No: P8833) and 100 U/ml of Penicillin G and 100 μg/ml Streptomycin. Puromycin selectively eliminates cells that do not express p-glycoprotein (Weksler, Subileau et al. 2005). In addition, it is also cytotoxic to cells like astrocytes that express the protein albeit at a lower level than seen in endothelial cells. Medium was changed every 48 hrs and cells were grown without puromycin after the first passage.

2.4 Immunofluorescence

Cells were grown to confluence on collagen-coated cover slips and rested for 2 days in medium complemented with serum but lacking growth factors. The monolayers were washed with PBS for 15 minutes with three changes of the buffer. For staining for tight junction proteins, methanol was added along the walls of the culture plate containing the cover slips and the plate was quickly transferred to -20°C for 10 min. Claudin 5 (Z43.JK), Occludin (ZMD.467) and ZO1 (Z-R1) were used in the immunostaining. Methanol was gently aspirated from the wells with chilled PBS being added simultaneously and this step was repeated three times to rid of residual methanol. The cover slips were then incubated with blocking buffer (0.5% BSA in PBS) for 15 min at RT. Cells were then incubated with the primary antibody for 2 hours at RT and washed in PBS for 15 min with three changes of the buffer. This was followed by incubation with rabbit anti-mouse or goat anti-rabbit IgG conjugated FITC (1:250, Vector Labs, Burlingame, CA) for 1 hour at RT. Cells were washed in PBS for 40 min with four changes of buffer and the cover slips were mounted on slides with DakoCytomation fluorescent medium (Carpinteria, CA) and analysed by fluorescent microscopy.

2.5 Characterisation of endothelial cells

The different endothelia were characterised for expression of the endothelial marker vWF by FACS analysis (Fig. 2.1). Expression of the TJ proteins occludin and claudin 5 were analysed by western blot (Fig. 2.2). In addition, expression of

the transferrin receptor and pgp-1, markers that confirm the brain endothelial phenotype of hCMEC/D3 cells (Weksler, Subileau et al. 2005) were also investigated. Localisation of the TJ proteins occludin, claudin 5 and ZO1 was observed by immunofluorescence microscopy in hCMEC/D3 cells (Fig. 2.3). Results shown are representative of three experiments.

The TJ protein occludin was detected migrating at 65 kDa and is strongly expressed by hCMEC/D3 cells, faintly expressed in lung endothelial cells and was absent in dermal endothelium (Fig. 2.2). The transferrin receptor was detected as a band migrating at 95 kDa; P-glycoprotein-1 was detected at 170 kDa, claudin-5 at 20–22 kDa (Fig. 2.2).

Localisation of the TJ proteins occludin, claudin 5 and ZO1 was observed by immunofluorescence microscopy in hCMEC/D3 cells (Fig. 2.3). The proteins were localised to the tight junctions. However, ZO1 and occludin were also seen in the nucleus.

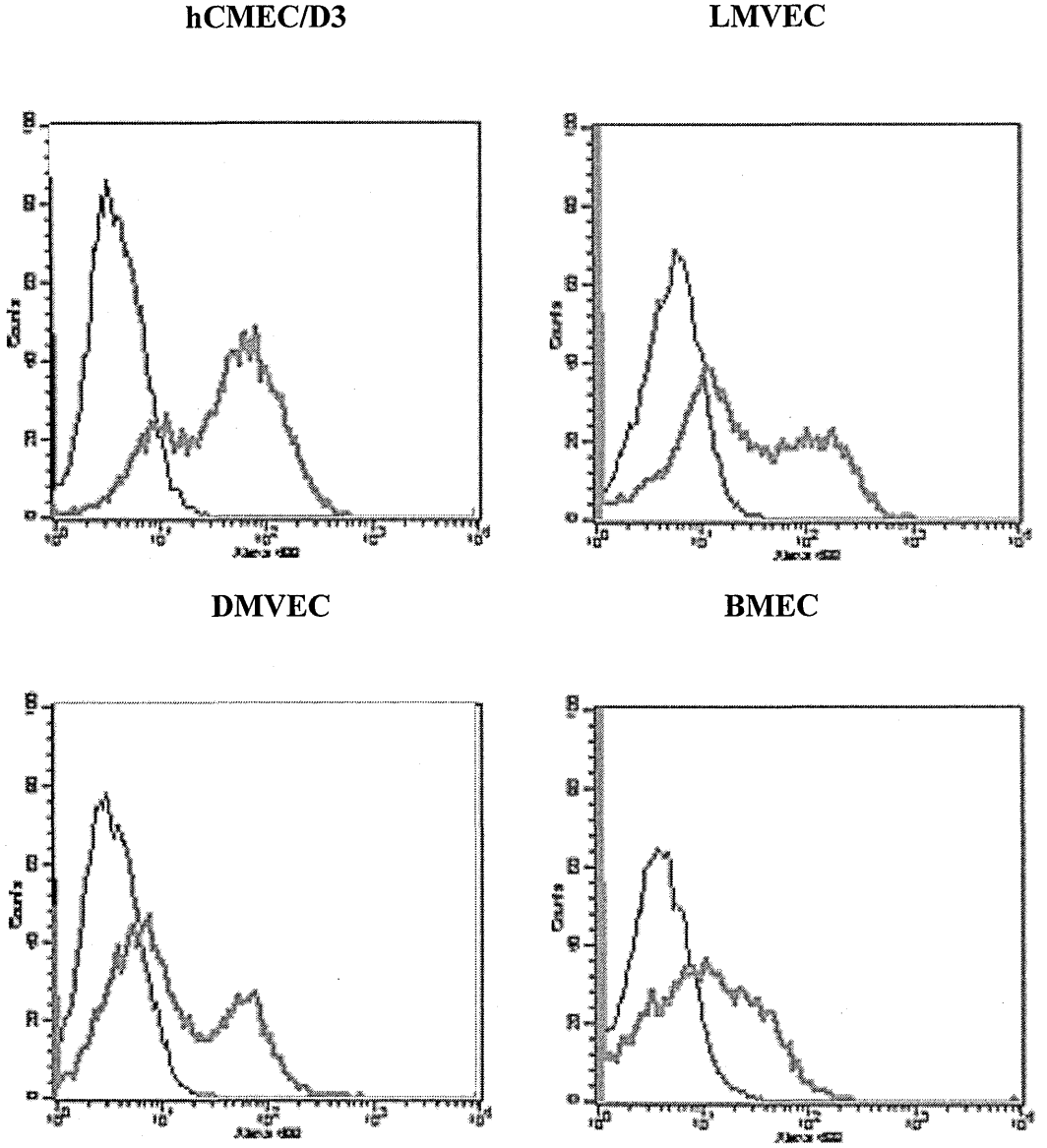
Localization of occludin at tight junctions is controlled by posttranslational modifications such as phosphorylation of the protein and also by the reorganization of the actin filaments during cell division (Sakakibara, Furuse et al. 1997, Kojima, Sawada et al. 1998). Serine/threonine phosphorylated form of occludin is selectively concentrated at the TJ whereas phosphorylation on tyrosine residues leads to its disassociation from the TJ complex. In hepatocytes that localization of occludin and actin simultaneously changed upon cell proliferation and there was a decrease in immunoreactivity of occludin at cell-cell contacts and increased cytoplasmic and nuclear localization (Kojima, Sawada et al. 1998). In

bovine aortic endothelial cells, occludin expression has been shown in the nucleus but when these cells are subjected to cyclic strain, the protein localizes to cell borders (Collins, Cummins et al. 2006). However, the function of occludin in the nucleus is not known. It has been suggested that occludin may interact with ZO1 to modulate expression of other TJ proteins but there is no supporting evidence.

It has been documented that ZO1 interacts with ZONAB, a TF homologous to Y-box proteins (Balda and Matter 2000). The two proteins functionally interact in the nucleus to modulate the expression of the *erbB-2* proto-oncogene, indicating a role for regulation of gene expression for TJ proteins (Balda, Garrett et al. 2003).

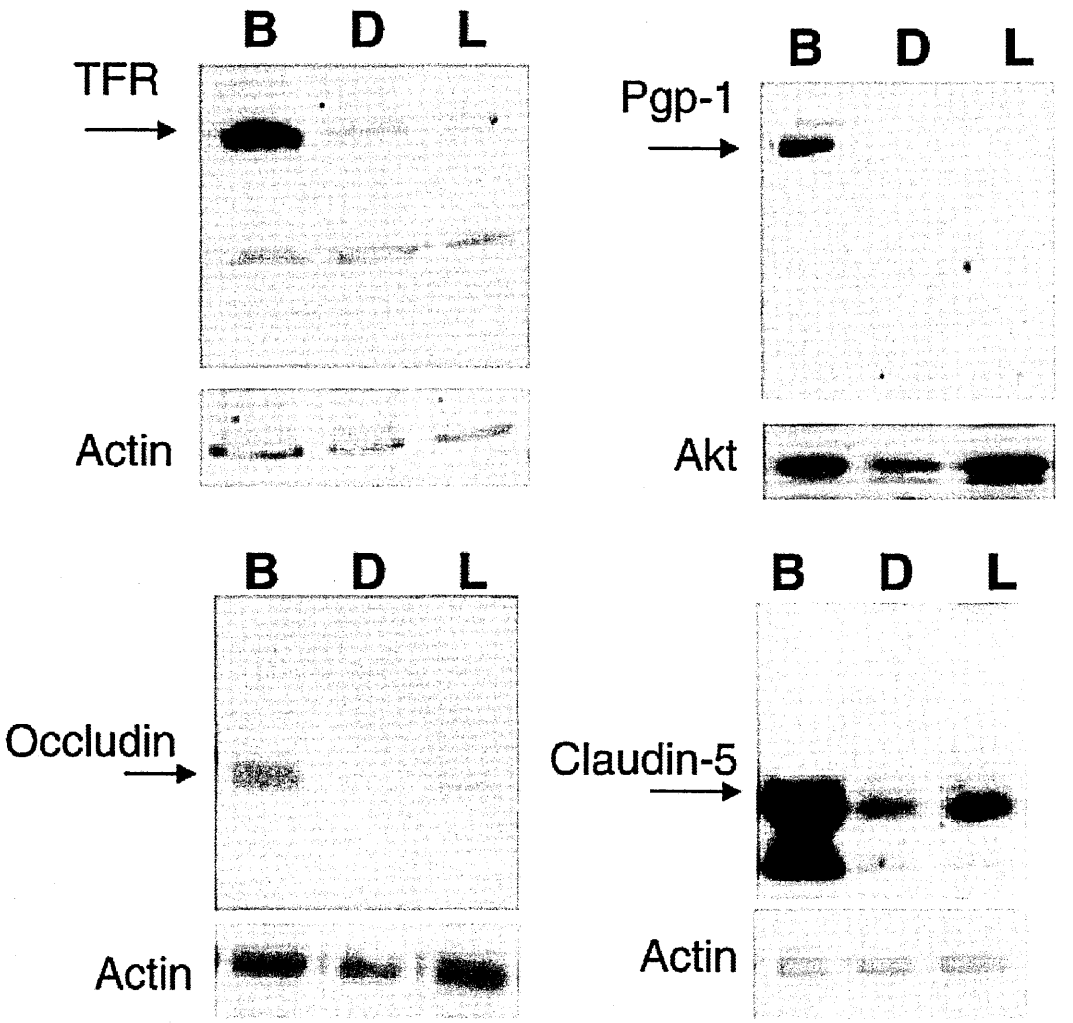
All the endothelial cells expressed the vWF marker the expression of which is restricted to endothelial cells and cells of megakaryocytic origin. The von Willebrand factor mediates adhesion of platelets to endothelial cells at site of injury.

Figure 2-1 Expression of the Endothelial Marker vWF in Different Endothelia



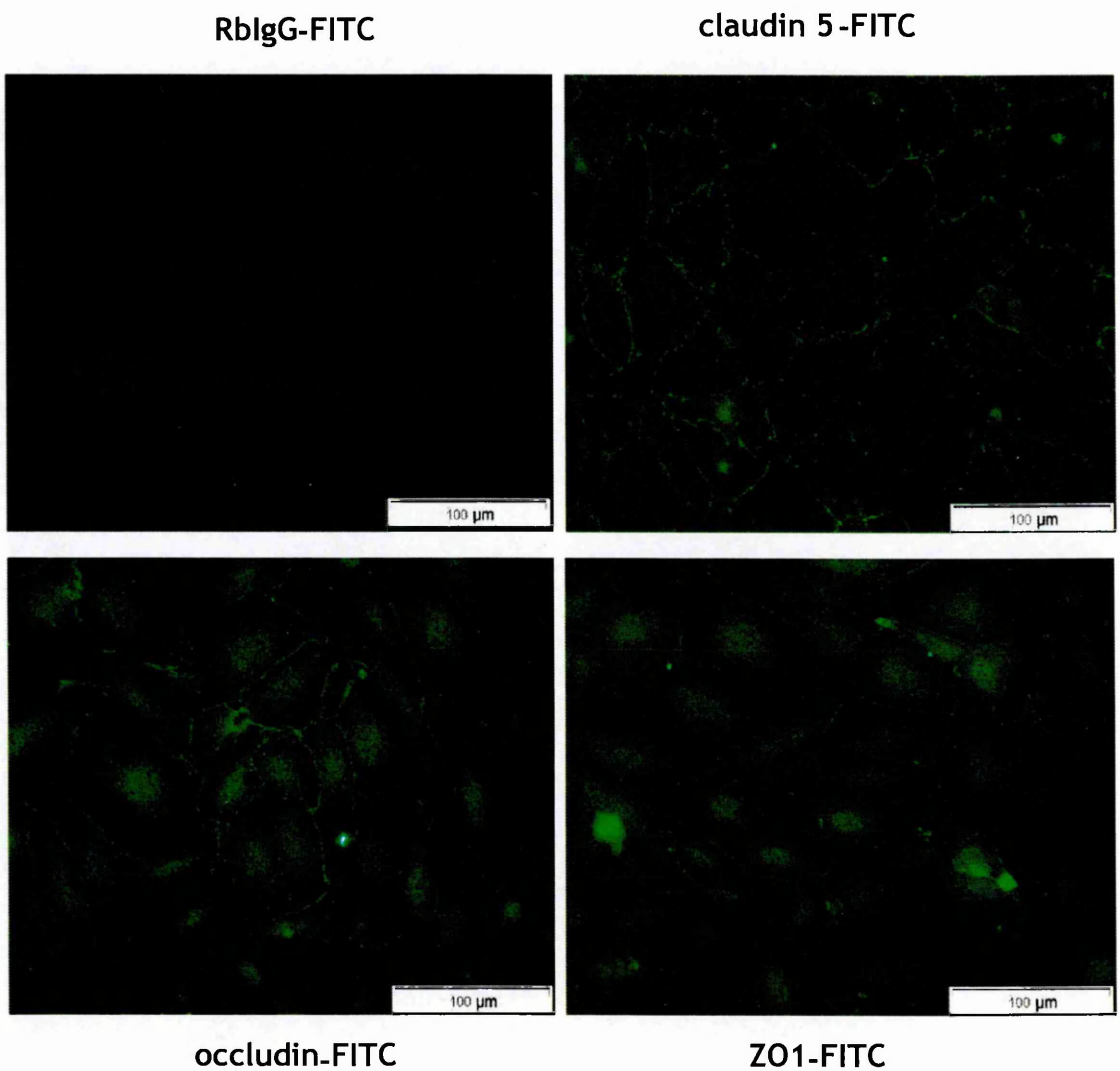
hCMEC/D3, lung (LMVEC), dermal (DMVEC) and bone marrow (BMEC) cells were grown to confluence and immunostained for expression of the endothelial marker vWF (grey histograms). Black histograms indicate cells stained with the control antibody (RbIgG)

Figure 2-2 Expression of the TJ Protein occludin in Different Endothelia Analysed by WBA



Whole cell lysates (20μg) from hCMEC/D3 (B), dermal (D) or lung (L) endothelial cells were separated on 10% SDS-PAGE gels. Blots were probed for transferrin receptor (TFR), p-glycoprotein-1 (pgp-1), occludin and claudin-5. Arrows indicate the expected Mr of each of these markers. Blots were stripped and reprobed for actin or Akt ascontrols for equal protein loading.

Figure 2-3 Localisation of occludin, claudin 5 and ZO1 at the TJ in hCMEC/D3



hCMEC/D3 cells were grown to confluence on coverslips and rested for 48 hours. Following fixation with methanol, the cells were immunostained for expression of the TJ proteins-occludin, claudin 5 and ZO1 or control antibody (RbIgG) and analysed by fluorescent microscopy.

Chapter 3

Activity of human occludin promoter

3 Activity of occludin promoter *in vitro*

3.1 Introduction

The activity of the occludin promoter has been determined in the human intestinal cell line, HT-29/B6 (Mankertz, Tavalali et al. 2000). The authors concluded that region of the promoter spanning nucleotides 1402-1853 (451 bp) is sufficient for the promoter activity. Further, the work also identified the minimal promoter which comprises of 208 bp and extends from 1573-1853. Inversion of these sequences abrogated activity of the promoter and hence luciferase activity leading to the conclusion that the promoter sequence is unidirectional.

We hypothesised that activity of the full length and specific regions of the occludin promoter may not be similar between cells of epithelial and endothelial origin. To investigate the regions of the promoter essential for function in brain endothelial cells, we constructed a series of constructs in which different lengths of the occludin promoter were ligated into the reporter vector, pGlowTOPO®, which is a promoter deficient GFP expression vector. This vector can be used to analyse in addition to other functions, cell-specific promoter function and deletions within a promoter. CMV promoter cloned similarly served as a control for transfection efficiency. The reporter vector with no added sequence was used as a negative control. The longest construct we examined was the full length promoter (occ-FL). The shortest construct was F8, which contained only 120 bp of the promoter. Promoter activity and strength of different promoter fragments can be determined by assaying GFP activity after ectopic expression in cells of

interest.

This chapter presents data on the functional analysis of the human occludin gene promoter in brain and non-brain endothelium.

Aims:

1. To analyse the activity of the occludin promoter in brain and non-brain endothelial cells

2. To determine the regulation of promoter activity in brain and non-brain endothelium

3. To determine the smallest region of the promoter sufficient for promoter activity (minimal promoter).

3.2 Materials and Methods

3.2.1 Generation of reporter plasmids

The full length and specific fragments of the occludin promoter were PCR amplified using primers described before. PCR reactions were run on 1% agarose gels and fragments migrating at the appropriate size were excised from the gel, extracted and purified (SpinPrep Gel DNA kit, Novagen) The fragments were ligated into pCR2.1-pGlow-TOPO® reporter vector (Invitrogen, Paisley, UK) following manufacturers instructions. Colonies obtained after transformation of TOP10F` E Coli cells were grown in LB medium with ampicillin and the DNA isolated (Wizard Plus SV Mini-Prep, Promega) was screened by double digestion with restriction enzymes to release the insert. The DNA was further subjected to PCR amplification using M13 primers and specific primers for the insert.

FRAGMENT	PRIMERS FP-RP	OCCLUDIN Ch. 5 NT_006713	SIZE (bp)
0	1-225	19380963-1938118	225
1	202-338	19381165-1938130	136
2	312-619	19381275-19381582	307
3	598-864	19381561-19381827	256
4	842-1091	19381805-19382054	249
5	1068-1238	19382031-19382201	170
6	1218-1524	19382181-19382487	306
7	1505-1754	19382468-19382717	249
8	1733-1853	19382696-19382816	120

3.2.2 Transient transfections

hCMEC/D3, DMVEC and LMVEC cells were transfected with occludin DNA promoter fragments cloned in pGlow TOPO vector using the reagent Lipofectamine™ 2000 as per manufacturer's instructions (Invitrogen, Paisley, UK).

Briefly, 2-6 x 10^5 cells were plated per well on 6 well plates in 2 ml of EGM2-MV medium and cultured until 60% confluent. For each transfection condition, 2 µg or 5 µg of DNA was diluted in 250 µl of basic EGM-2 medium in a sterile eppendorf. Similarly, 10 µl of Lipofectamine™ 2000 was diluted in 250 µl medium for each transfection condition and incubated for 5 min at RT. 250 µl of the diluted Lipofectamine™ 2000 reagent was added to each tube containing the diluted DNA, mixed gently by rocking and incubated for 20 min at RT to allow DNA-Lipofectamine™ 2000 complexes to form. The medium was aspirated and cell monolayers were washed with HBSS with Ca^{2+} and Mg^{2+} for 10 min with two changes of the buffer. The DNA-Lipofectamine™ 2000 complexes (~500µl) was added directly to each well of the 6 well plates containing cells and swirled so the reagent covered the cell monolayer and were incubated at 37°C for 6 hrs. The DNA-Lipofectamine™ 2000 complexes were then removed from the wells and EGM2-MV medium without antibiotics but with reduced serum (1%) and growth factors was added and cells cultured for a further 48-96 hours before analysis for GFP expression by FACS analysis.

3.2.3 FACS analysis

Transfected cell monolayers were washed once in HBSS without Ca^{2+} and Mg^{2+} and then detached from the matrix by incubation with 0.25% trypsin-EDTA at 37°C for 5 min. Cells were then centrifuged at 1500 rpm for 5 min and cell pellets resuspended gently in excess chilled PBS and centrifuged at 1500rpm for 5 min. After an additional wash, cell pellets were resuspended in chilled PBS for analysis by FACS.

Transfection of the brain and lung microvascular endothelial cells lead to the generation of heterogeneity in the cell population. The forward scatter and side scatter profiles of cell populations that have been successfully transfected showed two populations of cells readily distinguished by the differences in the forward scatter profiles (Fig. 3.1). These two distinct populations can also be differentiated in the expression of the transgene. Smaller cells corresponding to lower forward scatter profile and gated as region R2 represent the transfected population. The untransfected population has been gated into region 1. Gate 3 (R3) on the FACS profiles represent both the R1 and the R2 gates.

Figure 3-1 FACS Profiles of Transfected Cells

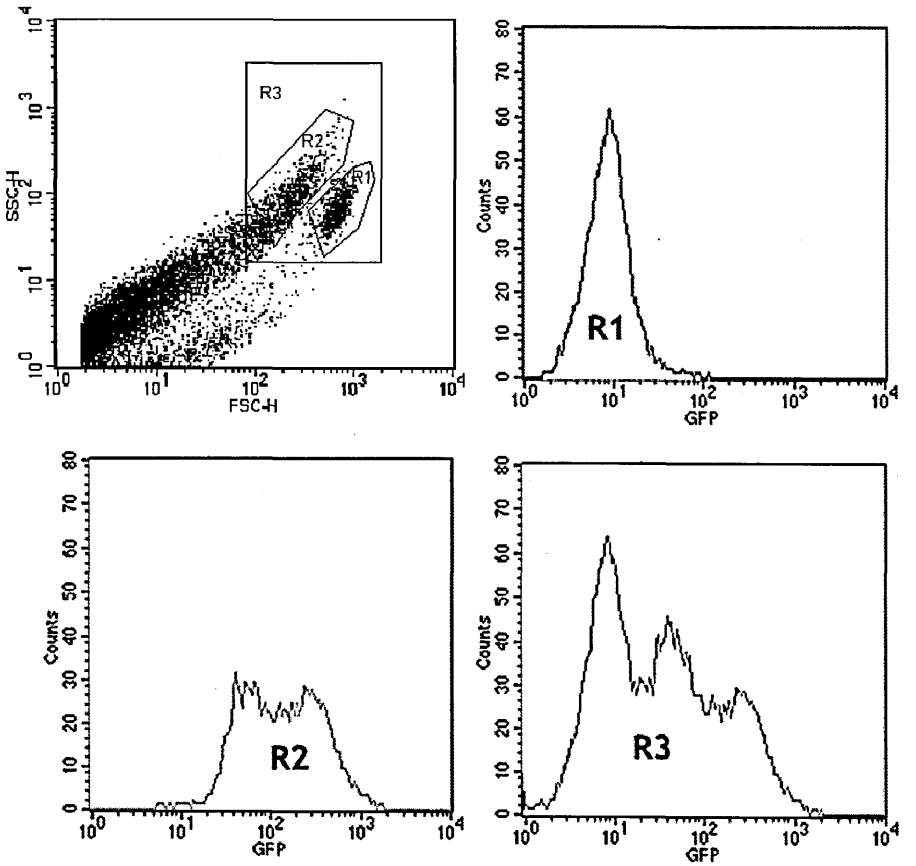
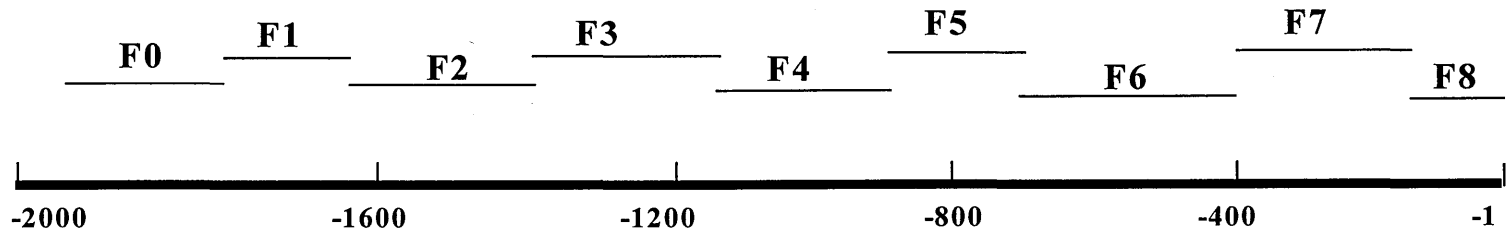


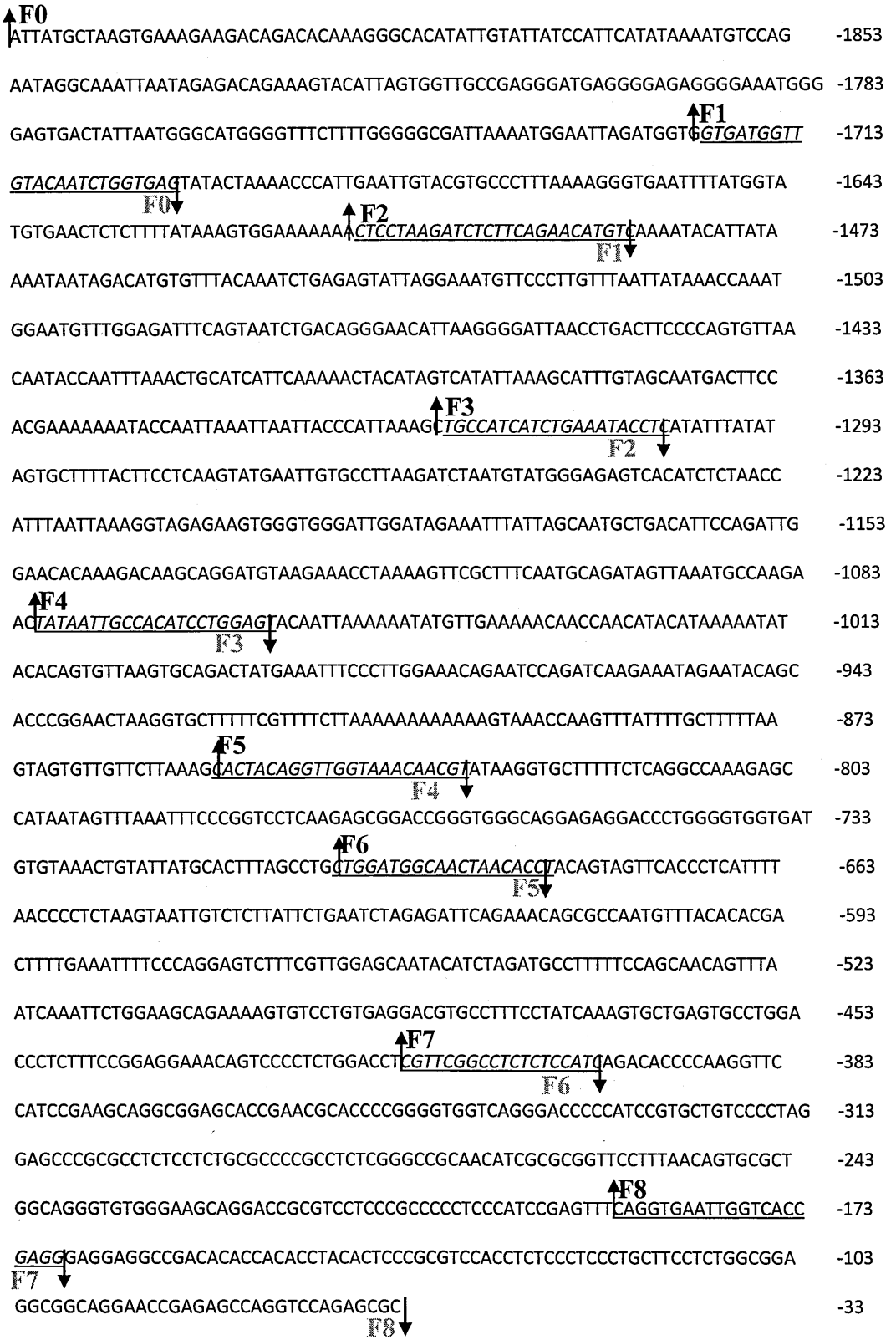
Figure 3-2 Organisation of occludin promoter



FRAGMENT	PRIMERS FP-RP	OCCLUDIN Ch. 5 NT_006713	SIZE (bp)
0	1-225	19380963-1938118	225
1	202-338	19381165-1938130	136
2	312-619	19381275-19381582	307
3	598-864	19381561-19381827	256
4	842-1091	19381805-19382054	249
5	1068-1238	19382031-19382201	170
6	1218-1524	19382181-19382487	306
7	1505-1754	19382468-19382717	249
8	1733-1853	19382696-19382816	120

Figs. 3.2 and 3.3: Schematic showing the organisation of the different promoter fragments used in the deletion constructs. In Fig. 3.3, upright or downright arrows indicate the start and end of an individual fragment respectively. Underlined and italicised sequences are overlapping sequences between two fragments.

Figure 3-3 Schematic of the Occludin Promoter showing Fragments used in Deletion Constructs



3.3 Results

3.3.1 Activity of Full Length Human Occludin Promoter in Brain and Non-Brain Endothelium

The three constructs containing either no sequence (black histograms, empty vector) or the full length promoter (Figs. 3.4 and 3.5) or the CMV promoter (Figs. 3.6 and 3.7) were transiently transfected into hCMEC/D3 (Figs. 3.4 and 3.6) and LMVEC cells (Figs. 3.5 and 3.7). Cells were assayed for GFP expression 56 hours (hCMEC/D3) or 72 hours (LMVEC) post transfection.

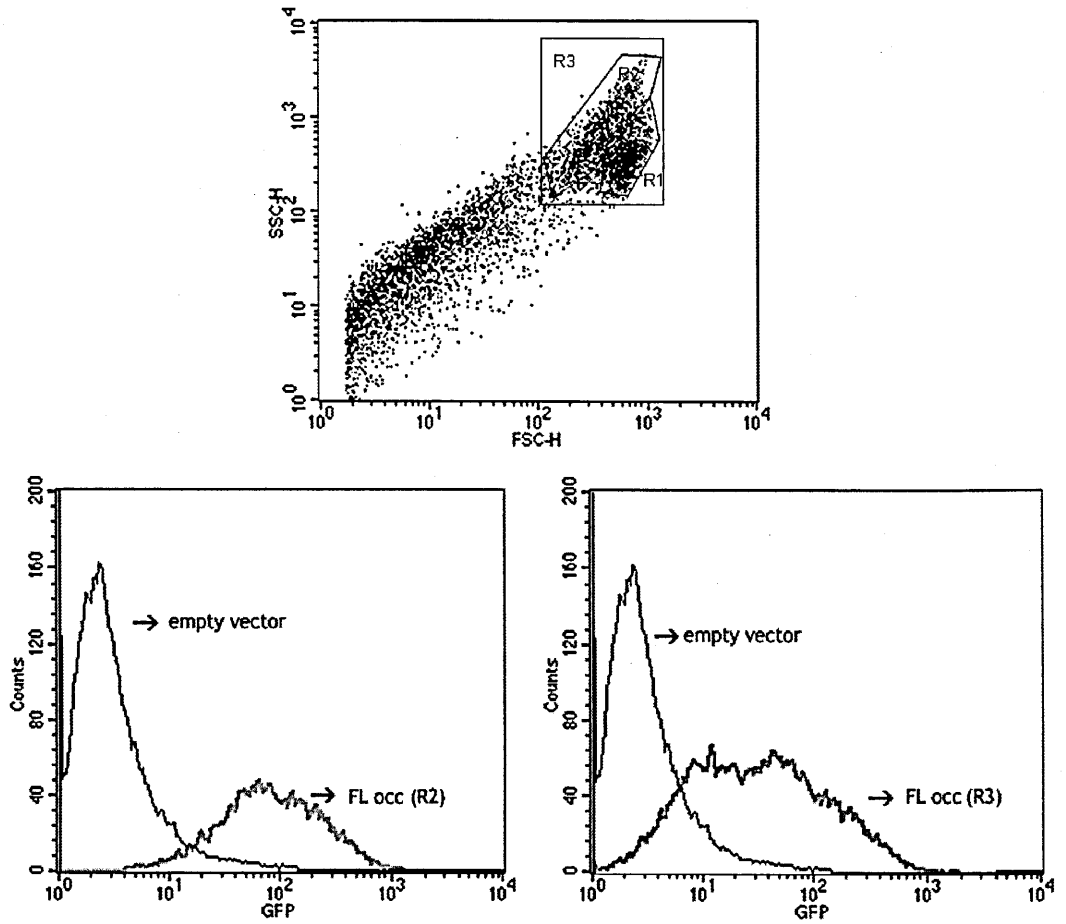
Results clearly indicate the expression of GFP driven by the CMV promoter in hCMEC/D3 and LMVEC cells is similar though seen at different time points (Figs. 3.6 and 3.7). Between experiments, we encountered different transfection efficiency rates between the transformed and the primary cells. It was easier to ectopically introduce foreign DNA into hCMEC/D3 cells but the primary LMVECs were resistant to transfection requiring higher amounts of DNA and longer culture durations post transfection.

We also encountered higher cell death in the primary cell line and poor transfection efficiency with increasing passage number. In addition, independent of the cells and conditions of culture, there was batch variation with the lipofectamine reagent leading to different ranges of transfection efficiencies.

However, when the transfection efficiencies were similar in the two cell types, the activity of the CMV promoter is comparable in both brain (Fig. 3.6) and non-brain endothelial cells (Fig. 3.7).

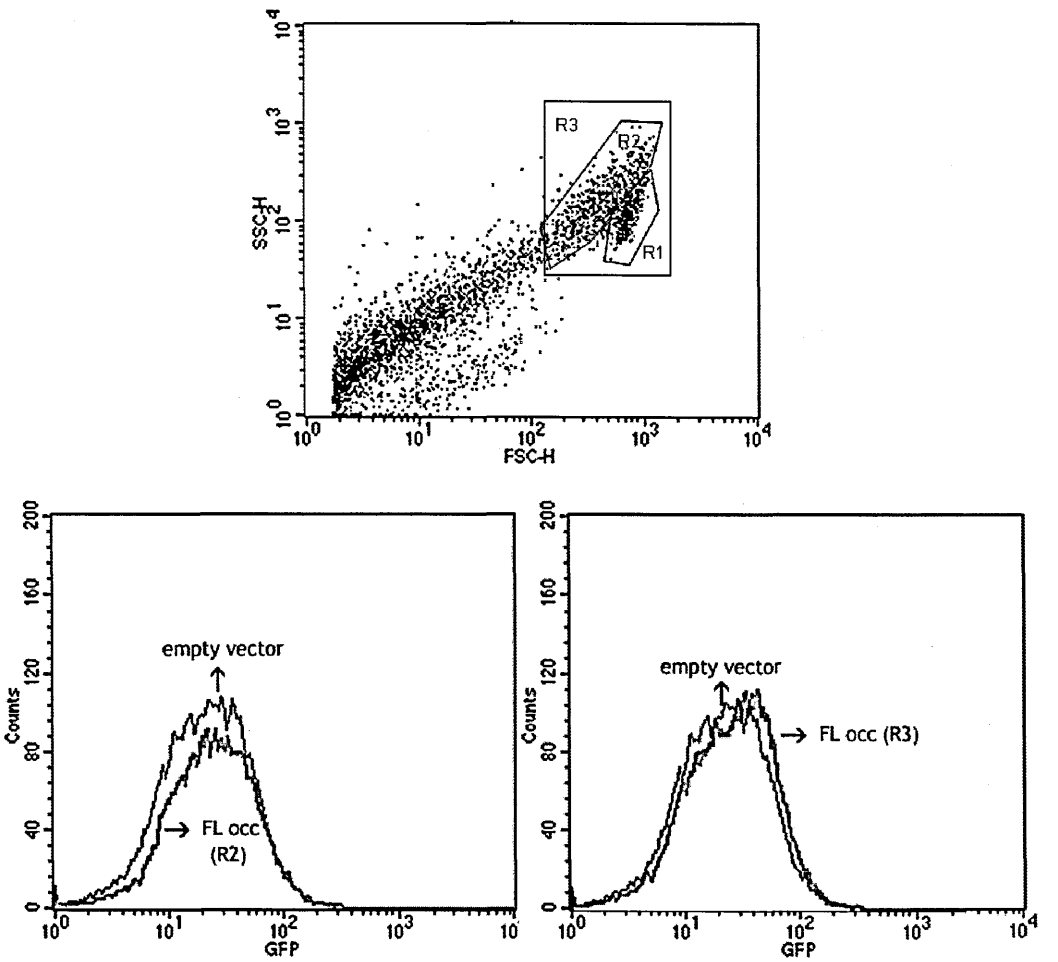
However, the GFP expression mediated by the full length occludin promoter in lung endothelial cells (Fig. 3.5) is similar to that driven by the reporter alone (empty vector). In contrast the occludin promoter is active in the brain endothelium (Fig. 3.4). These results indicate the differential activation of regulatory elements mediate expression from the occludin promoter in brain and non-brain endothelium.

Figure 3-4 Activity of Occludin Promoter in Brain Endothelium



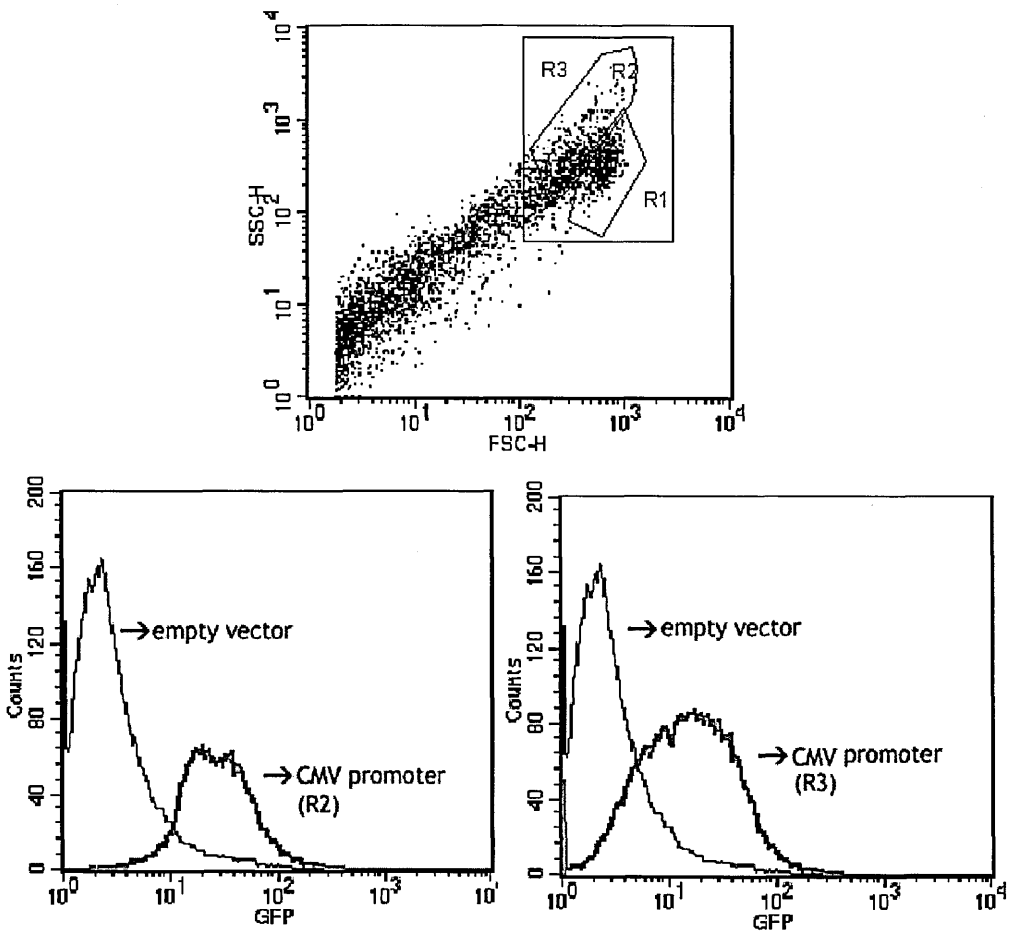
Colour histograms show GFP expression mediated by the human occludin promoter in the transfected (green histograms, R2) and the entire population (red histograms, R3) which includes the untransfected population represented in gate R1 (red histograms). Expression driven by the empty vector is represented in black histograms.

Figure 3-5 Activity of Occludin Promoter in Non-Brain Endothelium



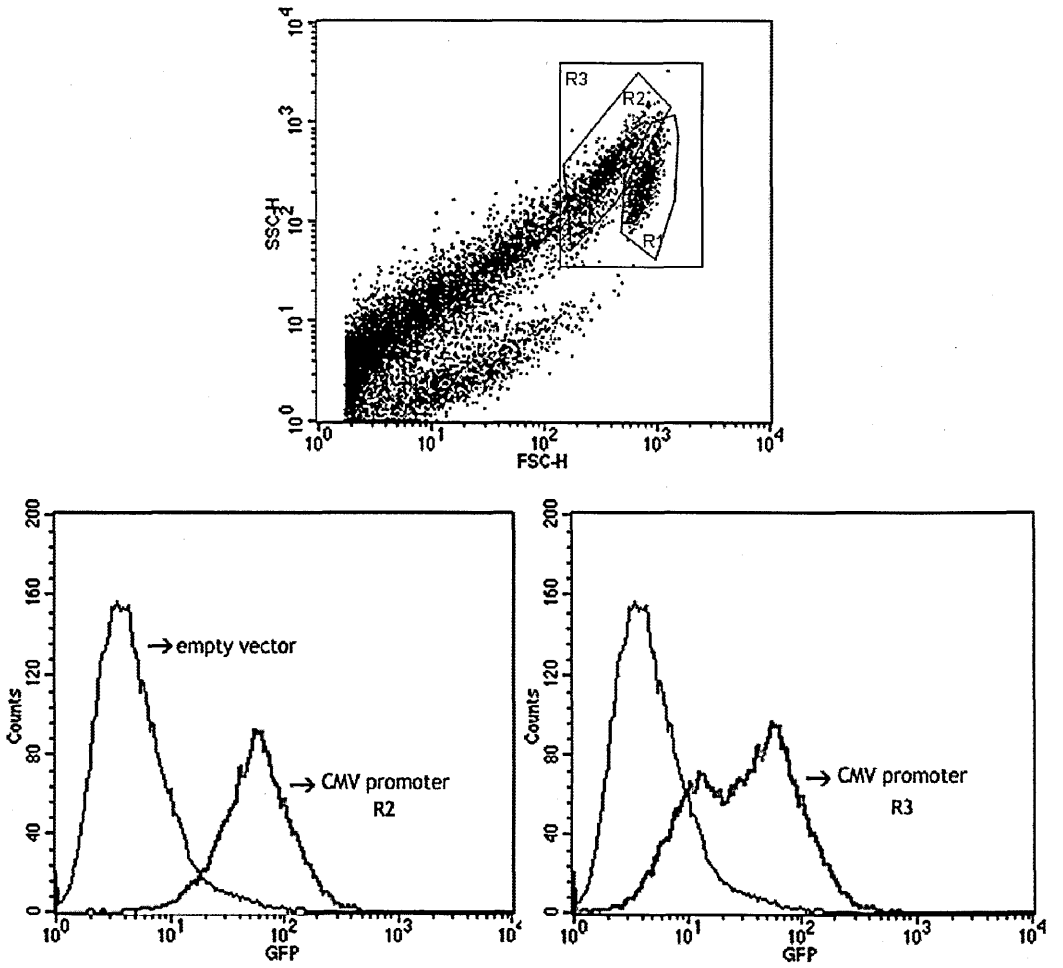
Colour histograms show GFP expression mediated by the full length occludin promoter in the transfected (green histograms, R2) and the entire population (red histograms, R3) which includes the untransfected population represented in gate R1 (red histograms). Expression driven by the empty vector is represented in black histograms.

Figure 3-6 Activity of CMV Promoter in Brain Endothelium



Colour histograms show GFP expression mediated by the CMV promoter in the transfected (green histograms, R2) and the entire population (red histograms, R3) which includes the untransfected population represented in gate R1 (red histograms). Expression driven by the empty vector is represented in black histograms.

Figure 3-7 Activity of CMV Promoter in Non-Brain Endothelium



Colour histograms show GFP expression mediated by the CMV promoter in the transfected (green histograms, R2) and the entire population (red histograms, R3) which includes the untransfected population represented in gate R1 (red histograms). Expression driven by the empty vector is represented in black histograms.

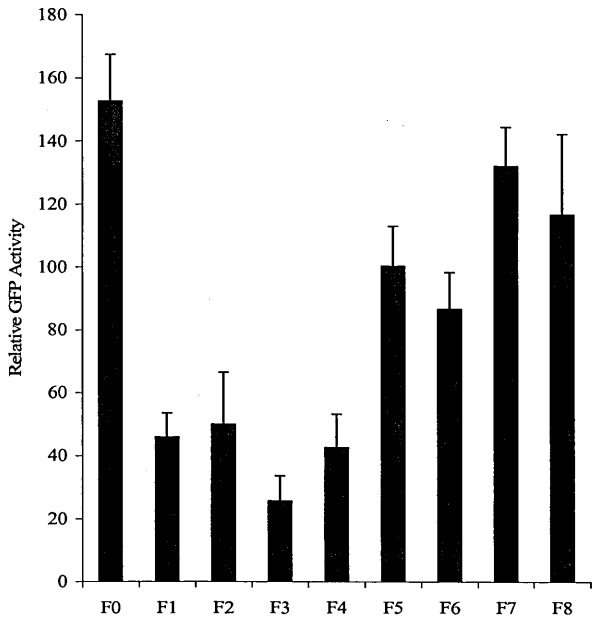
3.3.2 Activity of human occludin promoter fragments in brain (hCMEC/D3) and lung (LMVEC) endothelium

We also tested a series of constructs with fragments of the human occludin promoter in brain and non-brain endothelium. The nine constructs (Fig. 3.1) containing different promoter sequences and/or the empty vector (black histogram, empty vector) were transiently transfected into hCMEC/D3 (Figs. 3.10-3.18, panels A-C) and LMVEC (Figs. 3.10-3.18, panels D and E) cells. Cells were assayed for GFP expression 56 hours (hCMEC/D3) or 72 hours (LMVEC) post transfection.

Results clearly indicate in hCMEC/D3 cells, the expression of GFP is driven by all of the occludin promoter fragments that we have used in the experiments in hCMEC/D3. However, the intensity of the GFP generated was different with the various fragments leading to the conclusion that the promoter strengths vary between the fragments.

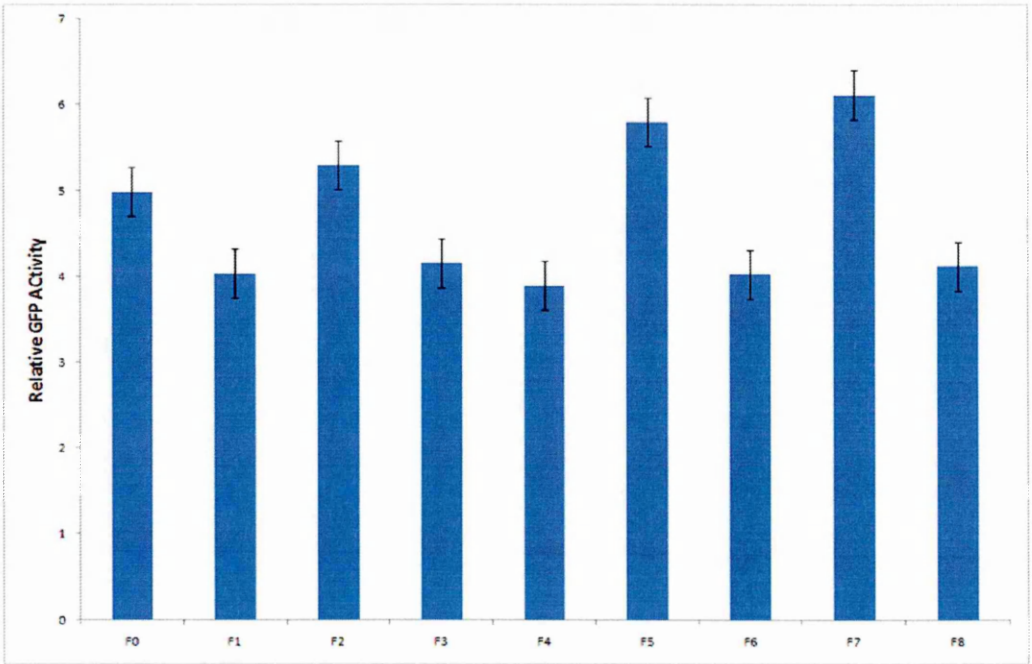
However, the GFP expression mediated by the occludin promoter fragments in lung endothelial cells (LMVEC) is similar to that driven by the reporter alone (empty vector). These results indicate the differential activation of regulatory elements mediate expression from the occludin promoter in brain and non-brain endothelium.

Figure 3-8 Activity of occludin promoter fragments in pGLOW-Topo in hCMEC/D3 cells



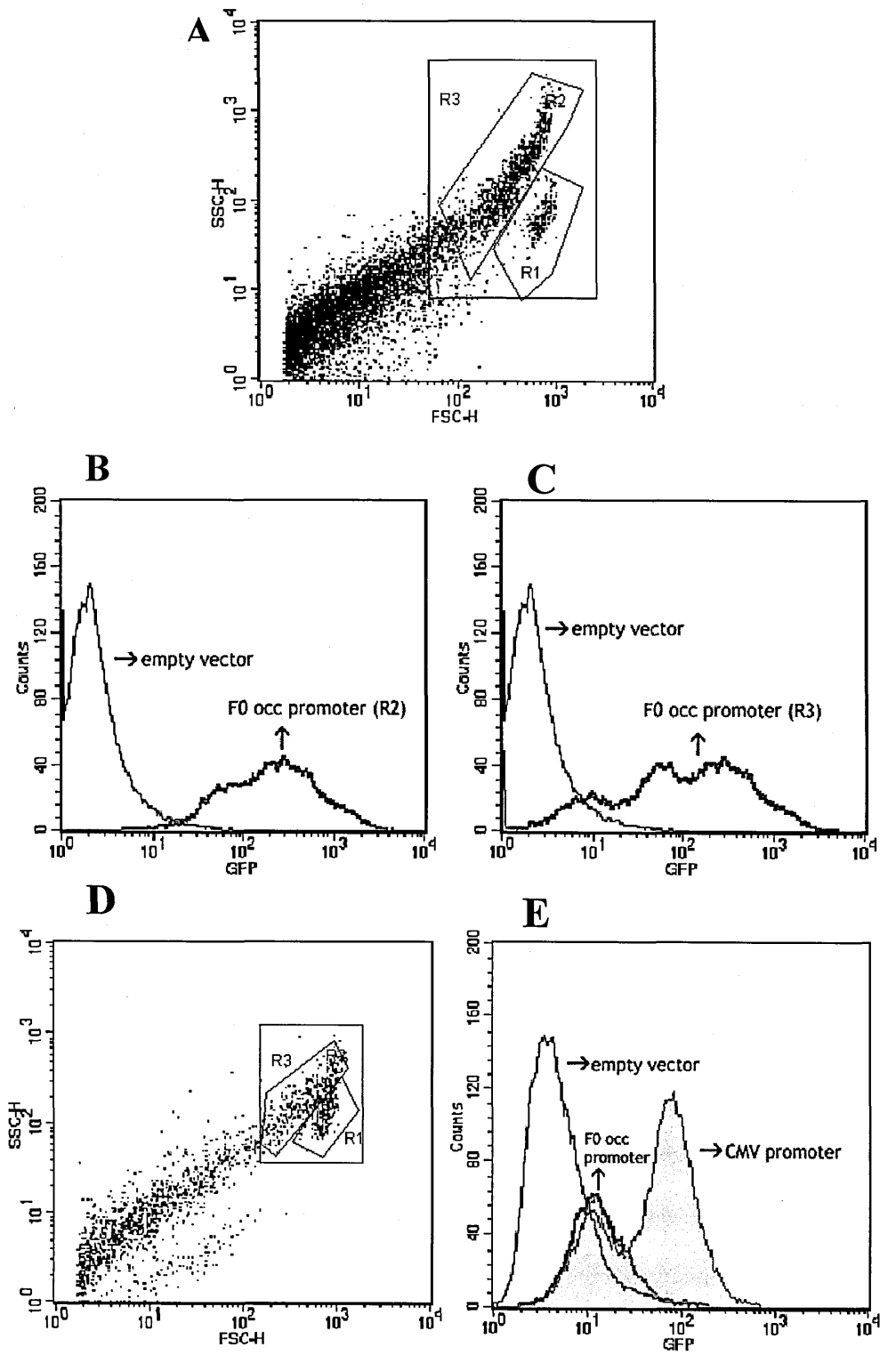
Relative GFP activity from occludin promoter fragments. hCMEC/D3 cells were transfected with the reporter plasmids (F0- F8) and cultured for 56 h. GFP activities were measured and the relative values of each reporter was calculated and normalized to that of the empty vector-transfected cells to derive the median fluorescence of transfected cells. The values represent the mean ± SD of the median fluorescence values from three independent experiments for each reporter. The data was analysed by ANOVA ($p<0.001$) followed by Tukey's multiple comparison test. Promoter activity from fragments F1, F2, F3 and F4 is significantly lower than that from the minimal promoter F8 ($p<0.01$ in all cases), but the activity of F0, F5, F6 and F7 is not significantly different from that of F8 ($p>0.05$).

Figure 3-9 Activity of Occludin Promoter Fragments in pGlow-TOPO in Lung Endothelial Cells



Relative GFP activity from occludin promoter fragments. Lung endothelial cells cells were transfected with the reporter plasmids (F0- F8) and cultured for 72 h. GFP activities were measured and the relative values of each reporter was calculated and normalized to that of the empty vector-transfected cells to derive the median fluorescence of transfected cells. The values represent the mean ± SD of the median fluorescence values from three independent experiments for each reporter.

Figure 3-10 Activity of F0 in hCMEC/D3 and LMVEC cells

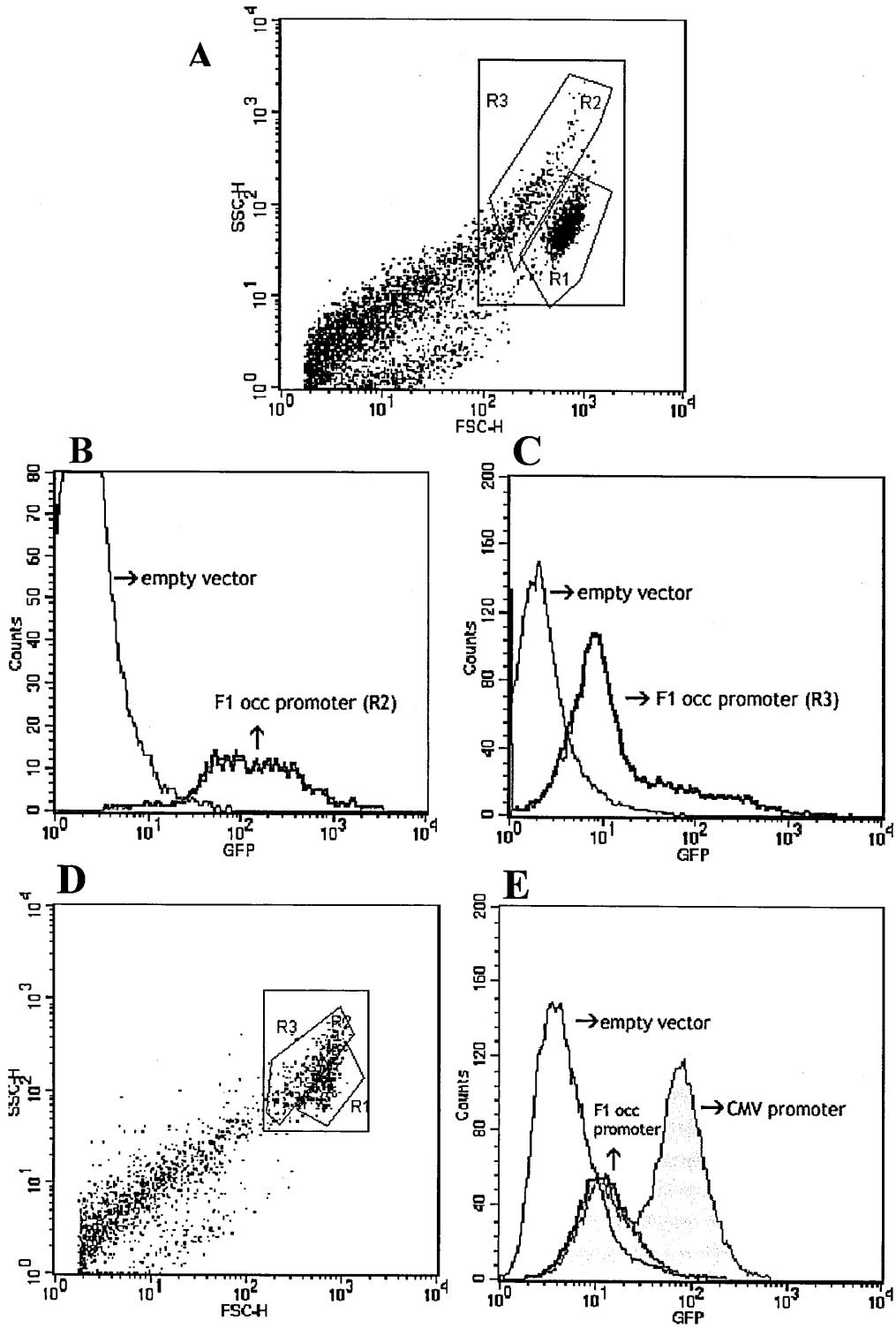


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F0. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-11 Activity of F1 in hCMEC/D3 and LMVEC cells

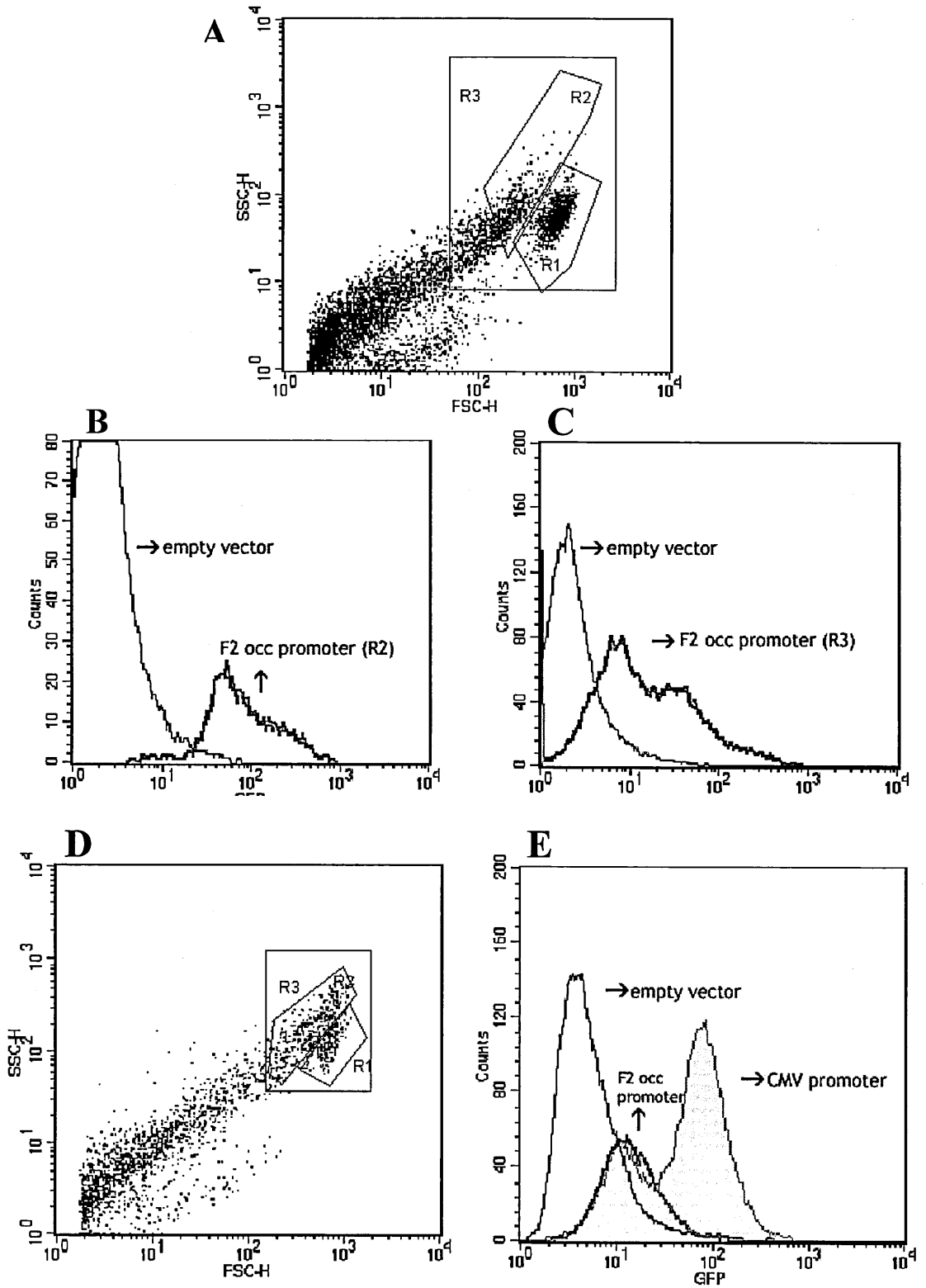


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F1. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-12 Activity of F2 in hCMEC/D3 and LMVEC cells

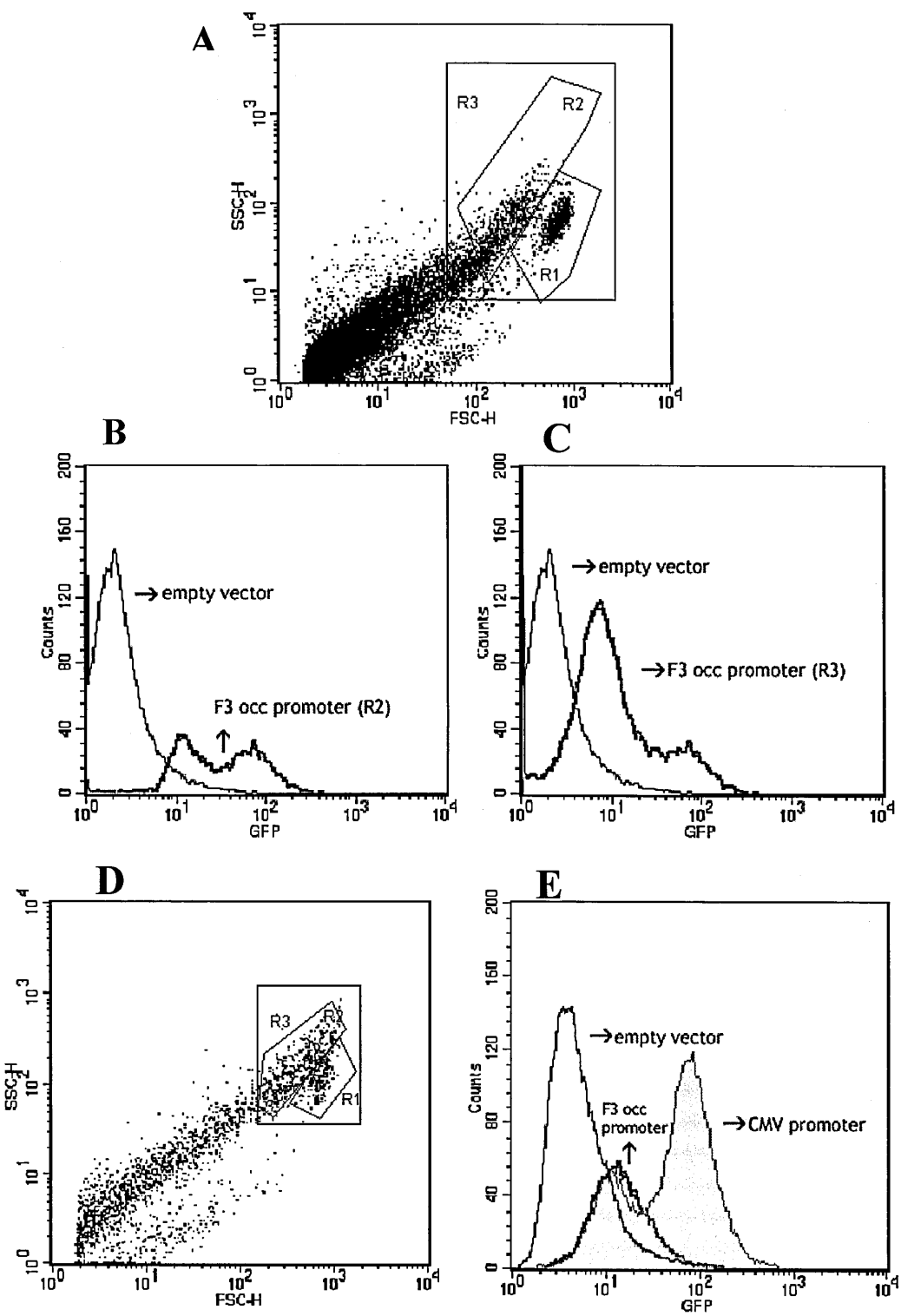


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F2. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-13 Activity of F3 in hCMEC/D3 and LMVEC cells

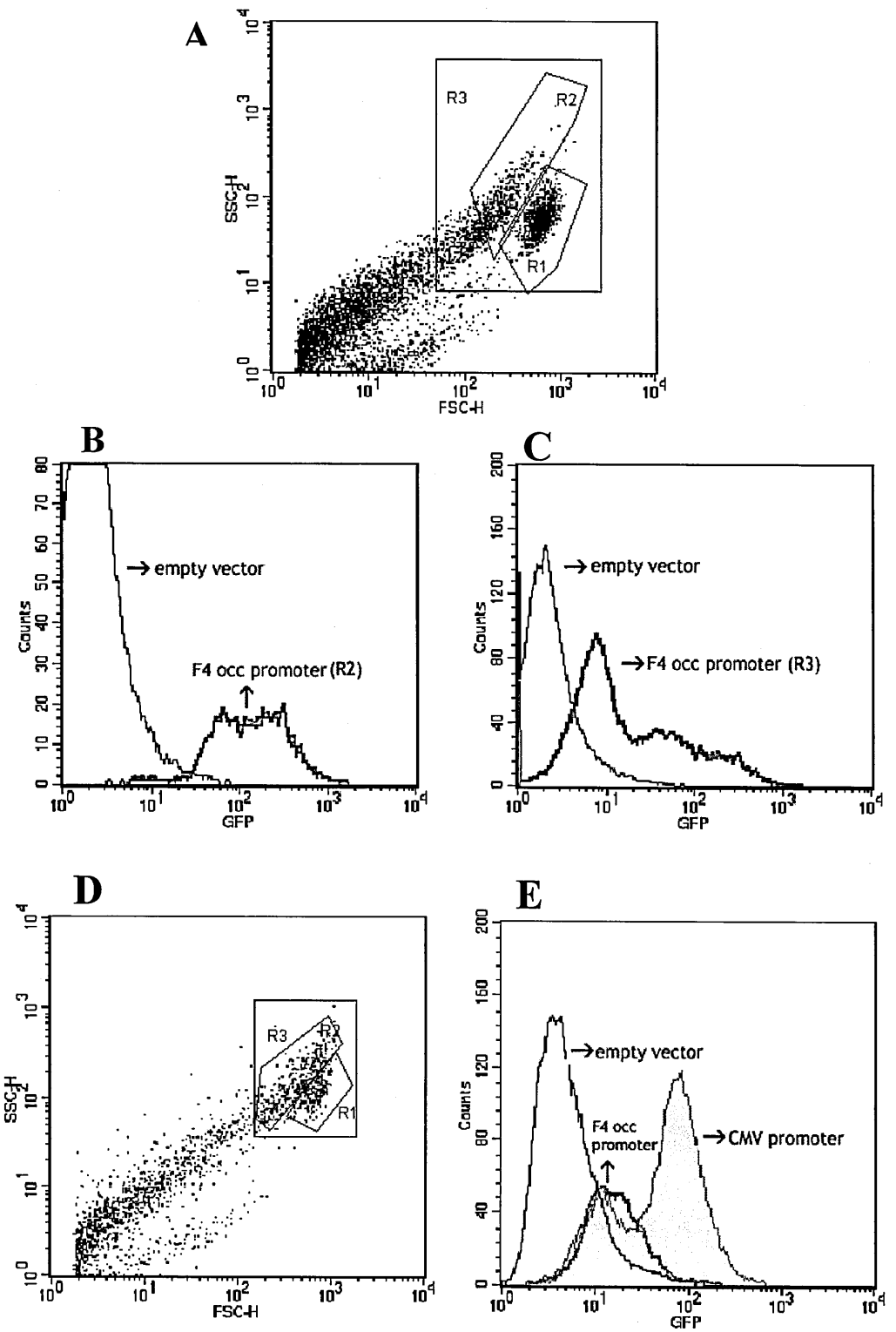


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F3. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-14 Activity of F4 in hCMEC/D3 and LMVEC cells

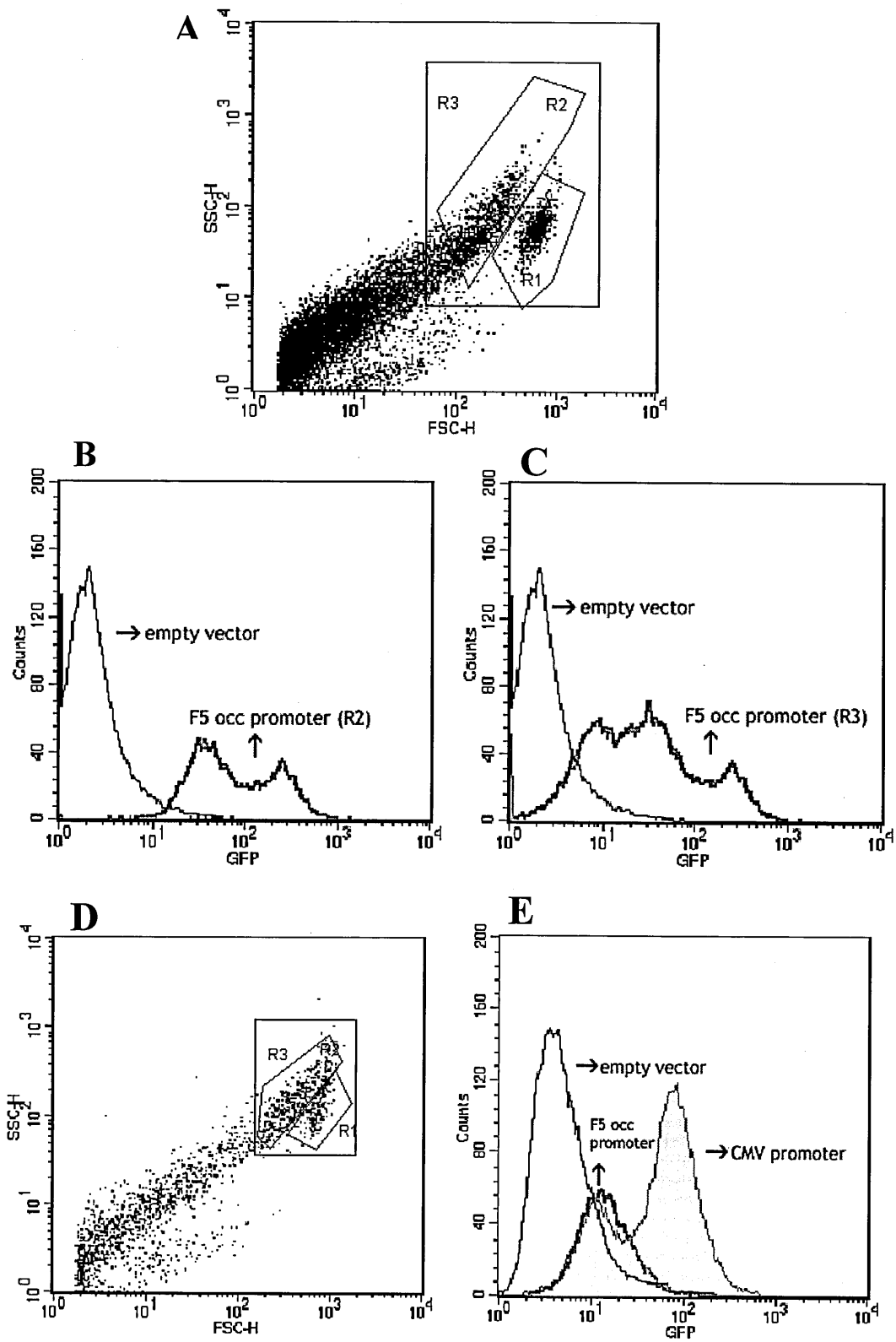


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F4. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-15 Activity of F5 in hCMEC/D3 and LMVEC cells

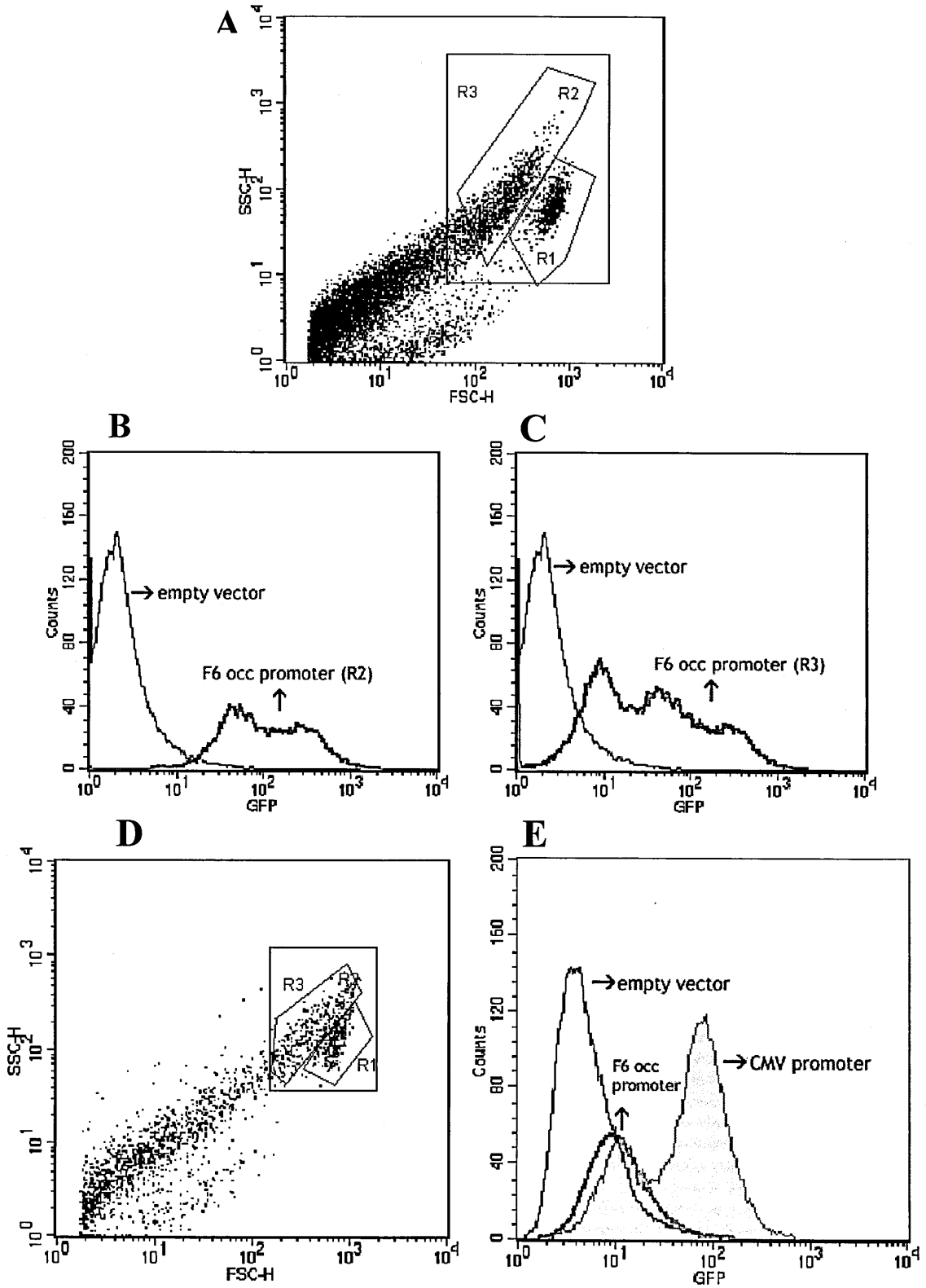


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F5. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-16 Activity of F6 in hCMEC/D3 and LMVEC cells

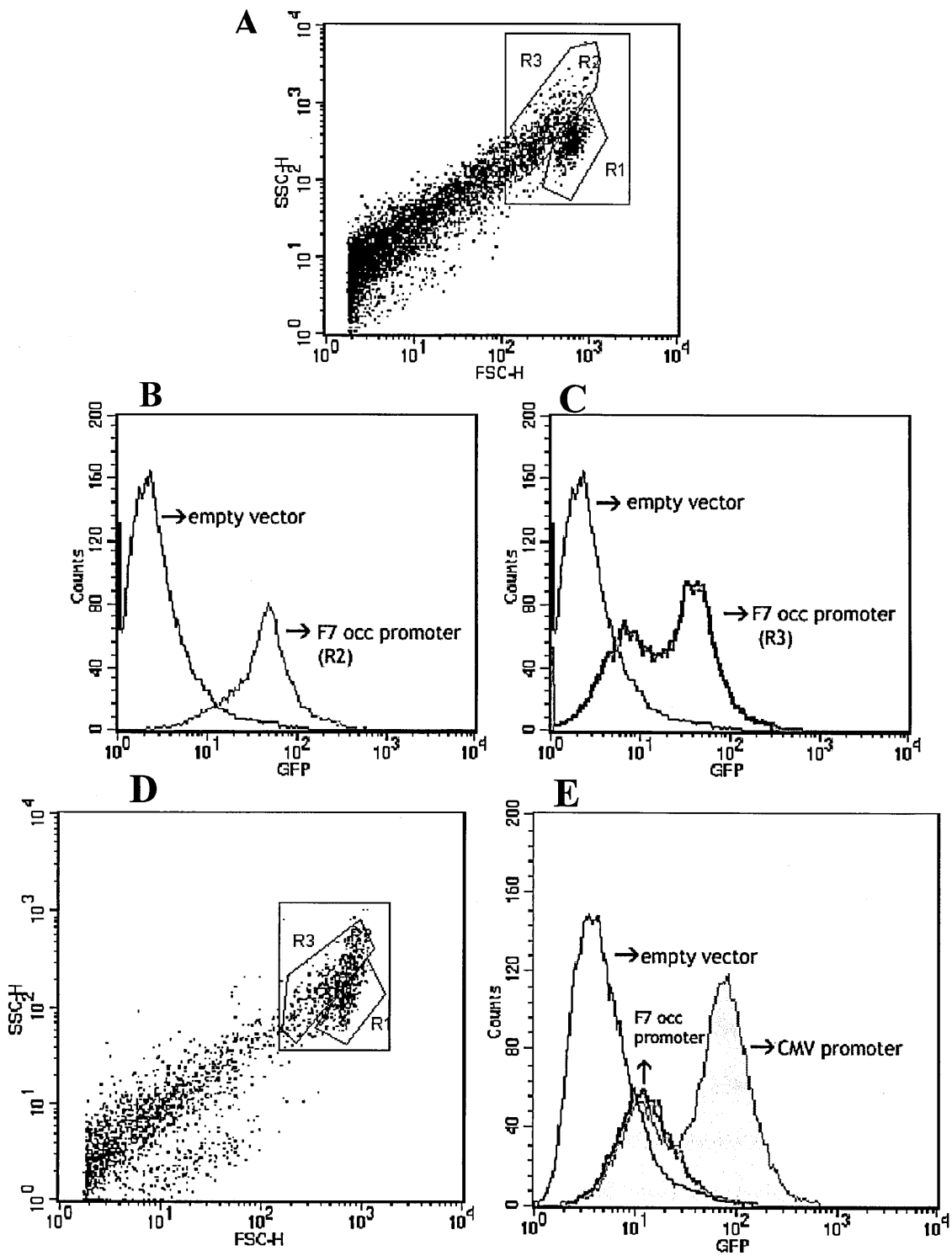


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F6. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

Figure 3-17 Activity of F7 in hCMEC/D3 and LMVEC cells

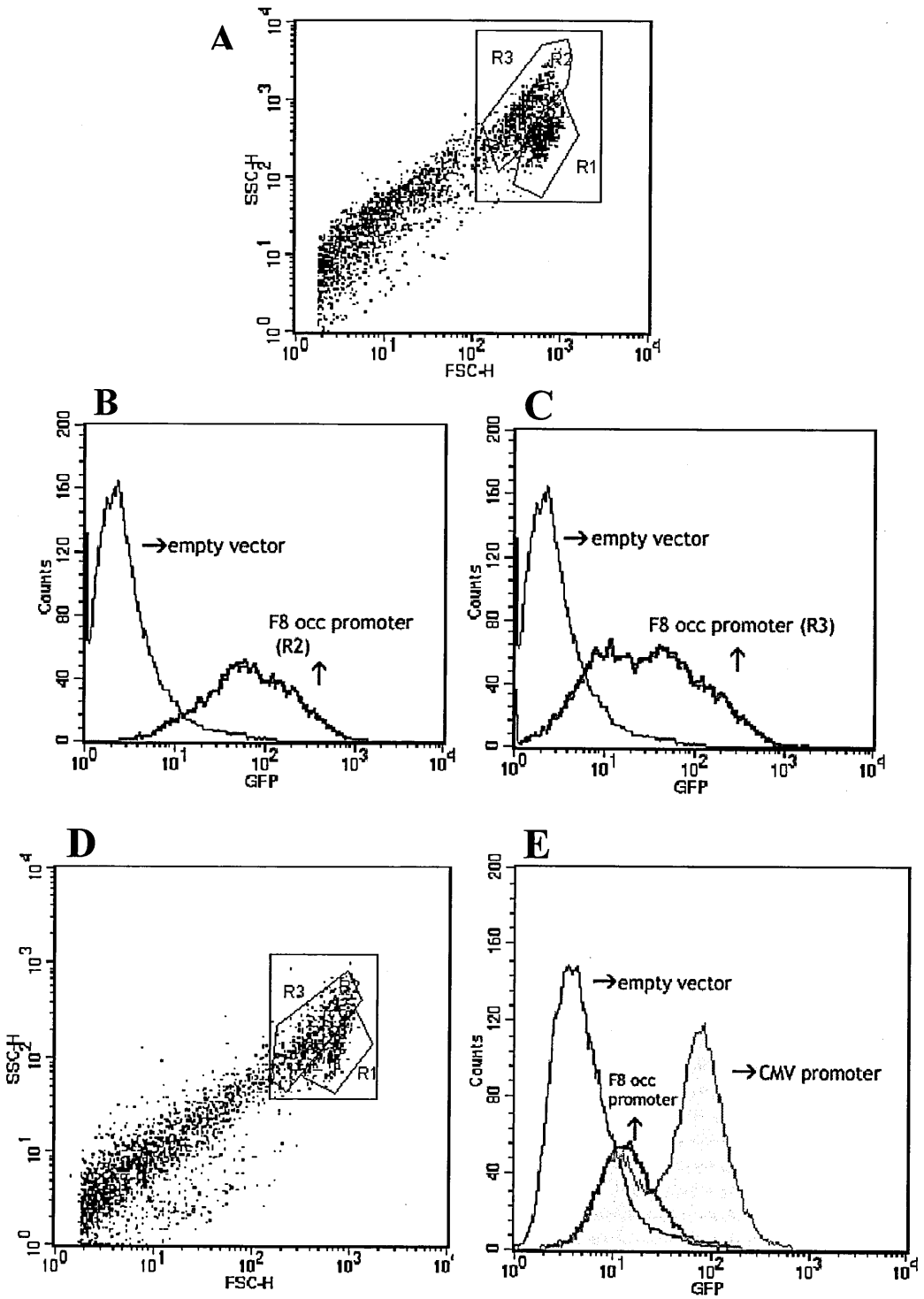


Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F7. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in

Figure 3-18 Activity of F8 in hCMEC/D3 and LMVEC cells



Histogram FACS plots of transiently transfected hCMEC/D3 (B and C) or LMVEC (E) showing GFP expression (coloured histograms) driven by reporter vector containing occludin promoter fragment F8. Control (grey histograms) denotes empty vector.

Forward and Side scatter profiles are shown in A (hCMEC/D3) and D (LMVEC). R1: untransfected cells, R2: transfected population, R3 represents R1+R2.

The entire population (R3) has been taken to represent GFP expression in LMVEC.

3.4 Discussion

This chapter details the characterization of the human occludin gene promoter activity in brain and non brain endothelial cells. Analysis of promoter regulation is one among the many strategies available to study regulation of genes and evaluation of promoter activity of tissue specific genes helps to understand mechanisms behind development and maintenance of differentiated phenotypes.

Full length and various fragments of the promoter were cloned upstream of GFP in the pGlow TOPO reporter vector that lacks promoter and enhancer sequences. The activity of the full length promoter and the fragments were monitored by GFP expression in transfected cells, expression of GFP indicating the activity. The present work set out to determine the effect of promoter length on activity, isolate minimal region showing strong promoter activity and determine differences in promoter activity between brain and non-brain endothelium.

The full length promoter was active in brain endothelium but not in lung endothelium. In hCMEC/D3 cells, its activity was comparable to that of the CMV promoter although variability of GFP expression was high between experiments. Cells transfected with plasmid DNA containing CMV promoter expressed GFP 24-36 hours post transfection but GFP expression driven by the occludin promoter was not visible until 56 hours post transfection. Surprisingly, occludin promoter fragments F0, F5, F6 and F8 displayed activity similar to that of the full length promoter leading to the conclusion that there isn't a single defined minimal promoter region. It appears promoter fragments which are rich in GC residues

display maximal promoter activity. These fragments exhibited binding sites for YY1 and the Sp family TFs (discussed in detail in Chapter 4). Other unidentified binding sites for factors that activate or repress occludin expression may also contribute to this result.

Occludin protein expression is differentially regulated in endothelium. It is highly expressed in brain endothelial cells and absent in lung endothelial cells. It is however expressed in epithelial cells where it participates in the formation of tight junctions. The activity of the promoter in epithelial cells has been analysed by Mankertz et al using a luciferase reporter vector system (Mankertz, Tavalali et al. 2000). The data shows the region of 1400-1853 bp to be sufficient for transcription as maximum promoter activity was driven by deletion constructs containing this region. The data in the epithelial cells also identifies a minimal promoter which is the region extending from 1645-1853 bp. However, our data does not clearly define a region in the occludin promoter that exhibits maximal promoter activity. Nevertheless, the present data suggests that occludin promoter is actively repressed in non brain endothelium-there was no 'leakage' of promoter activity. Shorter versions of the promoter were also inactive. These results indicate differential regulation of the protein in endothelia that contributes to tissue specific expression of occludin.

Chapter 4

Analysis of Transcription Factors Binding to Occludin Promoter *in vitro*

4 Analysis of Transcription Factors Binding to Occludin Promoter *in vitro*

4.1 Introduction

The promoter region of the human occludin gene was identified by Mankertz *et al* in 2000 (Mankertz, Tavalali et al. 2000). In addition to promoter activity there are several studies that have reported the modulation of the protein expression by transcription factors via a direct interaction with the promoter.

Expression of occludin may be regulated at the transcriptional level by a number of different factors, including those in the Ras pathway. In the human intestinal cell line HT-29/B6, the inflammatory cytokines, tumour necrosis factor-α, and interferon-γ regulate promoter activity (Mankertz, Tavalali et al. 2000).

In an elegant study, Wang *et al* (2007) demonstrated that expression of oncogenic Raf 1 in Pa4 epithelial cells increases expression of occludin by direct interaction with the occludin promoter. In addition, Raf 1 also upregulates the expression of the well documented transcriptional repressor, slug which also interacts with an E-box in the occludin promoter to repress transcription (Wang, Wade et al. 2007).

Dokladny *et al* have shown that Caco-2 cells upregulated occludin protein in response to stress induced by heat. HS-induced activation of heat shock factor-1 (HSF-1) resulted in nuclear translocation of cytosolic HSF-1 and binding to its target motif in the occludin promoter (Dokladny, Ye et al. 2008). In 2003, the transcriptional repressor snail was shown to repress transcription from the

occludin promoter. Snail along with the related slug belong to the Snail superfamily of transcriptional repressors. In 293T epithelial cells, snail interacts *in vitro* with the single E-box present in the occludin promoter (Ikenouchi, Matsuda et al. 2003; Forster, Silwedel et al. 2005).

Brain endothelial cells treated with glucocorticoids lead to an increase of occludin at protein and mRNA levels by activation of the glucocorticoid receptor (GR) and its binding to putative glucocorticoid responsive elements in the occludin promoter (Forster, Silwedel et al. 2005).

However, the analyses have been restricted mainly to epithelial cell systems and the present chapter represents the detailed investigation of the regulation of occludin promoter in endothelial cells and aims to identify important transcription factors regulating expression via a direct or indirect interaction with the promoter.

4.2 Analysis of the Occludin Promoter for Putative TF Binding Sites

One of the elements in studying the process of gene regulation is investigating the binding of TFs to cis-elements, short sequences of DNA which influence the expression from the gene. TFs each recognize a family of cis-regulatory DNA sequences usually about 4-10 bases long with varying degrees of conservation at each position. There are approximately 2000 known different TFs from about 100 species. They regulate spatial and temporal gene expression by binding to DNA and either activating or repressing action of an RNA polymerase. Like other proteins, TFs are composed of evolutionary units called domains, which belong to

families that can occur in many different proteins and various domain combinations. The precise description of TFs in DNA-binding is useful in a wide variety of studies. Examples include ChIP–chip assays, protein chip or yeast one-hybrid. This information is also valuable for studies involving gene regulation comparing multiple genomes or gene regulation networks. Hence it is useful to have a program, which can predict putative transcription factor binding sites in a given sequence of DNA based on binding motifs.

Transcription Element Search Software- TESS is a web based software tool for predicting possible transcription factor binding sites in a given DNA sequence. It can identify binding sites using site or consensus strings and positional weight matrices from the TRANSFAC, JASPAR, IMD, and the CBIL-GibbsMat databases. One of the disadvantages of using bioinformatics is that motif searching eliminates useful data when gene regulation is complex. For instance, the expression pattern of a particular gene may be due to a combination of different regulatory elements conferring different effects at different times (Xu, Unseren et al. 2000). In addition, a TF may be constitutively expressed but regulated strictly post-transcriptionally. A transcription factor's function may require cofactors that vary in expression temporally or spatially in a way that is not correlated with the TF itself. The program would not be able to predict the binding sites of such TFs nor would the algorithm make successful predictions concerning TFs that do not vary in expression.

The occludin promoter was analysed using this web program and results summarised (Appendix-1). Literature from groups who have published target-

binding sites based on mutagenesis assays was also considered. For example Forster *et al.*, 2005 investigated glucocorticoid receptor (GR) binding sites in the human occludin promoter. According to their study, the human occludin promoter contains several pentamer sequences located (332–336, 361–365, 399–403, 448–452, 781–785, 1073–1077, 1325–1329 and 1427–1431) bp upstream of the 5′ flanking region, which may be glucocorticoid-responsive elements (GREs) and on which glucocorticoids can influence gene expression.

4.3 Organisation of the Occludin Promoter

For use in EMSAs, we generated 15 individual overlapping gene segments of <200bp. The double stranded occludin promoter probes were divided into three categories based on the presence/absence of target sites for Sp family or YY1 on each of the probes (Fig. 4.1, Table 4.1). Eight of the probes have potential target sites for Sp-family transcription factors and six probes respectively for YY1. Four of the probes have sites for both of the TFs (Table 4.1). To determine which regions of the occludin promoter may interact with DNA-binding proteins in different endothelia, each probe was analysed by EMSA using nuclear extracts from hCMEC/D3 and lung endothelium. Data for each individual fragment shown is a representative image of data that has been repeated at least three times.

Figure 4-1 Schematic of the occludin promoter showing fragments used in EMSA

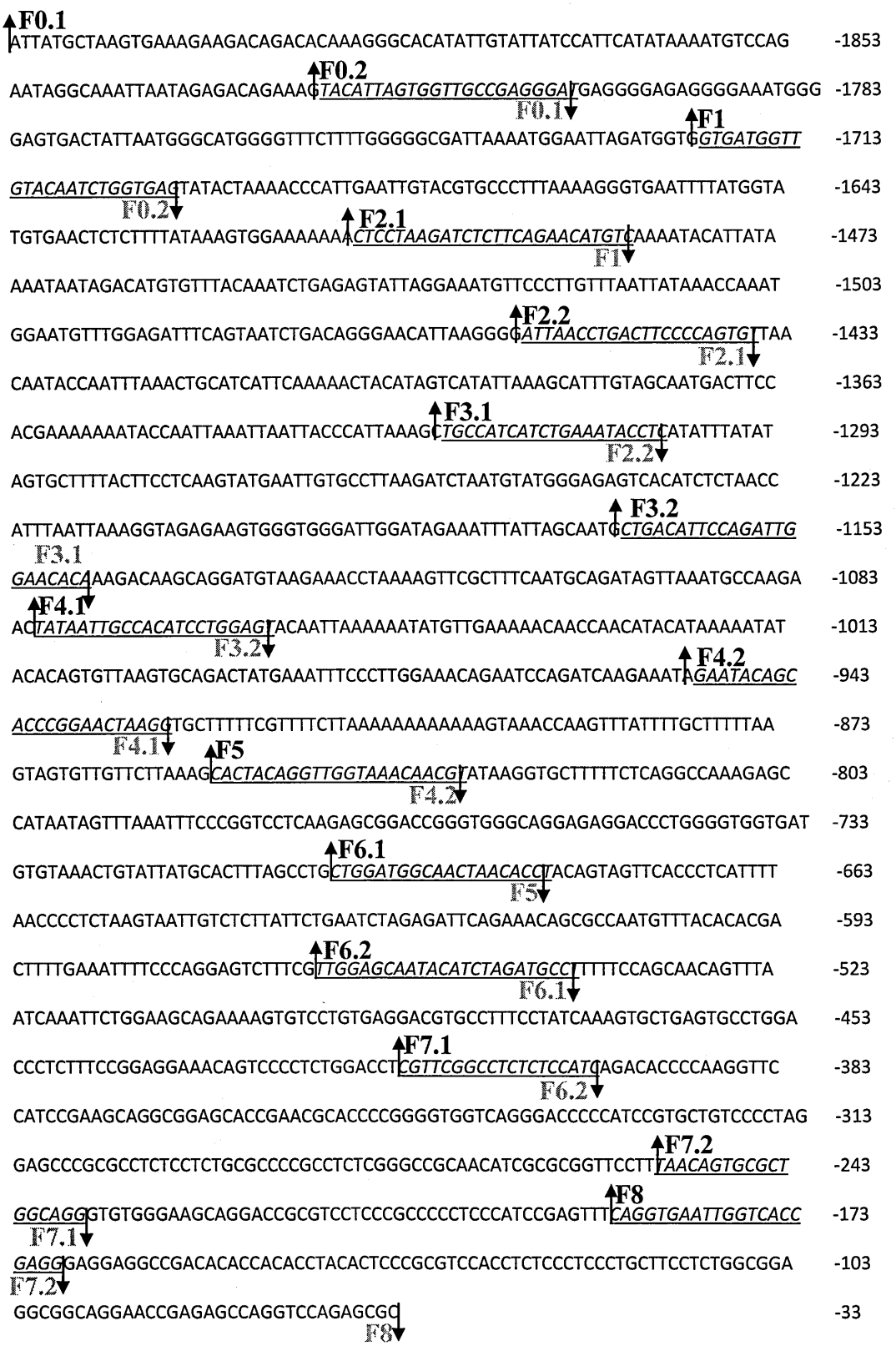
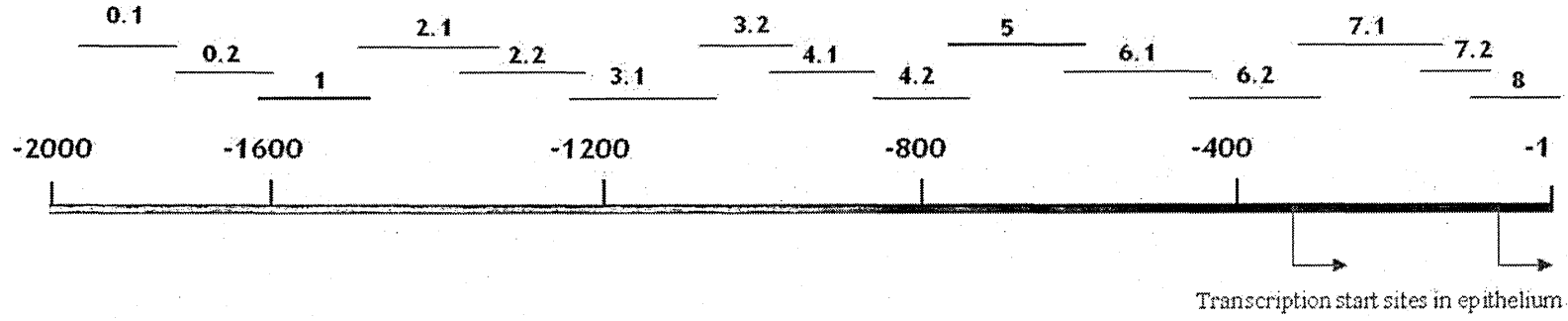


Table 4-1 Occludin promoter fragments used in EMSA



Fragment	Position on Chr5 NT_006713	Size (bp)	*Target sites for Sp family	*Target sites for YY1
0.1	19380963-19381083	120	X	X
0.2	19381054-19381188	128	X	X
1	19381165-19381301	136		X
2.1	19381275-19381450	175		X
2.1	19381428-19381582	154		X
3.1	19381561-19381740	179	X	X
3.2	19381717-19381827	110		
4.1	19381805-19381957	152		X
4.2	19382031-19382201	119		X
5	19382031-19382201	170	X	X
6.1	19382181-19382362	181		X
6.2	19382321-19382487	166	X	
7.1	19382468-19382649	181	X	
7.2	19382631-19382717	86	X	
8	19382696-19382816	120	X	

Fig. 4.1, Table 4.1: Schematic showing the organisation of the different promoter fragments. In Fig. 4.1, upright or downright arrows indicate the start and end of an individual fragment respectively. Underlined and italicised sequences are overlapping sequences between two fragments. Table 4.1 shows fragments containing potential Sp or YY1 target sites or both as identified by TESS (*).

4.4 Materials and Methods

4.4.1 Preparation of nuclear and cytosolic extracts from endothelial cell cultures

Buffers

Solution A: 10 mM Hepes (pH 7.8), 10 mM KCl, 1 mM DTT*, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM PMSF, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.3 mg/ml Leupeptin made up in water.

Solution B: 10 ml nonidet-40 in 100 ml distilled water (final concentration is 10% v/v).

Solution C: 50 mM Hepes, 50 mM KCl, 0.1mM DTT, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM PMSF, 0.2 mM NaF, 0.4 mM PMSF, 0.2 mM Na_3VO_4,10% v/v Glycerol, made fresh before assay and stored on ice or at 4°C.

Fully confluent and rested cell cultures of endothelial cells in 175 cm^2 flasks were used for the assay. Medium was discarded and cell monolayers were washed twice in chilled PBS and drained thoroughly. 1 ml of fresh PBS was added and cells were scraped into this volume by using a cell scraper (Cat no: 541080, Greiner). Cells were pipetted from the flask into a clean and sterile eppendorf tube and centrifuged at 1200 rpm for 10 min at 4°C. The supernatant was discarded and pellet was gently resuspended in 400 µl of solution A and incubated on ice for 15 min. 25 µl of solution B was added and mixed vigorously by vortexing for 15 sec followed by centrifugation at 14000 rpm for 1 min. The supernatant which is the cytosolic extract was quickly removed into another eppendorf and left on ice. The

pellet which is the nucleic fraction was resuspended in 50 µl of solution C and incubated for 40 min at 4°C on a cell mixer. The tube was then centrifuged at 14000 rpm for 10 min at 4°C and supernatant collected, the protein concentration estimated and aliquots stored at -80°C.

4.4.2 Assay for estimation of protein concentration

The Bio-Rad DC protein assay, a modification of the Lowry method was employed to determine the protein concentration of the nuclear and cytosolic extracts. Serial dilutions of the BSA protein standard were prepared to generate a range of protein concentrations: 2, 1.5, 1, 0.75, 0.5 and 0.25 mg/ml. 5 µl of the standards and the sample were pipetted into a 96-well flat bottomed micro titre plate. 25 µl of reagent S/A (20 µl of reagent S + 1 ml of reagent A) was added to each well followed by addition of 200 µl reagent B. Plate was read in an ELISA reader at 640 nm and the protein concentration of the samples was determined using the standard curve.

4.4.3 Generation of occludin promoter fragments for use in EMSA analysis

Primers were designed to amplify regions of 100-150 bp in the occludin promoter. Occludin promoter is 1853 bp in length and was obtained from Joachim Mankertz, whose work had earlier established and characterised the promoter (Mankertz, Tavalali et al. 2000).

Primers were designed using the Custom Primers-OligoPerfect™ Designer on the

Invitrogen (http://www.invitrogen.com) website following the parameters below:

1. Length of the primer ranging from 17-28 bases.
2. GC composition restricted to 50-60%.
3. Primers preferably ending (3') in a G or C, or CG or GC.
4. Melting temperatures (Tm) ranging from 55-80°C and the primers constituting the primer pair having similar melting temperatures.
5. No internal complementarity.
6. Restrict runs of three or more C's or G's at the 3'-ends of primers.

Desalted primers were synthesised at 50 nM scale using oligonucleotide synthesis services at Invitrogen. Appropriate amount of deionised water was added to achieve a final concentration of 100 μM. Primer pairs were generated by diluting individual forward and reverse primers together to a final concentration of 10 μM in water and were stored at -20°C. A PCR reaction contained 1 μl DNA template, 2 μl primer pairs, 1.2 μl 25 mM $MgCl_2$, 2 μl 10 x Taq buffer, and 11.5 μl water and 0.5 units' high fidelity Taq polymerase. PCR was carried out by 1 step at 94°C for 10 min, followed by 30 cycles of: 72°C 1 min, 50°C 30 sec, 72°C 30 sec, and finally 1 step of 72°C for 7 min. PCR products were diluted in blue/orange loading dye (Cat no: G1881, Promega) and were run on 1% agarose gels with 0.1μg/ml ethidium bromide. 1 kb and 100 bp DNA ladders were loaded and fragments migrating at the appropriate size were excised from the gel using a clean scalpel into autoclaved eppendorfs. The gel slices were weighed and DNA extracted and purified using the SpinPrep Gel DNA kit according to manufacturer's instructions (SpinPrep Gel DNA kit, Novagen). Concentration of

the DNA was maintained at 1μg/μl for use in EMSA analyses.

Table 4-2 Forward Primers

FP 1	5'ATT ATG CTA AGT GAA AGA AGA CAG ACA C 3'
FP 97	5'ATC CCT CGG CAA CCA CTA ATG TAC 3'
FP 202	5'GTA CAT TAG TGG TTG CCG AGG GAT 3'
FP 312	5'CTC CTA AGA TCT CTT CAG AAC ATG TC 3'
FP 465	5'GAT TAA CCT GAC TTC CCC AGT GT 3'
FP 754	5'GCT GAC ATT CCA GAT TGG AAC AC 3'
FP 842	5' CTA TAA TTG GCA CAT CCT GGA G 3'
FP 972	5' GAA TAC AGC ACC CGG AAC TAA G 3'
FP 1068	5'GCA CTA CAG GTT GGT AAA CAA CG 3'
FP 1218	5'TGC TGG ATG GCA ACT AAC AC 3'
FP 1358	5'GTT GGA GCA ATA CAT CTA GAT GCC 3'
FP 1505	5'TCG TTC GGC CTC TCT CCA T 3'
FP 1668	5'TTA ACA GTG CGC TGG CAG 3'
FP 1733	5'TCA GGT GAA TTG GTC ACC GAG 3'

Table 4-3 Reverse Primers

RP 120	5' ATC CCT CGG CAA CCA CTA ATG TAC 3'
RP 225	5' TCA CCA GAT TGT ACT ACC ATC CA 3'
RP 338	5' GAC ATG TTC TGA AGA GAT CTT AGG AG 3'
RP 487	5'ACA CTG GGG AAG TCA GGT TAA TC 3'
RP 598	5'GCT GCC ATC ATC TGA AAT ACC 3'
RP 619	5'GGT ATT TCA GAT GAT GGC AGC 3'
RP 777	5'GTG TTC CAA TCT GGA ATG TCA GC 3'
RP 864	5'CTC CAG GAT GTG CCA ATT ATA G 3'
RP 994	5'CTT AGT TCC GGG TGC TGT ATT C 3'
RP 1091	5'CGT TGT TTA CCA ACC TGT AGT GC 3'
RP 1238	5'GTG TTA GTT GCC ATC CAG CA 3'
RP 1382	5'GGC ATC TAG ATG TAT TGC TCC AAC 3'
RP 1524	5'ATG GAG AGA GGC CGA ACG A3'
RP 1686	5'CTG CCA GCG CAC TGT TAA 3'
RP 1754	5'CTC GGT GAC CAA TTC ACC TGA 3'
RP 1853	5'GCG CTC TGG ACC TGG CTC T 3'

4.4.4 Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay (EMSA) of DNA-protein interactions is a sensitive method for the detection of sequence-specific (transcription factors) or non-sequence specific (histones) proteins in cellular extracts that can bind a particular gene segment *in vitro*. It can also be used to study the binding activities of purified or recombinant sequence-specific DNA-binding proteins. Additionally, it can be used to determine the affinity, abundance, association and dissociation rates and binding specificities of DNA binding proteins.

The basis of the EMSA assay is based on the observation that protein: DNA complexes migrate slower than free DNA molecules when subjected to separation on non-denaturing polyacrylamide or agarose gel electrophoresis. In an EMSA, a ^{32}P-labelled DNA fragment is incubated with a candidate transcription factor present in a nuclear extract or as a pure protein. The protein-DNA complexes are separated from free (unbound) DNA by electrophoresis through a non-denaturing polyacrylamide gel. The protein retards the mobility of the DNA fragments to which it binds; thus, the free DNA migrates faster through the gel than does the DNA-protein complex. Appearance of more than one or more slowly migrating bands indicates the presence of more than one sequence specific DNA-binding protein in the extract.

Buffers and reagents

150 mM STE buffer pH 8.0: 0.438 g of NaCl was dissolved in 40 ml TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and the pH was adjusted to 8.0 and

the volume made up to 50 ml with 1x TE buffer, pH 8.0.

T4 Polynucleotide Kinase and 10x buffer (Cat no: M4101) - Promega

Nuclease free water (Cat no: P1193)-Promega

[γ-^{32}P]ATP Amersham (discontinued from Dec 2007)

4.4.4.1 Phosphorylation reaction and purification

Phosphorylation was carried out using 2 µl (1µg/µl) consensus oligonucleotide, 1 µl T4 Polynucleotide Kinase (5 U/µl), 1 µl 10x Buffer, 2.5µl [γ-^{32}P] ATP and 3.5 µl nuclease-free water in a sterile micro centrifuge tube. The reaction was incubated at 37°C for 30 min following which 1µl of 0.5 M EDTA was added to stop the labelling reaction. The volume was made up to 50 µl with 1x STE buffer and advanced to the next step.

Illustra ProbeQuant G-50 Micro Columns containing Sephadex™ (Amersham) were used for the removal of incorporated labelled nucleotides from the DNA labelling reaction. Briefly, the Sephadex G-50 resin in the column was vortexed for a few seconds followed by centrifugation at 735×g for 1 min to resuspend the resin and remove the excess storage buffer. The column was inserted into a sterile eppendorf and the 50 µl volume from the labelling reaction was applied to the centre of the resin bed. The tube was centrifuged for 2 min at 735×g and the eluted volume containing the purified labelled oligonucleotide was used immediately or stored at -20°C for subsequent assays.

4.4.4.2 Gel preparation

30% Acrylamide/Bis Solution, 29:1 (Bio-Rad, Cat no: 161-0157)

10x Tris-Borate-EDTA buffer-DNase and RNase free, (Cat no: T4415)

20% APS (Sigma, Cat no: A3678)

TEMED (Sigma, Cat no: T9281)

Gels were made up with 8 ml 30% Acrylamide/Bis Solution 29:1, 1.5 ml 10x TBE buffer, and 0.4 ml 10% APS, 0.1 ml TEMED and 50 ml water. The gels were allowed to set in the plates for about 2 hours at room temperature before using them in the experiment. Gels were pre-run at 120 V for 30 min in chilled 0.25x TBE buffer for equilibration. Temperature of the running apparatus was maintained at less than 15°C.

4.4.4.3 DNA binding reaction

Binding buffer: 8% Ficoll® (Cat no: F2637, Sigma) in 40 mM Hepes (Cat no: H3375, Sigma) pH 7.5.

Poly (deoxyinosinic-deoxycytidylic) acid sodium salt (Poly dI:dC): (Cat no: P4929, Sigma) 1 mg/ml solution were generated in water and aliquots of 50 µl were frozen at -20°C.

10 mM $MgCl_2$: (Cat no: M8266, Sigma) and **10 mM DTT**: (Cat no: D9779, Sigma).

Loading dye: 250 mM Tris-Cl, pH 7.8, 0.2% bromophenol blue, 40% glycerol.

The DNA binding reation was carried out with 10 µl binding buffer, 1 µl poly-dI: dC, 0.2 µl 10 mM $MgCl_2$, 0.2 µl 10 mM DTT, 1 µl labelled probe, adjusted to 20 µl with water and 5 µg nuclear protein extract. The reaction without the labelled oligo was assembled in sterile, U-bottomed 96 well plate for 20 min at room temperature on a shaker. For super shift and cold inhibitor assays, 2 µl of antibody (200 µg/0.1ml) or 2 µl of the ds DNA oligonucleotide (35 pMol/µl) were added respectively after this incubation and further incubated for 30 min at 4°C with gentle rocking.

Following this incubation, 1 µl of the labelled oligonucleotide was added to each condition and further incubated at RT for 30 min. After addition of 1 µl of the loading dye (250 mM Tris-Cl, pH 7.8, 0.2% bromophenol blue, 40% glycerol) DNA-protein complexes were loaded in pre-cooled and pre-run 6% native polyacrylamide (29:1) gels in 0.25x TBE for 5 hours at 120V. The gel plates were opened and the gel placed on a sheet of Whatman® 3 MM filter paper and then covered with plastic film and dried under vacuum at 70°C on a gel dryer and then exposed to Kodak X-Omat film (Amersham) at -80°C.

Antibodies used in super shift EMSA were purchased from Santa Cruz Biotechnology and 2 µg was used per reaction.

Table 4-4 Antibodies used in EMSA

Antibody	Species of Ab	Immunogen (human)	Clone/PAD*
GATA-1	Mouse	120-235 aa's of Gata-2	CG2-96
c-Myb	Rabbit	500-640 aa's of c-Myb	H-141
Pit1	Goat	Peptide mapping at the N-terminus of Pit1	N-20

Sp1	Rabbit	528-548 aa's within an internal region of Sp1	PEP2
Sp3	Rabbit	Peptide mapping at the C-terminus of Sp3	D-20
TFIID	Rabbit	1-300 aa's representing full length TFIID p36	SI-1
YY1	Rabbit	aa's 1-414 representing full-length YY1	H-414

Consensus and mutant oligonucleotides in EMSA analysis were purchased from Santa Cruz Biotechnology and 2 μl was used per reaction.

Table 4-5 Cold Block Oligonucleotides

ds DNA probe	Sequence
AP-1 consensus	5' CGCTTGATGACTCAGCCGGAA 3' 3' GCGAACTACTGAGTCGGCCTT 5'
AP-1 mutant	5' CGC TTGATGACT TGGCCGGAA 3' 3' GCGAACTACTGAACCGGCCTT 5'
CBF consensus	5' AGACCG TACGTGATTGGTTAATCTCTT 3' 3' TCTGGCATGCACTAACCAATTAGAGAA 5'
CBF mutant	5' AGACCGTACGAAATACGGGAATCTCTT 3' 3' TCTGGCATGCTT TATGCCCTTAGAGAA 5'
TFIID consensus	5' GCAGAGCATATAAAATGAGGTAGGA 3' 3' CGTCTCGTATATTTTACTCCATCCT 5'
TFIID mutant	5' GCGGAGCAGCTAAAATGAGGTAGGA 3' 3' CGTCTCGTCGATTTTACTCCATCCT 5'
MEF-2 consensus	5' GATCGCTCTAAAAATAACCCT GTCG 3' 3' CTAGCGAGATTTTTATTGGGACAGA 5'
MEF-2 mutant	5' GATCGCTGTAAACATAACCCT GTCG 3' 3' CTAGCGACATTTGTATTGGGACAG C 5'
c/EBP consensus	5' TGCAGATTGCGCAATCTGCA 3' 3' ACGTCTAACGCG TTAGACGT 5'
c/EBP mutant	5' TGCAGAGACTAGTCTCTGCA 3' 3' ACGTCTCTGATCAGAGACGT 5'
Sp1 Consensus	5' ATTCGATCGGGGCGGGGCGAGC 3' 3' TAAGCTAGCCCCGCCCCGCTCG 5'
Sp1 mutant	5' ATTCGATCGGTTCGGGGCGAGC 3' 3' TAAGCTAGCCAAGCCCCGCTCG 5'
AP-2α consensus	5' GATCGAACTGACCGCCCGCGGCCCGT 3' 3' CTAGCTTGACTGGCGGGCGCCGGGCA 5'
AP-2α Mutant	5' GATCGAACTGACCGCTTGCGGCCCGT 3' 3' CTAGCTTGACTGGCGAACGCCGGGCA 5'
YY1 consensus	5' CGC TCC CCG GCC ATC TTG GCG GCT GGT 3' 3' GCG AGG GGC CGG TAG AAC CGC CGA CCA 5'

YY1 mutant	5' CGCTCCGCGATTATCTTGGCGGCTGGT 3' 3' GCGAGGCGCTAATAGAACCGCCGACCA 5'
GR consensus	5'GACCCTAGAGGATCTGTACAGGATGTTCTAGAT3' 3'CTGGGATCTCCTAGACATGTCCTACAAGATCTA 5'
GR mutant	5'GACCCTAGAGGATCTCAACAGGATCATCTAGAT3' 3'CTGGGATCTCCTAGAGTTGTCCTAGTAGATCTA
Ets-1/PEA3 consensus	5' GATCTCGAGCAGGAAGTTCGA 3' 3' CTAGAGCTCGTCCTTCAAGCT 5'
Ets-1/PEA3 mutant	5' GATCTCGAGCAAGAAGTTCGA 3' 3' CTAGAGCTCGTTCTTCAAGCT 5'
c-Myb consensus	5' TACAGGCATAACGGTTCCGTAGTGA 3' 3' ATGTCCGTATTGCCAAGGCATCACT 5'
c-Myb mutant	5' TACAGGCATATCGGTTCCGTAGTGA 3' 3' ATGTCCGTATAGCCAAGGCATCACT 5'
CDP consensus	5' ACCCAATGATTATTAGCCAAT TTCTGA 3' 3' TGGGTTACTAATAATCGG TTAAAGACT 5'
GATA consensus	5' CACTTGATAACAGAAAGTGATAACTCT 3' 3' GTGAACTATTGTCT TTCACTATTGAGA 5'
GATA mutant	5' CACTTGATAACAGAAAGTCTTAACTCT 3' 3' GTGAACTATTGTCTTTCAGAATTGAGA 5'
IRF-1 consensus	5' GGAAGCGAAAATGAAATTGAC T 3' 3' CCTTCGCTTTTACTT TAACTGA 5'
MEF-2 consensus	5' GATCGCTCTAAAAATAACCCTGTCG 3' 3' CTAGCGAGATTT TTATTGGGACAGC 5'
NF-1 consensus	5' TTTTGGATTGAAGCCAATATGATAA 3' 3' AAAACCTAACTTCGGTTATACTATT 5'
NF-1 mutant	5' TTTTGGATTGAATAAAATATGATAA 3' 3' AAAACCTAACTTATTTTATACTATT 5'
NFκB consensus	5' AGTTGAGGGGACTTTCCCAGGC 3' 3' TCAACTCCCCTGAAAGGGTCCG 5'
NFAT-c consensus	5' CGCCCAAAGAGGAAAATTTGTTTCATA 3' 3' GCGGGT TTCTCCTTTTAAACAAAG TAT 5'

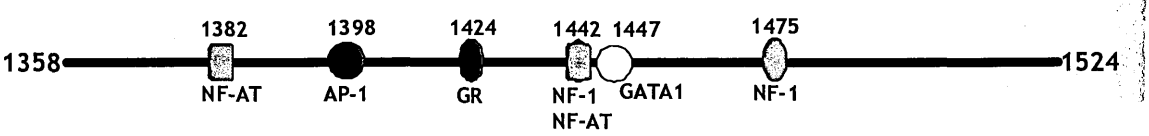
4.5 Results

4.5.1 Promoter Probes with Sp Target Sites

In this section, I shall discuss the interaction of occludin promoter probes containing putative target sites for the Sp family with nuclear protein from brain endothelial cells (hCMEC/D3) and/or lung endothelial cells (LMVEC, non-brain endothelium).

Fragment	Position on Chr5 NT_006713	Size (bp)	*Target sites for Sp1
6.2	19382321-19382487	166	X
7.1	19382468-19382649	181	X
8	19382696-19382816	120	X

4.5.2 Fragment 6.2



Fragment 6.2 spans the region 1358-1524 in the occludin promoter and includes binding sites for NF-AT (1382, 1442), AP-1 (1398), NF-1 (1442, 1475), GR (1424) GATA (1447) and Sp family (1491). There is an overlap of nineteen base pairs between probes F6.2 and F7.1 owing to the particular nature the fragments were amplified and includes the transcription start site as published by Van Itallie and Anderson (Van Itallie and Anderson 1997) but this short stretch does not exhibit any potential transcription factor sites.

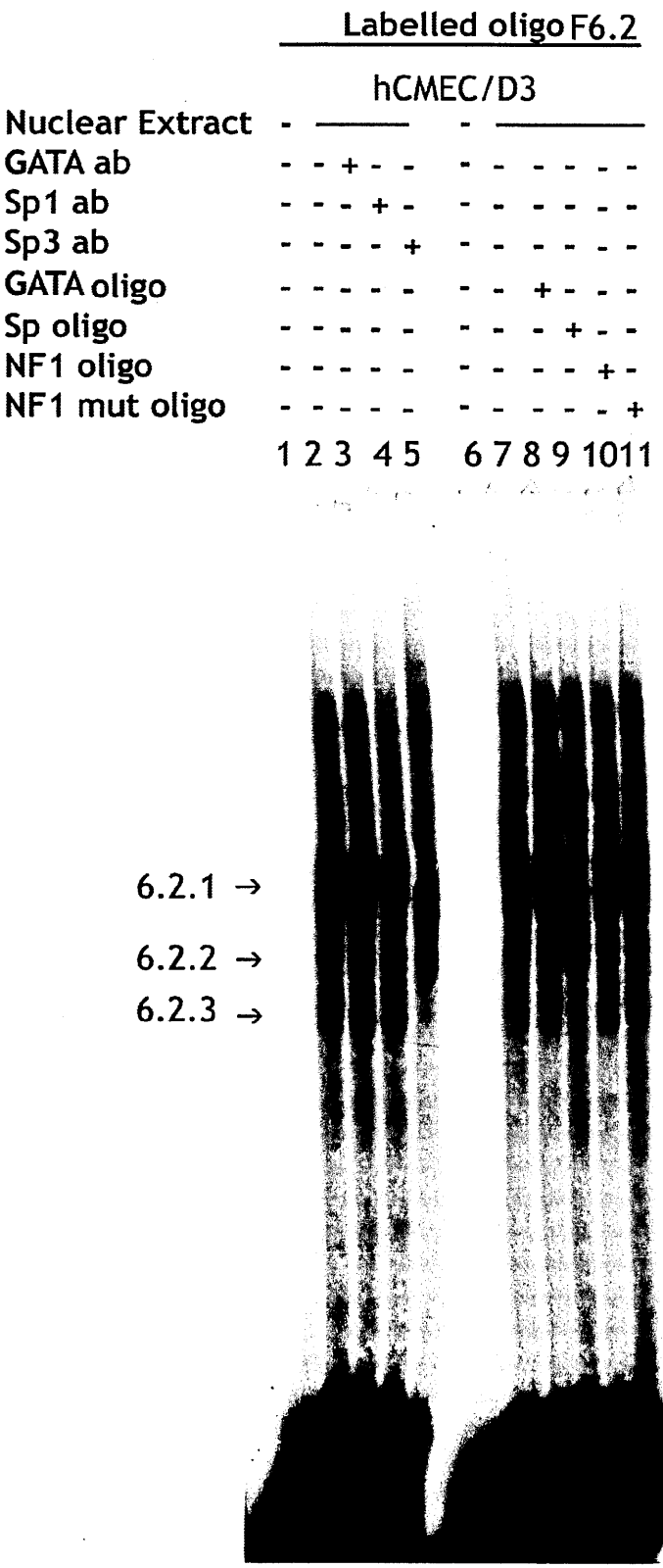
Activity of fragment 6.2 in brain endothelium (hCMEC/D3)

The probe interacted with nuclear protein from hCMEC/D3 cells to form four DNA-protein complexes (Fig 4.2). The highest mobility complex was specific for Sp family as it was blocked in the presence of 100 molar excess of unlabelled Sp1 competitor (lane 9) and the complex was shifted in the presence of an antibody to Sp3 (lane 4) but not Sp1 (lane 3) hence confirming the preferential binding of Sp3 and not Sp1 to this probe in the brain endothelial cells.

Table 4-6 Summary of F6.2 Activity in Brain Endothelium (hCMEC/D3)

Shifted band	Sp1	Sp3	GATA	NF-1
6.2.1	-	-	-	-
6.2.2	-	-	-	-
6.2.3	-	✓	-	-

Figure 4-2 Activity of Fragment 6.2 in Brain Endothelium



Nuclear protein bound to ^{32}P labelled probe F6.2 (lanes 2, 7) was challenged with antibodies or consensus oligonucleotides to GATA1 (lane 3 and 8), Sp1 (lane 4 and 9), Sp3 (lane 5 and 9). Sp3 antibody (lane 5) shifted the highest mobility complex and this was also blocked in the presence of a competitor (lane 9). Wild type (lane 10) and mutant competitors (lane 11) to the factor NF1 did not affect the DNA-protein complexes.

4.5.3 Fragment 7.1

Fragment 7.1 spans the occludin promoter region between 1505 and 1686 (including the first and last base). This region includes target binding sites for the TF, AP2 at 1590 and 1614; Sp family at 1551, 1572, 1632 and 1675; a single binding site for the TFs, GR, NF-AT, c-Ets and c-Myb site at 1601, 1610, 1663 and 1669 respectively.

The transcriptional role of AP1 has been well established for cytokine gene promoters. Functional association for the TFs, AP1 and Sp1 has been reported from the characterisation of the human GMCF gene promoter (Ye, Zhang et al. 1996). AP1 is a nuclear localised transcription factor that comprises gene products of the Fos and Jun families and binds to 5′-TGA (C/G) TCA-3′ target sequence (Bohmann, Keller et al. 1987). AP1 activity requires co-operation from the NF-AT or Elf group of TFs (Macian, Lopez-Rodriguez et al. 2001).

Ets transcription factors comprise about 20 members and are characterised by a conserved 85 amino acid ETS domain. Members bind to the consensus DNA sequence GGA (A/T) and binding is affected by presence of flanking sequences (Seth and Watson 2005) .

c-Myb is a nuclear localised oncogenic TF that is mainly expressed in cells of hematopoietic origin. It recognises the target motif 5'-VHYAACYR-3' (where V = A, C, or G, and H = A, C, or T). Ets-12 and CBF/PEBP2/AML1 are two examples of TFs associating with c-Myb to enhance transcription from target promoters (Gonda 1998).

Activity of F7.1 in brain endothelium (hCMEC/D3)

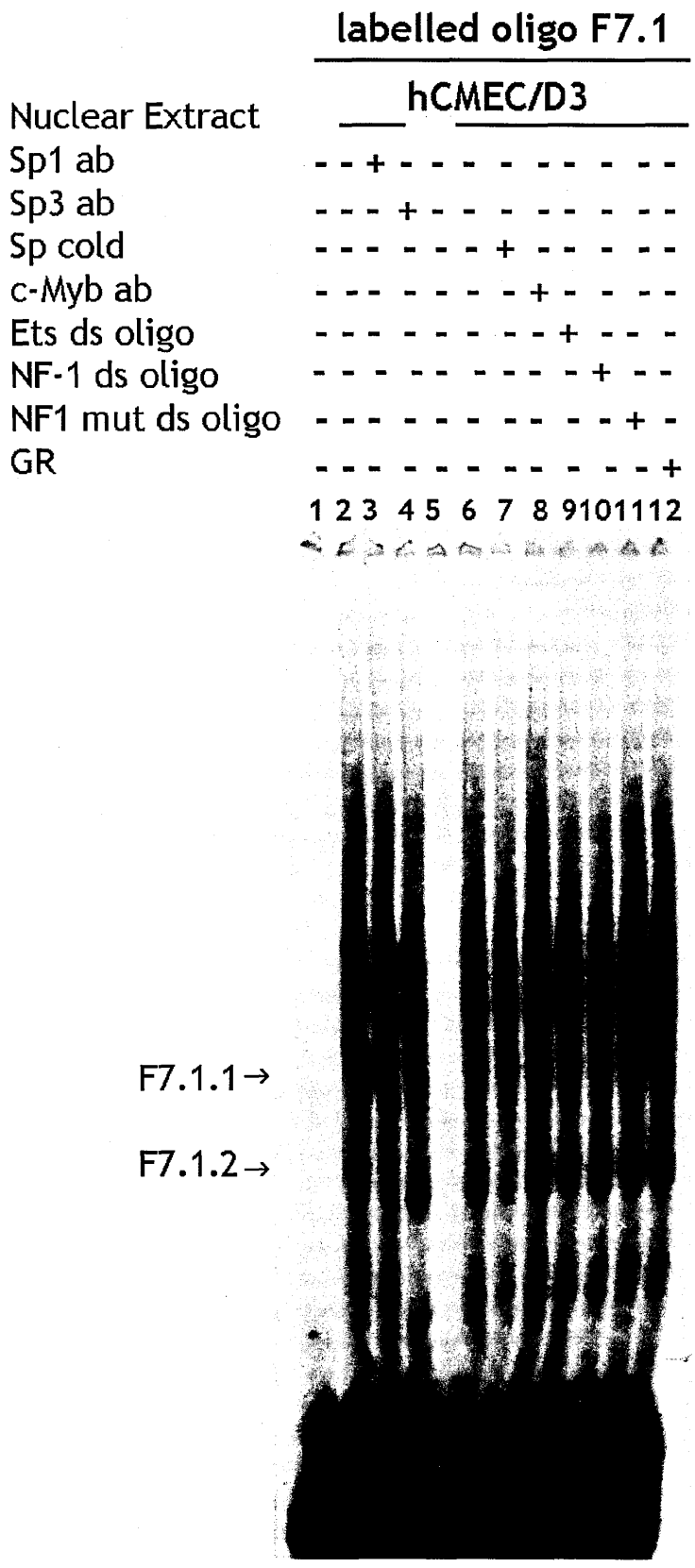
Probe F7.1 interacted with nuclear proteins from hCMEC/D3 cells to generate one low mobility and one high mobility complex of which the latter was abolished by addition of competitor to Sp1 (Fig 4.3, lane 7). Unlike the other promoter probes where Sp3 successfully competed out Sp1 for the binding sites, the converse was true as the DNA-protein complex was retarded by addition of an antibody to Sp1 (lane 3) and not Sp3 (lane 4).

Lung endothelial nuclear extracts did not generate any nuclear protein-DNA complexes with probe 7.1.

Table 4-7 Summary of Probe 7.1 in Brain Endothelium (hCMEC/D3)

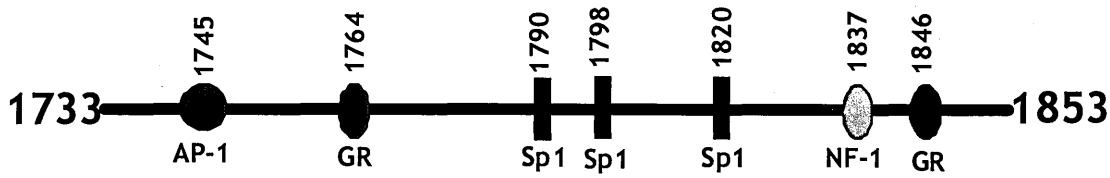
Shifted band	Sp1	Sp3	c-Myb
7.1.1	-	-	-
7.1.2	✓	-	-

Figure 4-3 Activity of Fragment 7.1 in Brain Endothelium



EMSA showing the characterisation of complexes (lanes 2, 6; indicated by arrows) formed between ^{32}P labelled probe F7.1 and hCMEC/D3 nuclear extracts. Addition of an antibody directed towards Sp1 (lane 3) or an unlabelled Sp1 consensus probe (lane 7) super shifted and/or retarded one of the complexes. Antibodies against Sp3 (lane 4), c-Myb (lane 8) or wild type (lane 10) and mutant competitors (lane 11) to the factor NF1 have no effect on formation of the complexes.

4.5.4 Fragment 8



Fragment 8 spans the nucleotide bases 1733-1853 in the occludin promoter. Using HT29/B6, a subclone of the human intestinal cell line HT-29, Mankertz *et al* (Mankertz, Tavalali et al. 2000) determined the minimal promoter activity of the occludin promoter resides in 208 bp upstream of the putative transcription start site at 1781 bp previously published by Ando-Akatsuka *et al* (Ando-Akatsuka, Saitou et al. 1996). Putative transcription factors with target binding sites on this fragment include the Sp family, GR and members of the CCAAT binding proteins as illustrated in the figure above. These transcription factors have been discussed in chapter 1.

Activity of fragment 8 in brain endothelium (hCMEC/D3)

Four nuclear protein-DNA complexes, 8.1, 8.2, 8.3 and 8.4 were seen in the presence of extracts from hCMEC/D3 (Fig. 4.4 and 4.5, lane 2). The subunit composition of the complexes was investigated using competitor probes and/or antibodies directed against transcription factors predicted to have binding sites on the probe.

In the presence of a cold competitor to Sp1 (Fig. 4.4, lane 18) which as previously described is also specific to Sp3 due to redundancy in binding targets between Sp

family members, the formation of complexes, 8.1, 8.2 and 8.3 was inhibited and in the presence of an antibody directed against Sp3 (Fig. 4.4, lane 4), the complexes, 8.1, 8.2, 8.4 were retarded. In an unexpected observation we have observed that protein binding to this region is blocked by 100-molar fold excess of competitor for NFκB and AP2-α (Fig. 4.5, lanes 26 and 30), in which formation of nuclear protein-DNA complexes 8.1 and 8.2 are blocked. Since NFκB consensus oligonucleotide blocks the same complexes as does the Sp1 competitor, it is likely that the transcription factor interacts with the Sp family. Whether this is a functional interaction is not known. Due to this observation we performed EMSA employing cold probes and antibodies against transcription factors that do not have putative target sites on the probe (lanes 5-9; 11-25; 27-29 and 31). With the exception of the AP-2α transcription factor, none of the other antibodies and competitors inhibited the formation or retarded the nuclear protein-DNA complexes.

Activity of fragment 8 in lung microvascular endothelium (LMVEC)

The F8 probe interacted with nuclear extracts from lung endothelial cells to form a single DNA-protein complex (Fig 4.6, lane 2) which was not super-shifted by antibodies to Sp1 (lane 3) or Sp3 (lane 4). Notably, the complexes formed by brain and non-brain endothelium are totally distinct, implying that lung endothelium lacks the active Sp3 complex, even though Sp3 is present in these cells, albeit at a lower level than in brain endothelium (Holloway, Sade et al. 2007). Furthermore, this complex is blocked in the presence of all of the unlabelled cold competitors (lanes 5-8) leading to the observation that the DNA-

protein complex is not sequence specific.

Table 4-8 Activity of F8 in Brain Endothelium (hCMEC/D3)

Shifted Band	Sp1	Sp3	NFκB	AP2-α
F8.1	-	✓	✓	✓
F8.2	-	✓	✓	✓
F8.3	-	✓	-	-
F8.4	-	-	-	-

Table 4-9 Activity of F8 in Non-Brain Endothelium (LMVEC)

Shifted Band	Sp1	Sp3	NFκB	AP2-α
F8.I	-	-	-	-

Figure 4-4 Activity of Fragment 8 in Brain Endothelium

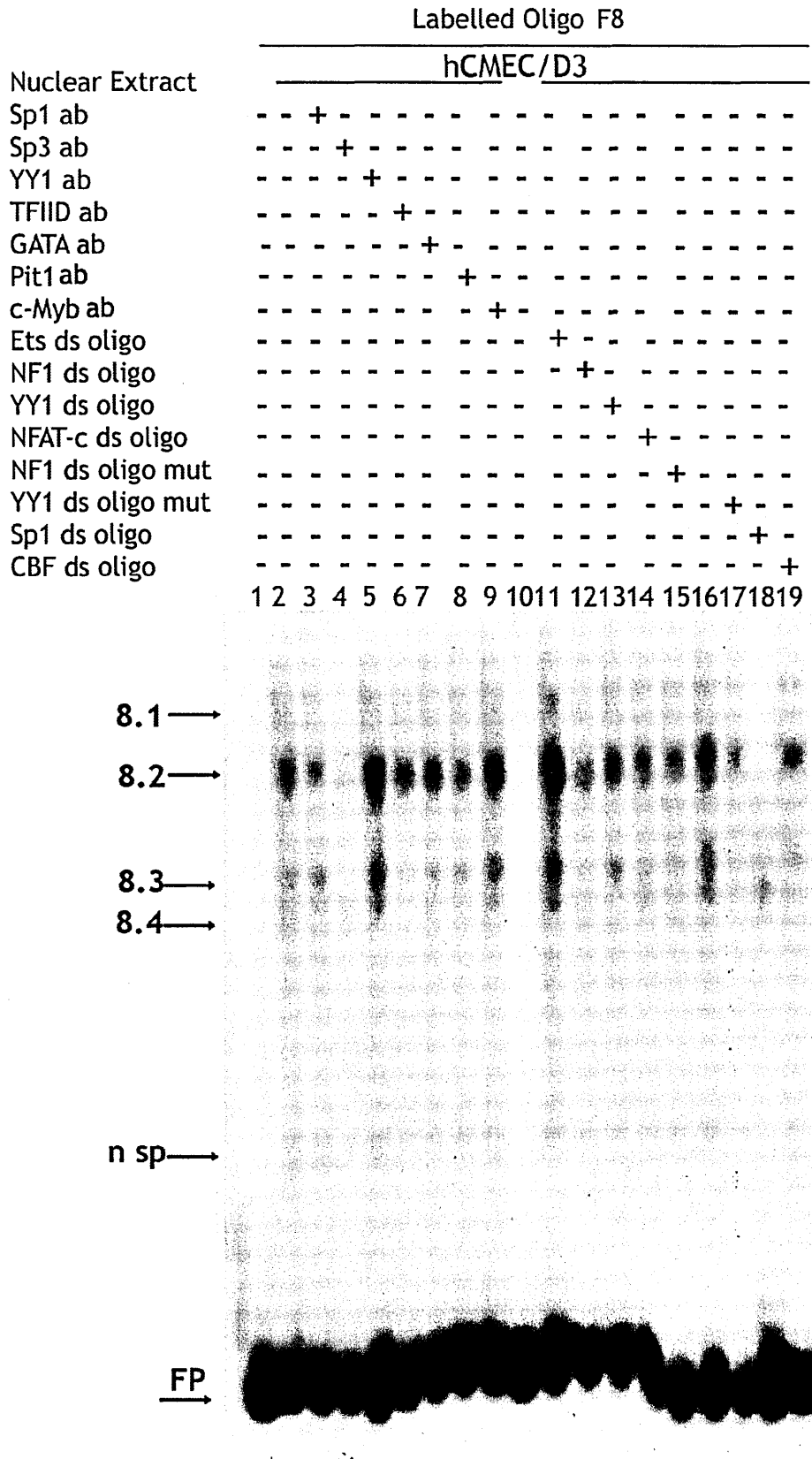
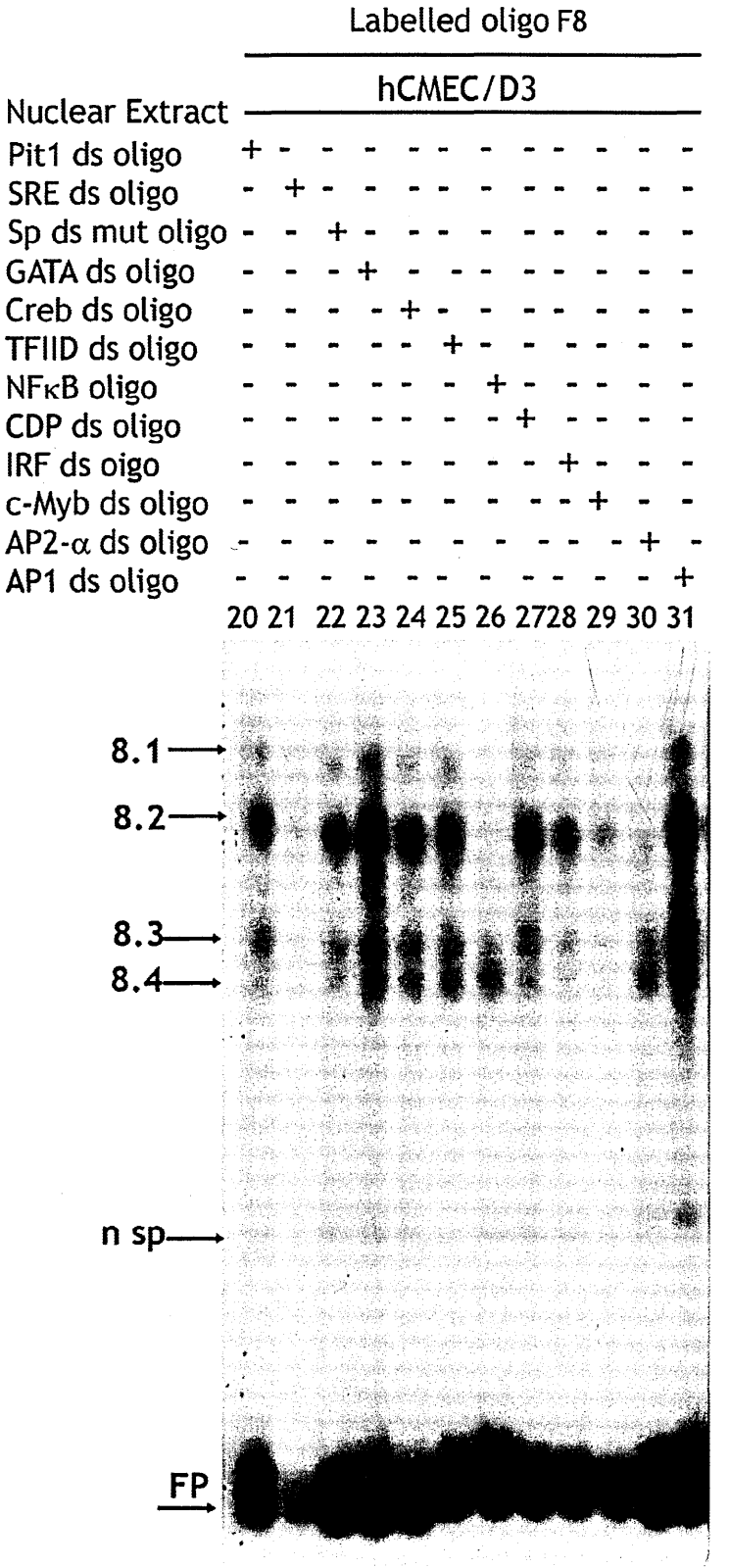


Figure 4-5 Activity of Fragment 8 in Brain Endothelium

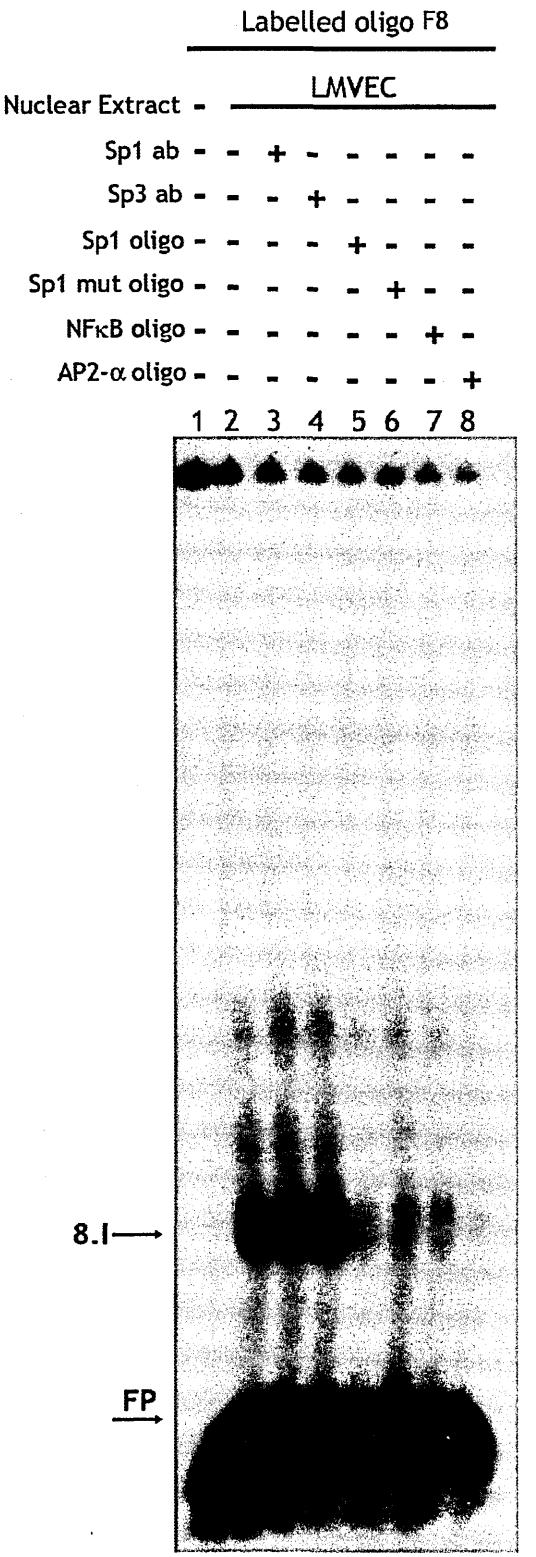


Figs. 4.4 and 4.5: EMSA analyses of complexes (8.1, 8.2, 8.3 and 8.4) formed with hCMEC/D3 (lanes 2-9 and 11-31) nuclear extracts and the promoter probe F8. Lanes 1 and 10 represent mobility of the probe in the absence of nuclear protein. The composition of the complexes was investigated using antibodies directed against transcription factors predicted to bind to the F8 sequence. Addition of an antibody to Sp3 (lane 4) or a competitor to Sp1 (lane 18) blocked the formation of all the nuclear protein DNA complexes in hCMEC/D3 cells. Addition of a consensus mutant Sp oligonucleotide reversed the loss of the complexes. Competitors to the transcription factors NFκB (lane 26) and AP2-α (lane 30) also blocked the formation of the nuclear protein-DNA complexes.

Antibodies to Sp1 (lane 4), YY1 (lane 5), TFIID (lane 6), GATA (lane 7), Pit1 (lane 8), c-Myb (lane 8) or presence of consensus or *mutant competitors*-YY1 (13, *16*), TFIID (25), GATA (lane 23), Pit1 (lane 20), c-Myb (lane 29), Ets (lane 11), NF1 (lanes 12, *15*), NFAT-c (lane 14), CBF (lane 19), SRE (lane 21), Creb (lane 24), CDP (lane 27), IRF (28) and AP-1 (lane 31) did not affect the formation of the complexes.

Fig. 4.6: EMSA analysis of nuclear protein derived from LMVEC cells bound to ^{32}P labelled probe F8. Antibodies, *wild type* or mutated oligonucleotides to Sp1 and 3 (lanes 3 and 4, *5*, 6), NFKB (lane *7*), AP-2α (lane *8*) were added to characterise the composition of the nuclear protein DNA complex (8.I).

Figure 4-6 Activity of Fragment 8 in Lung



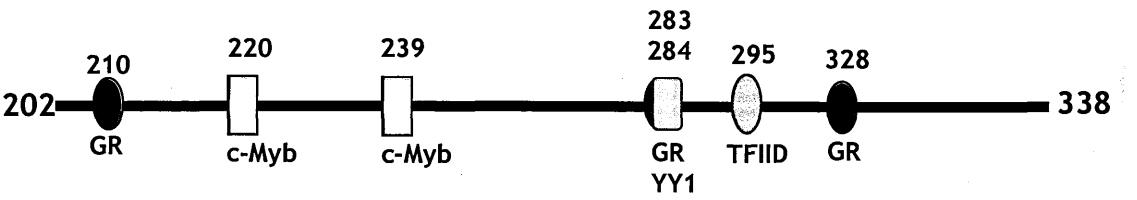
endothelium

Fig. 4.5: DNA-protein complexes with lung nuclear lysates (8.I) and probe F8 (lane 2) do not appear to be specific for any of the transcription factors (lane 3-Sp1; 4-Sp3) as they were blocked by the addition of consensus (5-Sp1; 7-AP1; 8-NFκB) or mutant (6-Sp1) oligonucleotides.

4.5.5 Promoter Probes with YY1 Target Sites

Fragment	Position on Chr5 NT_006713	Size (bp)	*Target sites for YY1
1	19381165-19381301	136	X
2.1	19381275-19381450	175	X
2.1	19381428-19381582	154	X
4.1	19381805-19381957	152	X
4.2	19382031-19382201	119	X
6.1	19382181-19382362	181	X

4.5.6 Fragment 1



F1 of the occludin promoter exhibits putative binding sites for the TFs GR (210, 283, 328), c-Myb (220, 239), YY1 (284) and TFIID (295). YY1 has been shown to interact with a complex containing the TFs Stat 5 and GR in the nucleus in the transcription of the Spi 2.1 gene (Bergad, Towle et al. 2000). c-Myb is capable of replacing GR in erythroid progenitors (Wessely, Deiner et al. 1997).

Activity of F1 in non-brain endothelium (LMVEC)

Two closely migrating nuclear protein-DNA complexes were seen when probe F1 was incubated with nuclear protein from lung endothelial cells (Fig. 4.7). Formation of complexes was unaffected in the presence of antibodies to the TFs c-Myb, TFIID, Pit1 and YY1.

Figure 4-7 Activity of Fragment 1 in Lung Endothelium

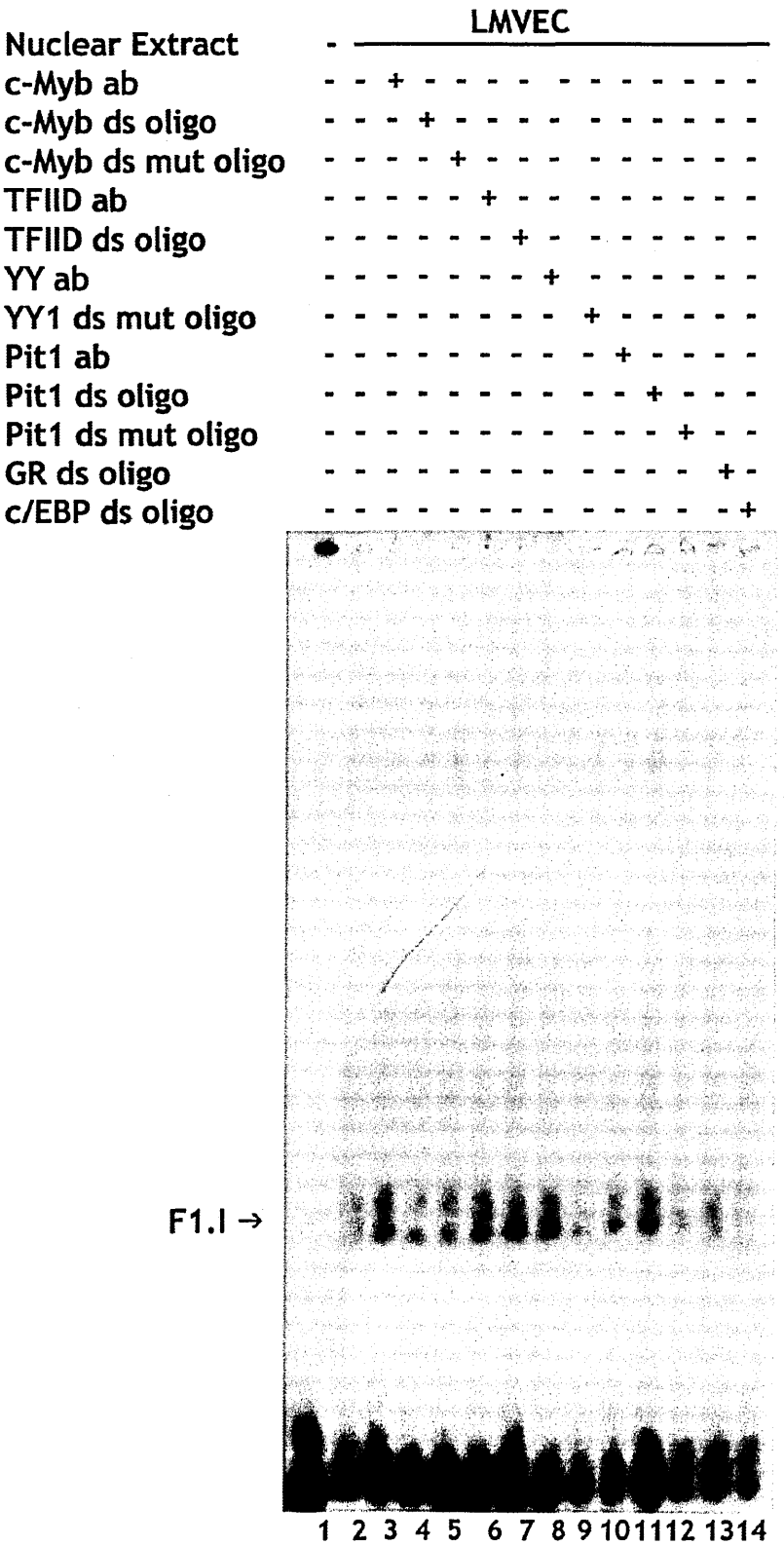
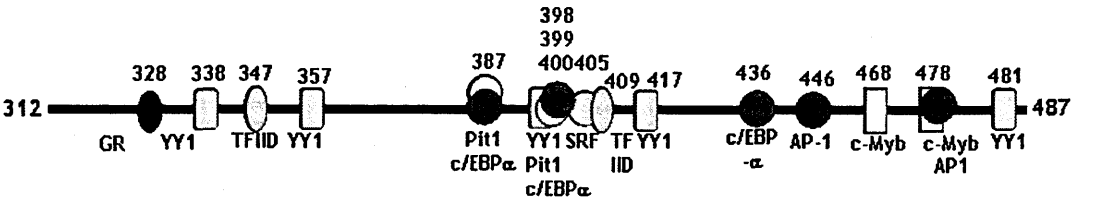


Fig. 4.7 EMSA analysis of nuclear protein derived from LMVEC cells bound to ^{32}P labelled probe F1. Antibodies, *wild type* or mutated oligonucleotides to c-Myb (lane 3 and *4*, 5), TFIID (lane 6 and 7), full-length YY1 (lane 8 and 9) or Pit1 (lane 10 and *11*, 12) were added to characterise the composition of the nuclear protein DNA complex (1.I).

4.5.7 Fragment 2.1



F 2.1 probe of the occludin promoter spans nucleotides 312 to 487 and exhibits putative binding sites for the TFs GR (328), c-Myb (468, 478), YY1 (338, 357, 417, 481) and TFIID (347, 409), SRF (405), AP1 (446, 478), Pit1 (387, 399), c/EBP-α (387, 400, 436).

Activity of F2.1 in brain endothelium (hCMEC/D3)

Nuclear protein from brain and non-brain endothelium interacted with probe F2.1 (Fig 4.8) to form a single nuclear protein-DNA complex which was not affected in the presence of consensus oligonucleotides or antibodies directed against any of the factors predicted to have binding sites on the probes.

Table 4-10 Summary of Activity of F2.1 in Brain Endothelium (hCMEC/D3)

Shifted band	YY1	c-Myb	TFIID	c/EBP	AP1
F2.1.1	-	-	-	-	-

Figure 4-8 Activity of Fragment 2.1 in Brain Endothelium

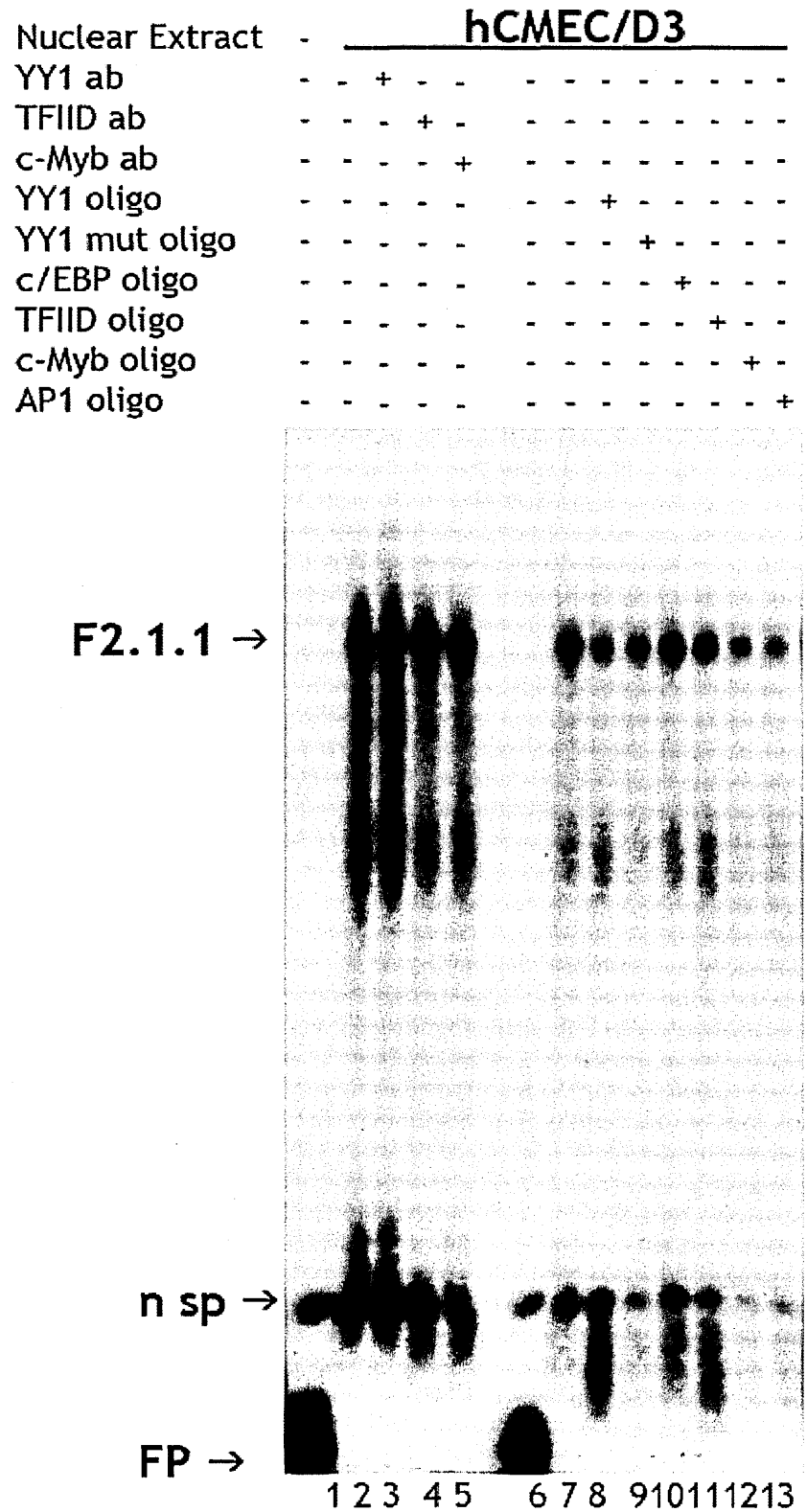
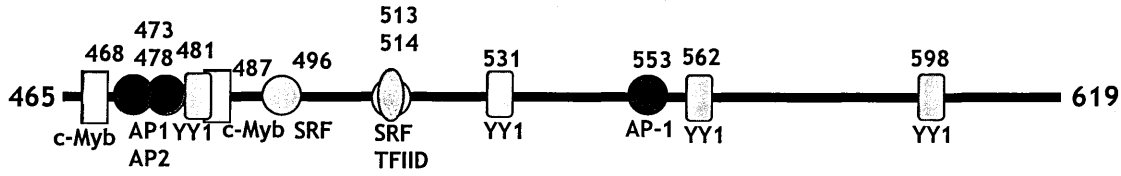


Fig. 4.8 Nuclear protein isolate from hCMEC/D3 cells was incubated with ^{32}P labelled probe F2.1. The DNA-protein complex (2.1.1, lanes 2, 7) was challenged with antibodies, *wild type* or mutated consensus oligonucleotides to YY1 (lanes 3, *8*, 9), TFIID (lanes 4, *11*), c-Myb (lanes 5 and *12*), AP1 (lane *13*) and c/EBP (lane *10*).

4.5.8 Fragment 2.2



F2.2 probe of the occludin promoter spans nucleotides 465 to 619 and exhibits putative binding sites for the TFs c-Myb (468, 487), YY1 (481, 531, 562, 598), TFIID (513), SRF (514), AP1 (478, 553), AP2 (473).

Activity of F2.2 in brain endothelium (hCMEC/D3)

Nuclear protein from brain and non-brain endothelium interacted with probe F2.2 (Fig 4.9) to form a single nuclear protein-DNA complex which was not affected in the presence of consensus oligonucleotides or antibodies directed against any of the factors predicted to have binding sites on the probes.

Table 4-11 Summary of Activity of F2.2 in Brain Endothelium (hCMEC/D3)

Shifted band	YY1	c-Myb	TFIID	SRF	AP1	AP2
F2.2.1	-	-	-	-	-	-

Figure 4-9 Activity of Fragment 2.2 in Brain Endothelium

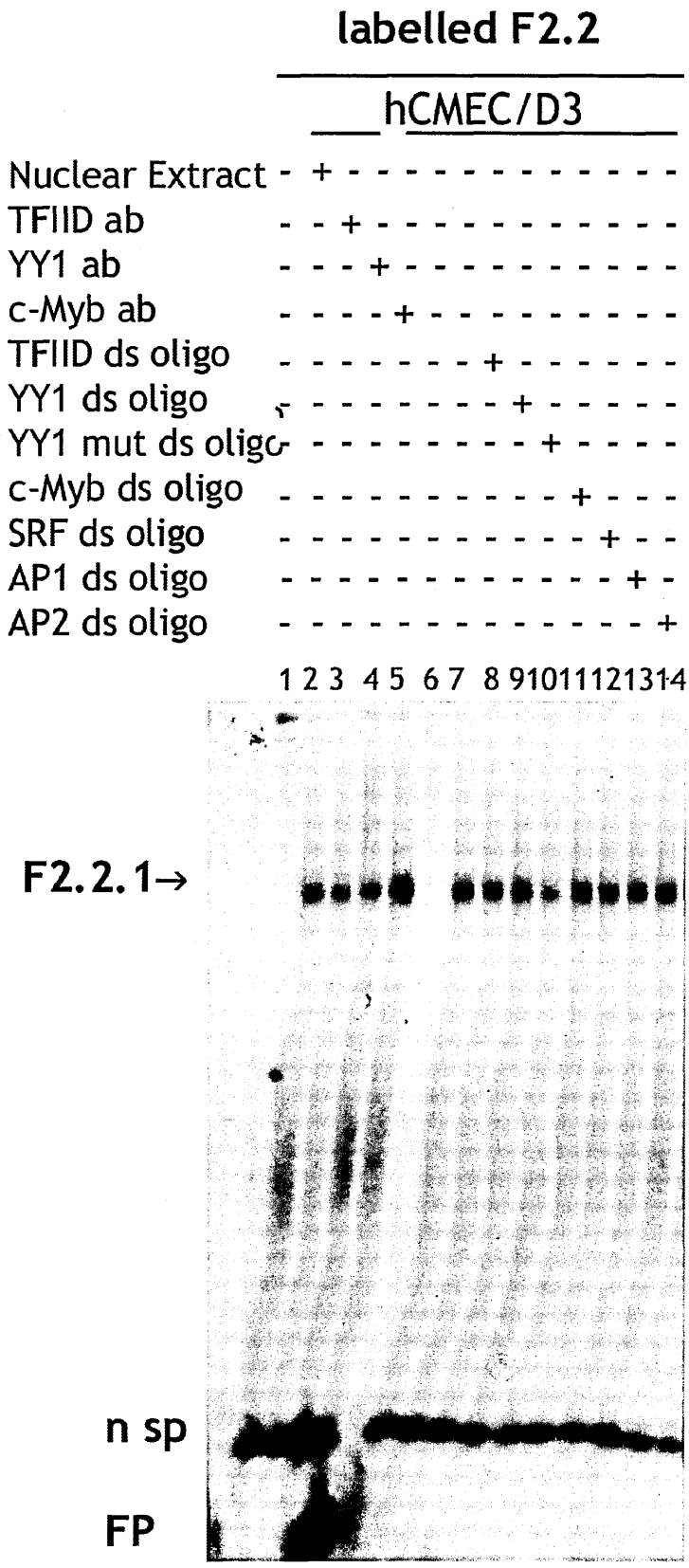


Fig. 4.9 Nuclear protein isolate from hCMEC/D3 cells was incubated with ^{32}P labelled probe F2.2. The DNA-protein complex (2.2.1, lanes 2, 7) was challenged with antibodies, *wild type* or mutated consensus oligonucleotides to YY1 (lanes 4, *9*, 10), TFIID (lane 3), c-Myb (lane *11*), SRF (lane 12), AP1 (lane *13*) and AP2 (lane *14*).

4.5.9 Fragments 4.1 and 4.2

Probes 4.1 and 4.2 span the occludin promoter between the nucleotides 842-994 and 972-1091 respectively. The region comprising nucleotides 876-1044 is conserved in human and mouse. The probes exhibit putative binding sites for the TFs Pit1, TFIID, YY1 and NF1.

Activity of 4.1 and 4.2 in brain and non brain endothelium

In the presence of nuclear protein from hCMEC/D3 and lung endothelial cells the probes generated a single nuclear protein-DNA complex in each condition (Fig. 4.10-F4.1.1 and F4.2.1). But, antibodies and/or competitor probes to the transcription factors predicted to bind to the sequence did not abrogate or restrict the migration of the complexes. Extensive smearing was seen in the lanes where nuclear extract from hCMEC/D3 cells was used. This smearing was also seen with nuclear protein from lung endothelial cells but it was less intense. There is a possibility that there could be a protein-DNA complex in the region of the smearing. Hence, poly dA: dT was employed instead of poly dI: dC which was effective in getting rid of the background. However, it also abrogated the formation of complex F4.1.1 (Fig. 4.11). This can be attributed to the fact the F4.1.1 is a low specificity complex. Nuclear protein-DNA F4.2.1 was seen but remained unaffected when challenged by antibodies and/or cold competitor probes to the TFs.

Figure 4-10 Activity of Fragments 4.1 and 4.2 in Brain and Lung Endothelium

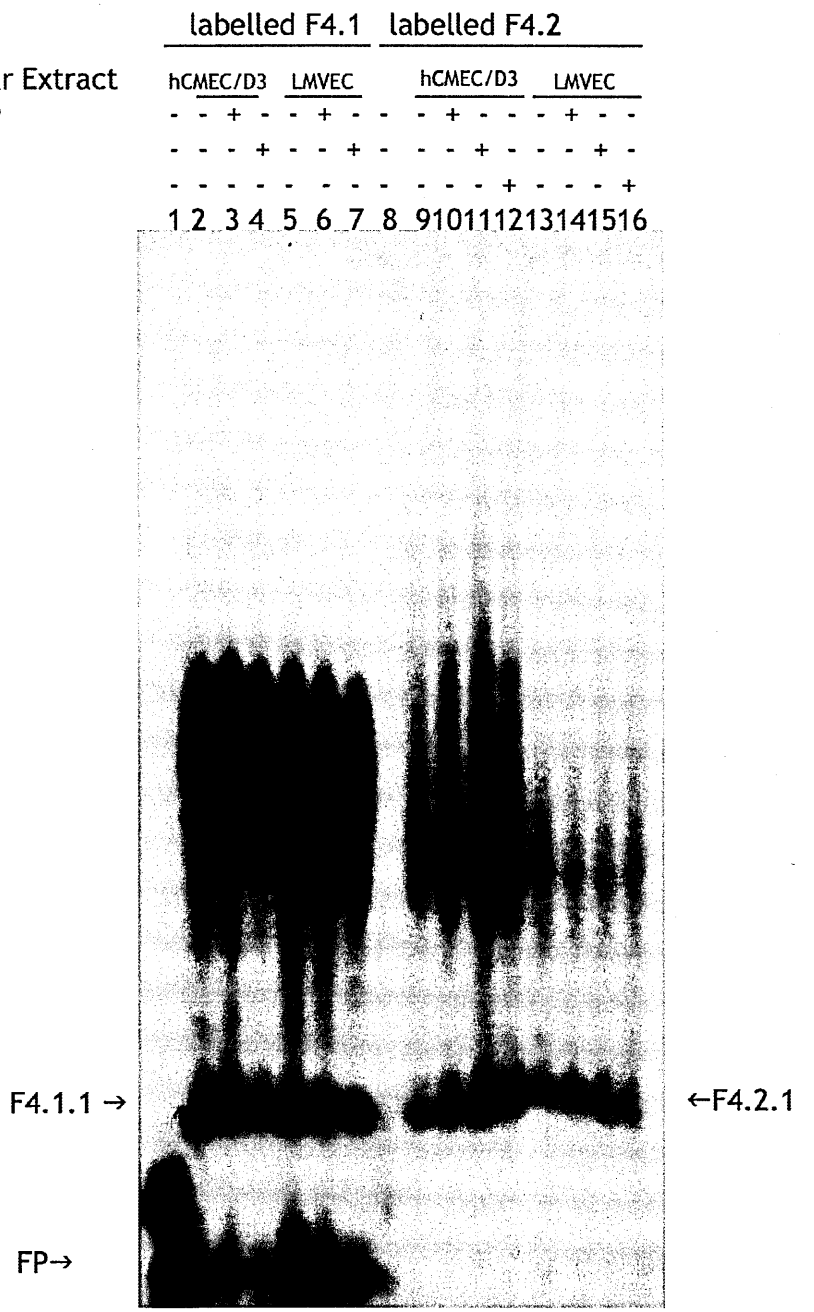


Figure 4-11 Activity of Fragments 4.1 and 4.2 in Brain Endothelium

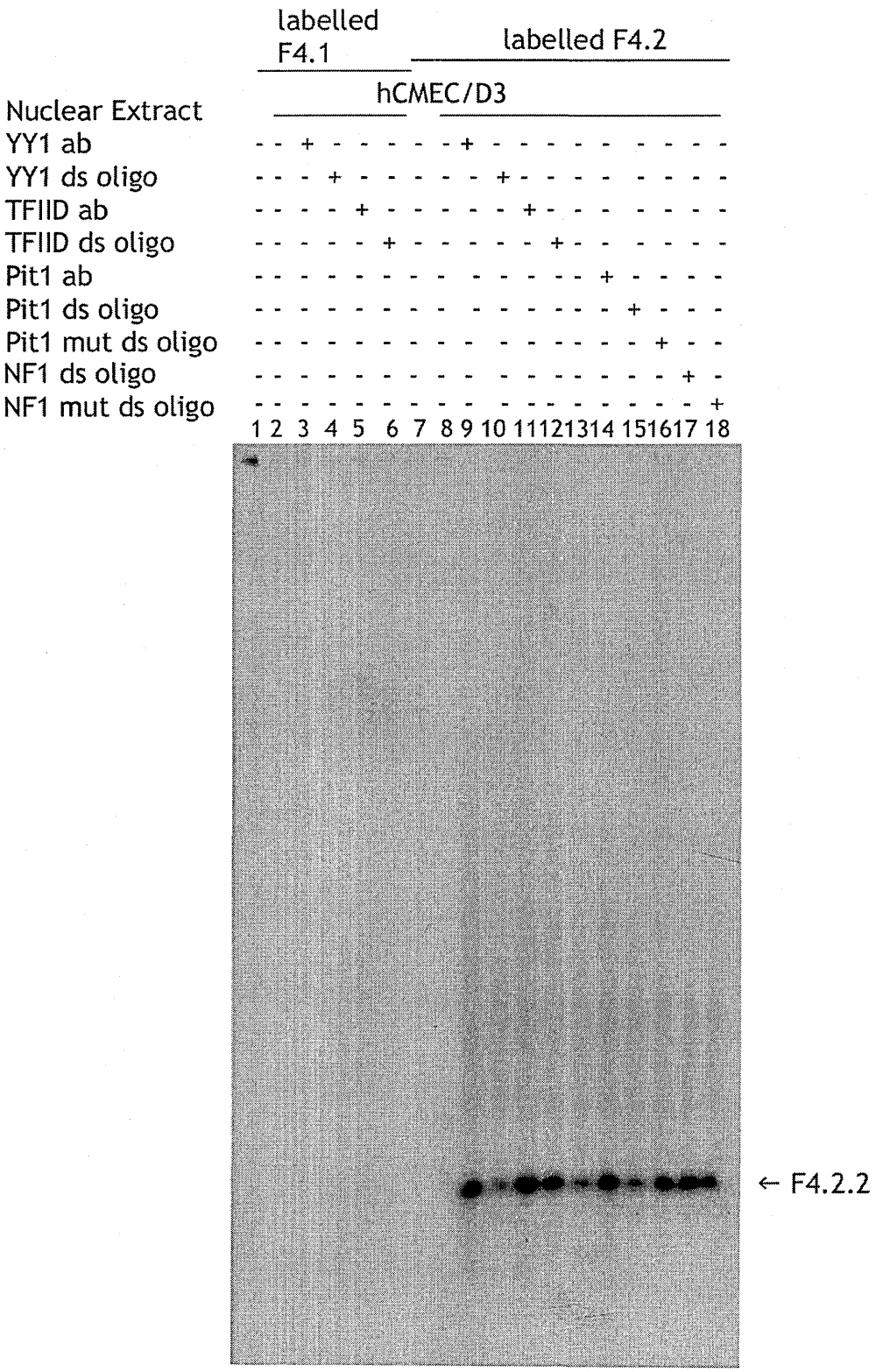
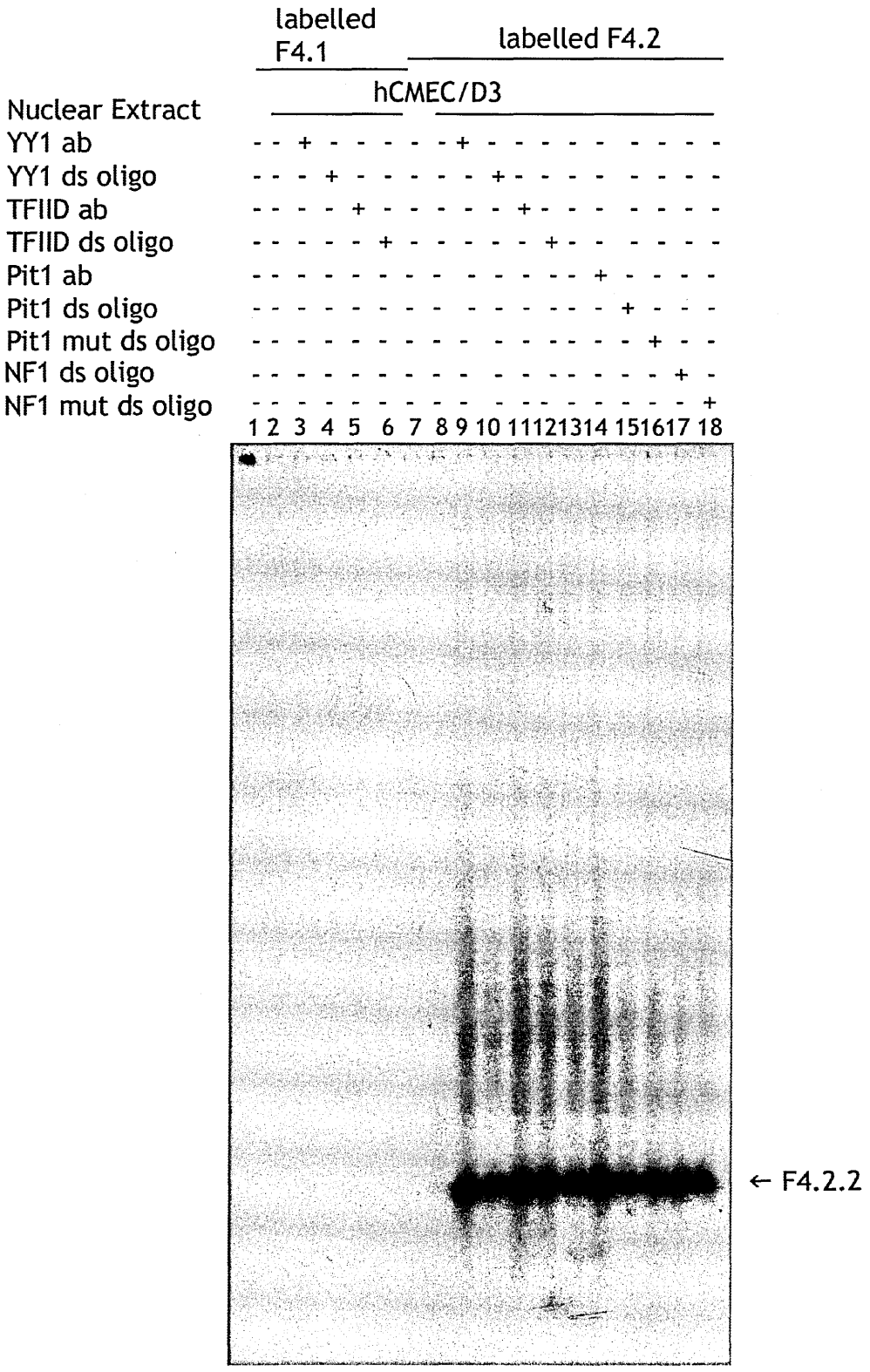


Figure 4-11 Activity of Fragments 4.1 and 4.2 in Brain Endothelium



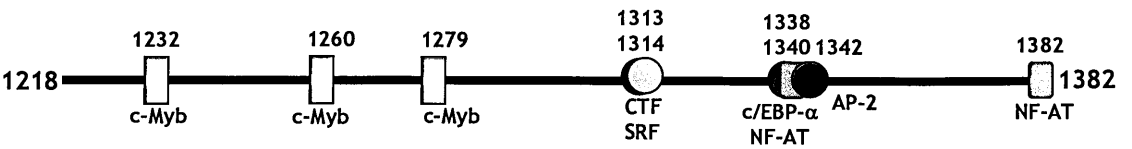
(lanes 5-7; 13-16) endothelial cells was incubated with ^{32}P labelled probe F4.1 (lanes 1-7) or F4.2 (lanes 8-16). The DNA-protein complexes (F4.1.1 and F4.2.1- lanes 2, 5, 9, 13) were challenged with antibodies to YY1 (lanes 3, 6, 10, 14), TFIID (lanes 4, 7, 11, 15) and Pit1 (lanes 12, 16).

Fig. 4.11: Nuclear protein isolated from hCMEC/D3 endothelial cells was incubated with ^{32}P labelled probe F4.1 (lanes 1-8) or F4.2 (lanes 9-18). The non-specific inhibitor poly dA:dT was used instead of poly dI:dC. The DNA-protein complexes were challenged with antibodies, *wild type* or mutated consensus oligonucleotides to YY1 (lanes 3, *4*, 9, *10*), TFIID (lanes 5, *6*, 11, *12*) and Pit1 (lanes 14, *15*, 16) and NF-1 (*17*, 18)

Table 4-12 Summary of Activity in Brain (hCMEC/D3) Endothelium

Shifted band	YY1	TFIID	Pit1	NF1
F4.1.1	-	-	-	-
F4.2.1	-	-	-	-

4.5.10 Fragment 6.1



F6.1 of the occludin promoter exhibits putative binding sites for the TFs c-Myb (1232, 1260, 1279), SRF (1314), NF-AT (1340, 1382), AP2 (1342) and c/EBP-α (1338).

Activity in brain endothelium (hCMEC/D3)

The promoter segment spanning nucleotide base pairs F6.1 interacted with the nuclear proteins from the hCMEC/D3 cells to generate six distinct migratory DNA-protein complexes (Fig. 4.12, lanes 2 and 6). Addition of an antibody to YY1 abrogated complex 6.1.5 in contrast to the competitor which proceeded to block the formation of all the complexes but a mutant form of the competitor failed to inhibit or retard the complex confirming the sequence specificity of this interaction. From the data it is likely the YY1 sites are occupied by other transcription factors.

Figure 4-12 Activity of Fragment 6.1 in Brain Endothelium

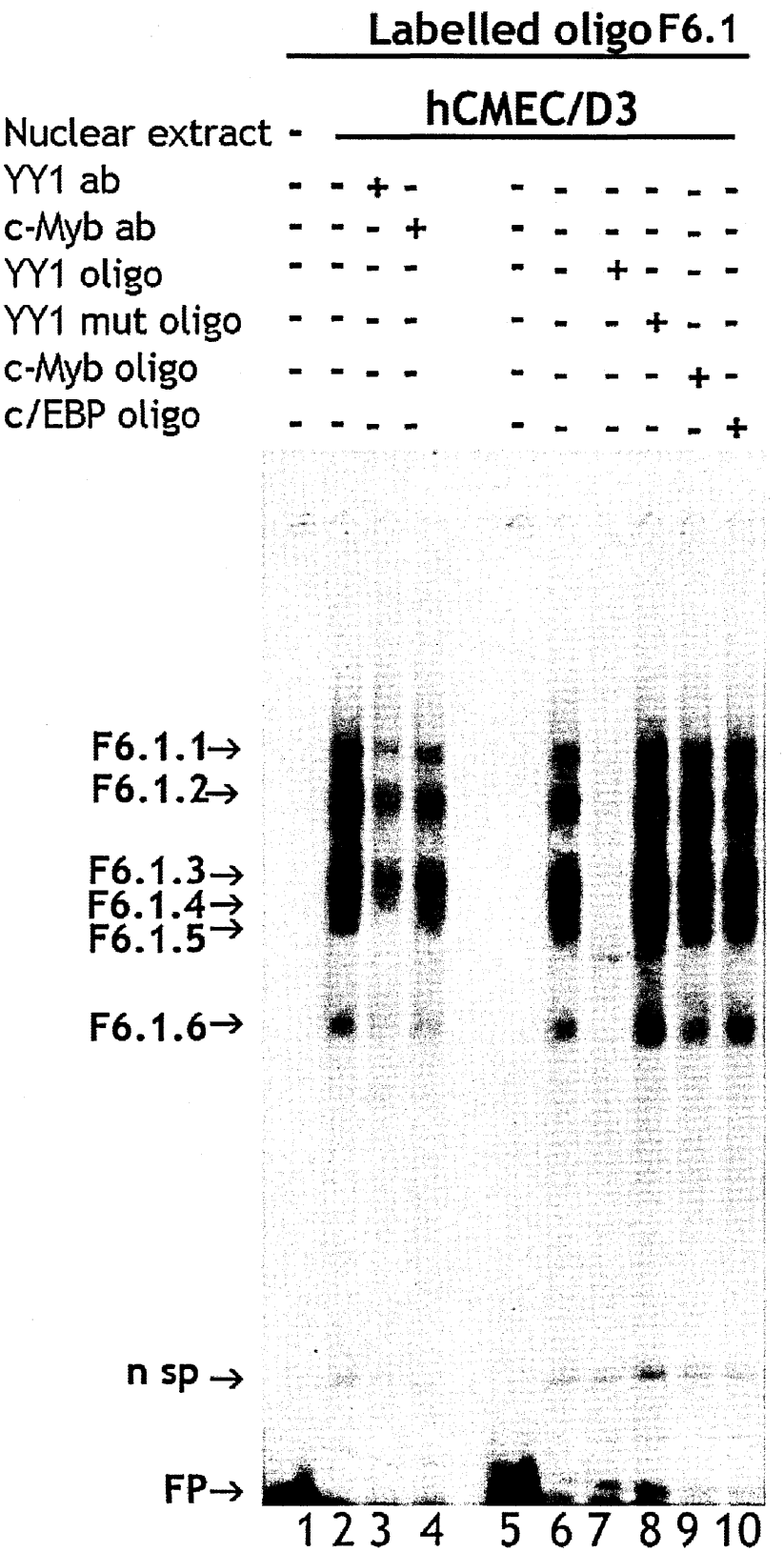


Fig. 4.12: Nuclear protein isolated from hCMEC/D3 cells was incubated with ^{32}P labelled probe F6.1. The DNA-protein complexes (6.1.1-6.1.6, lanes 2, 6) were challenged with antibodies, *wild type* or mutated consensus oligonucleotides to YY1 (lanes 3, *7*, 8), c-Myb (lanes 4 and 9) and c/EBP (lane *10*). Shifts 6.1.5 and 6.1.6 were retarded in the presence of an antibody to YY1 (lane 3) and the complexes were restored in the presence of an YY1 mutated oligonucleotide (lane 8) confirming the sequence specific interaction of YY1 with the probe. The consensus oligonucleotide however blocked the formation of all the complexes (lane 7) and it is possible these complexes are specific to transcription factors that are binding to the YY1 target site.

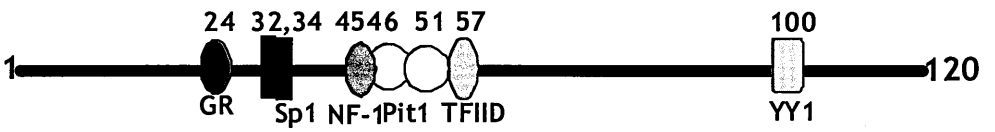
Table 4-13 Summary of Activity of F6.1 in Brain Endothelium (hCMEC/D3)

Shifted Band	YY1	c-Myb	c/EBP-α
6.11	-	-	-
6.12	-	-	-
6.13	-	-	-
6.14	✓	-	-
6.15	✓	-	-
6.16	-	-	-

4.6 Promoter Probes with Sp and YY1 Sites

Fragment	Position on Chr5 NT_006713	Size (bp)	*Target sites for Sp1	*Target sites for YY1
0.1	19380963-19381083	120	X	X
0.2	19381054-19381188	128	X	X
3.1	19381561-19381740	179	X	X
5	19382031-19382201	170	X	X

Fragment F 0.1



F0.1 of the occludin promoter exhibits numerous binding sites for diverse transcription factors as predicted by the TESS program. The reverse primer for F0.1 is also the forward primer for F0.2 hence there is an overlap of twenty three base pairs and so the probes share the predicted YY1 binding site at position 100. In addition to the YY1 site, F0.1 has response elements for TFs: interferon regulatory factors IRF1 and IRF 2, GR, members of the Sp family, Pit1 and TFIID.

IRF-1 and IRF-2 are highly homologous TFs that bind specifically to AAGTGA hexamer repeat motifs (Tamura, Yanai et al. 2008). IRF-1 recognizes this site to initiate transcription of downstream target genes in contrast to IRF-2 which binds competitively and represses transcription through its C-terminal repression domain. The IRF recognition DNA sequence is also found in the regulatory

regions of IFN-α and IFN-inducible genes so it is possible that occludin promoter region is a target for transcriptional modulation during inflammation and disease.

Activity of F0.1 in brain endothelium (hCMEC/D3)

The single nuclear protein-DNA complex formed between probe F0.1 and nuclear proteins from brain endothelial cells (F0.1.1) is not specific for any of the transcription factors with binding sites in the segment (Fig. 4.13)

Activity of F0.1 in lung endothelium (LMVEC)

However, probe F0.1 formed three complexes-F0.1.I, F0.1.II, F0.1.III with nuclear proteins from lung endothelium (Fig. 4.14, lane 2). Complex 0.1.III was formed by YY1, since addition of a full length antibody to YY1 (lane 7) super shifted the complex. Probes F0.1 and F0.2 share one common YY1 site but the nuclear protein-DNA complex formed with F0.2 is not specific for YY1 (Fig 4.15), implying that the single YY1 site in F0.1 is a genuine target for YY1. Complex 0.1.II was blocked by a TFIID consensus oligonucleotide (lane 6), but was not supershifted by a TFIID antibody. There is a potential TFIID site in F0.1 located between the Sp site and the YY1 site.

Hence these data imply that a DNA-binding protein occupies the TFIID site, but that it is not TFIID itself. Taken together the data suggests that the YY1 site in F0.1 is active in lung endothelium, but not brain endothelium and that two additional proteins from lung endothelium may bind to this region.

Figure 4-13 Activity of Fragment 0.1 in Brain Endothelium

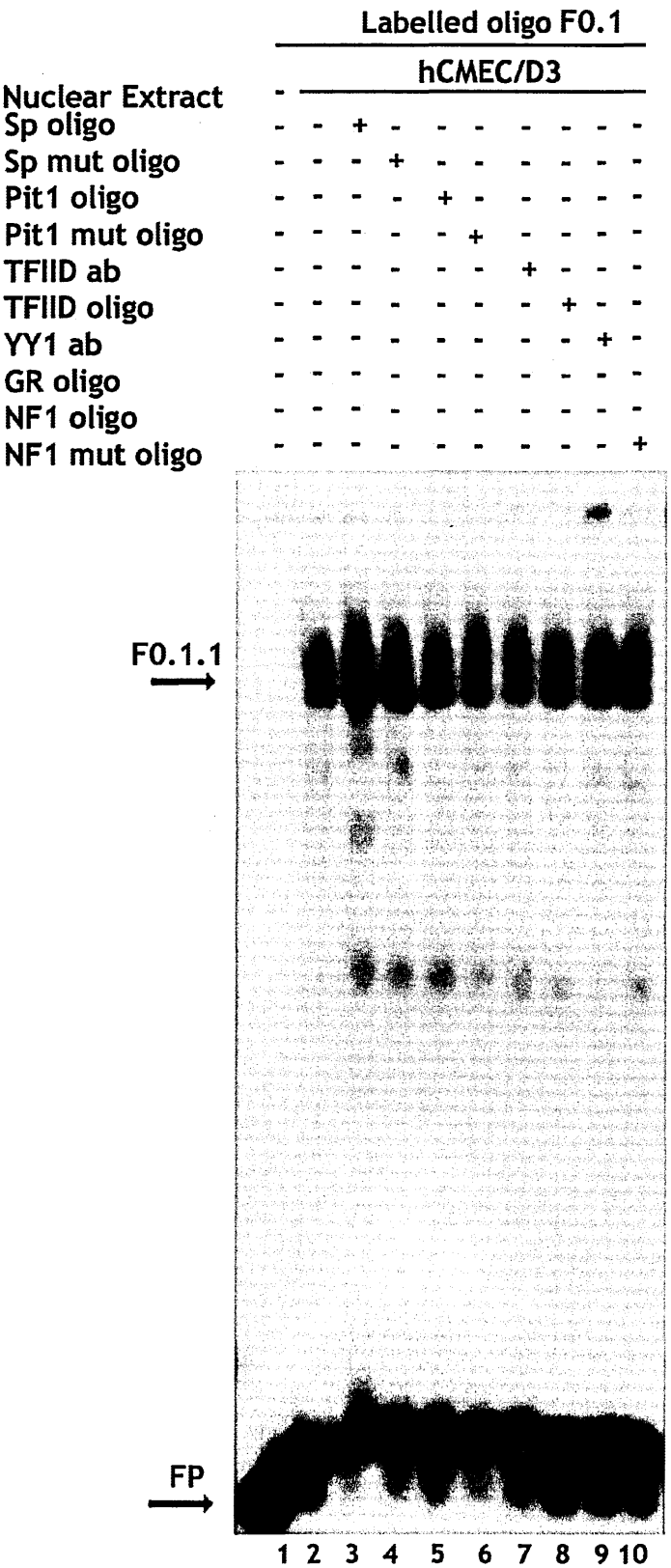


Fig. 4.13: EMSA analysis showing the binding of nuclear extracts from brain endothelial cells to ^{32}P labelled probe F0.1 in the presence of consensus oligonucleotides to Sp1 (lane 3), Pit1 (lane 5), TFIID (lane 8) or NF1 (lane 10) or with antibodies to TFIID (lane 7), full-length YY1 (lane 9) or with mutated consensus probes- Sp1 (lane 4) and Pit1 (lane 6).

Figure 4-14 Activity of Fragment 0.1 in Lung Endothelium

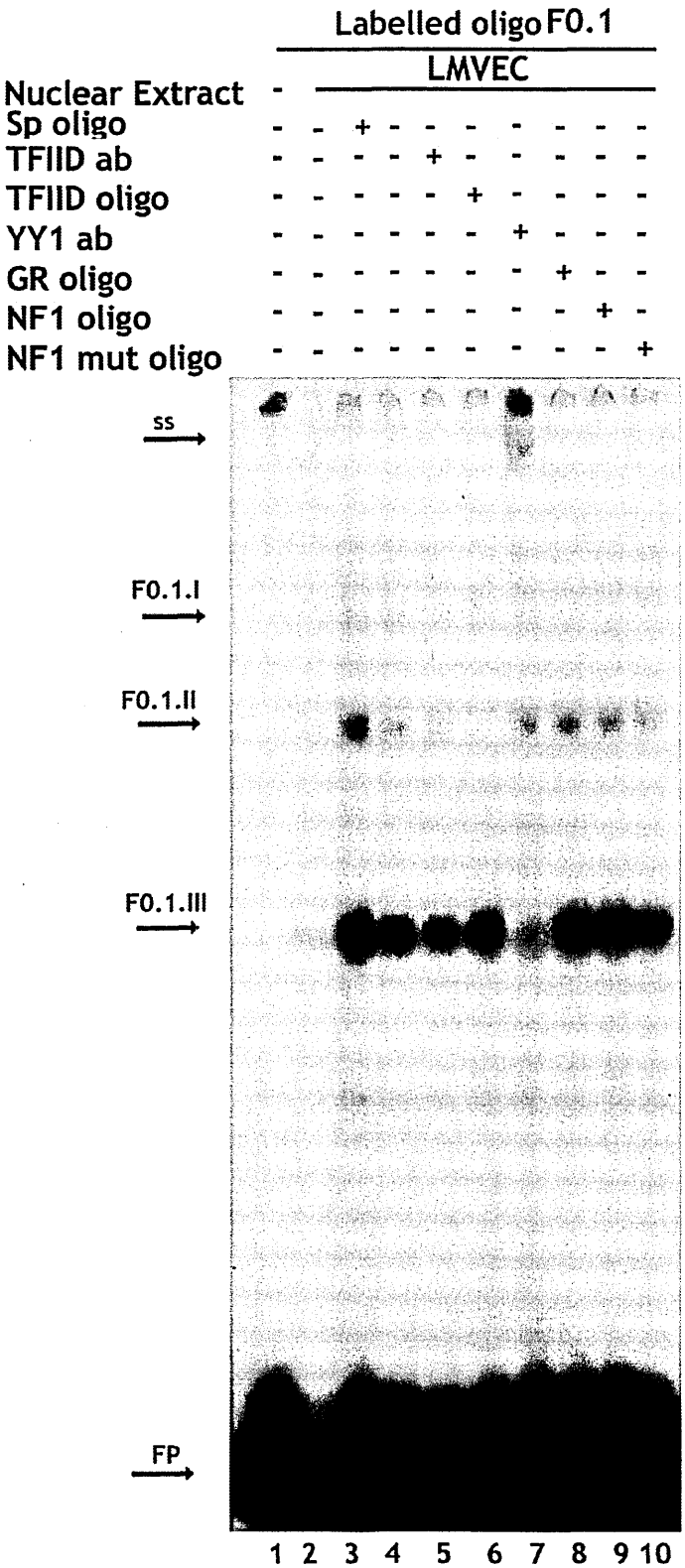


Fig. 4.14: EMSA analysis of nuclear protein complexes formed between radiolabelled probe F0.1 (0.1.I, 0.1.II, 0.1.III, lanes 2-10) in the absence (lane 1) or presence of nuclear proteins (lanes 2) isolated from lung endothelial cells. Consensus oligonucleotides to Sp1, Pit1, TFIID, GR, NF1 (lanes 3, 4, 6, 8, 9, 10 respectively) or antibodies to TFIID (lane 5) and full length YY1 (lane 7) were added to the binding reactions in order to investigate the composition of the complexes. Shift 0.1. III (ss, *) was super shifted in the presence of antibody to YY1, whereas shift 0.1.II was retarded by an antibody to TFIID confirming the presence of functional YY1 and TFIID proteins within LMVEC cell nuclei. ss denotes super shifted band.

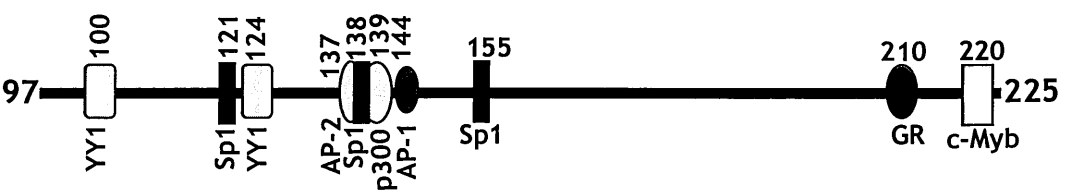
Table 4-14 Summary of F0.1 Activity in Brain Endothelium (hCMEC/D3)

Shifted Band	Sp	Pit1	TFIID	YY1
F0.1.1	-	-	-	-

Table 4-15 Summary of F0.1 Activity in non-brain endothelium (LMVEC)

Shifted Band	Sp	Pit1	TFIID	YY1
F0.1.I	-	-	✓	-
F0.1.II	-	-	-	✓
F0.1.III	-	-	-	-

Fragment F0.2



Fragment 0.2 shares an YY1 site at position 100 with F0.1 in addition to a predicted site for YY1 at position 124. Other TFs with putative target binding sites in this region include Sp (121, 138, 155), AP2 (137), p300 (139), GR (210) and c-Myb (220).

Figure 4-15 Activity of Fragment 0.2 in Brain Endothelium

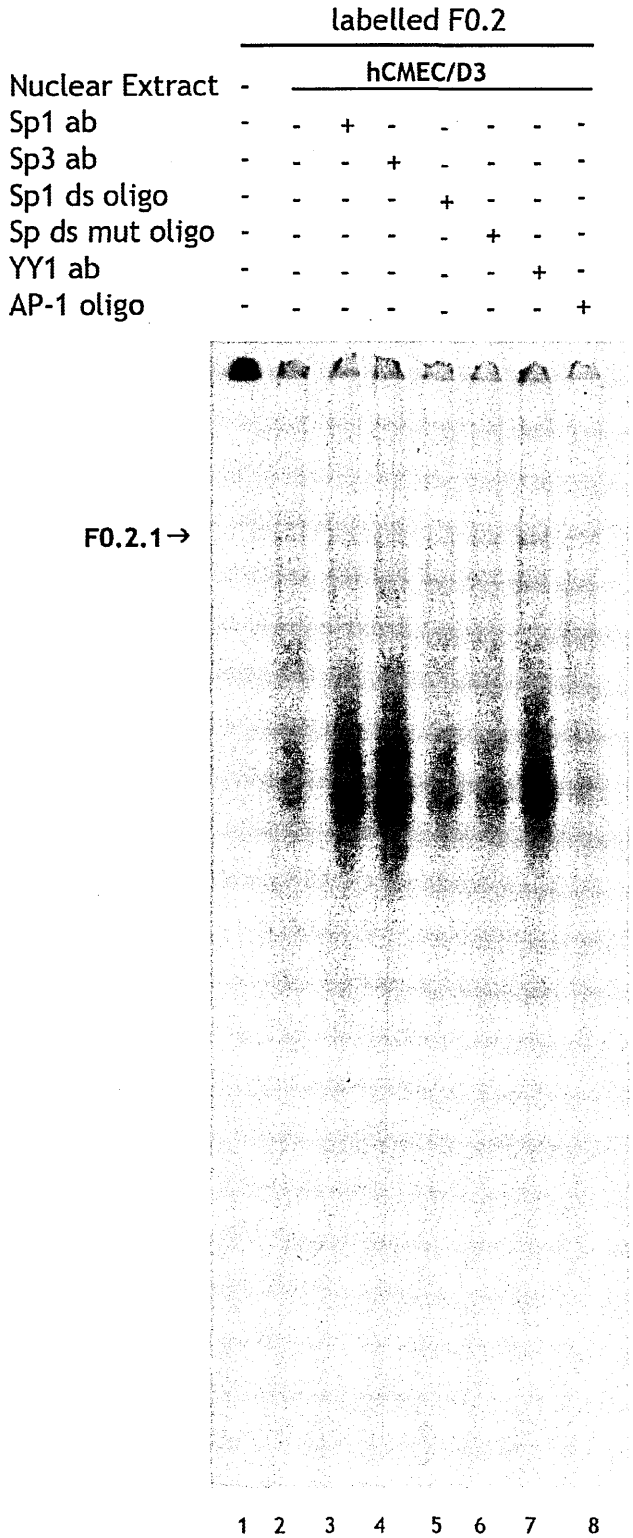


Fig. 4.15 EMSA analysis of nuclear extracts from brain endothelial cells with ^{32}P labelled probe F0.2 in the presence of antibodies to Sp1 (lane 3) or Sp3 (lane 4) or to full-length YY1 (lane 7) or with 30-molar excess of consensus oligonucleotides to Sp1 (lane 5), mutated Sp1 probe (lane 6) or to AP1 (lane 8).

Figure 4-16 Activity of Fragment 0.2 in Lung Endothelium

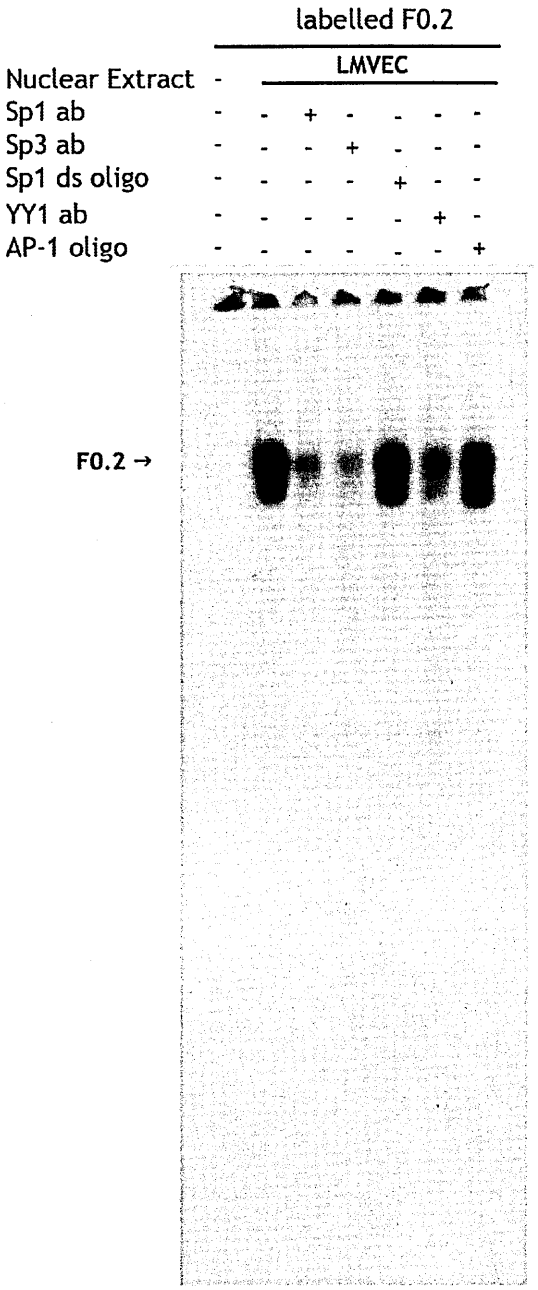
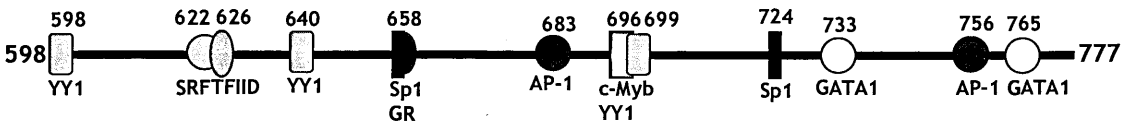


Fig. 4.16 EMSA analysis of nuclear extracts from lung endothelial cells with ^{32}P labelled probe F0.2 in the presence of antibodies to Sp1 (lane 3) or Sp3 (lane 4) or to full-length YY1 (lane 6) or with 30-molar excess of consensus oligonucleotides to Sp1 (lane 5) or to AP1 (lane 7)

4.6.1 Fragment 3.1



Activity of F3.1 in brain endothelium (hCMEC/D3)

The probe F3.1 showed three distinctly migrating DNA-protein complexes in the presence of nuclear proteins from hCMEC/D3 cells-F3.1.1, F3.1.2 and F3.1.3 (Fig 4.17). All of the complexes were competed out by the addition of 100-fold molar excess of non radio-labelled Sp1 consensus oligonucleotide (lane 8). However, different complexes were retarded in the presence of antibodies to Sp1 (lane 5, complex I) and Sp3 (lane 6, complex III). The DNA probe has in addition potential binding sites for other transcription factors including c-Myb, the antibody to this transcription factor affects the mobility of complex III (lane 7) but the binding is not specific as addition of an unlabelled competitor does not block the association (lane 9).

Table 4-18 Summary of F3.1 Activity in Brain Endothelium (hCMEC/D3)

Shifted Band	YY1	TFIID	Sp1	Sp3	c-Myb
F3.1.1	-	-	-	✓	✓
F3.1.2	-	-	-	-	
F3.1.3	-	-	-	✓	✓

Figure 4-17 Activity of Fragment 3.1 in Brain Endothelium

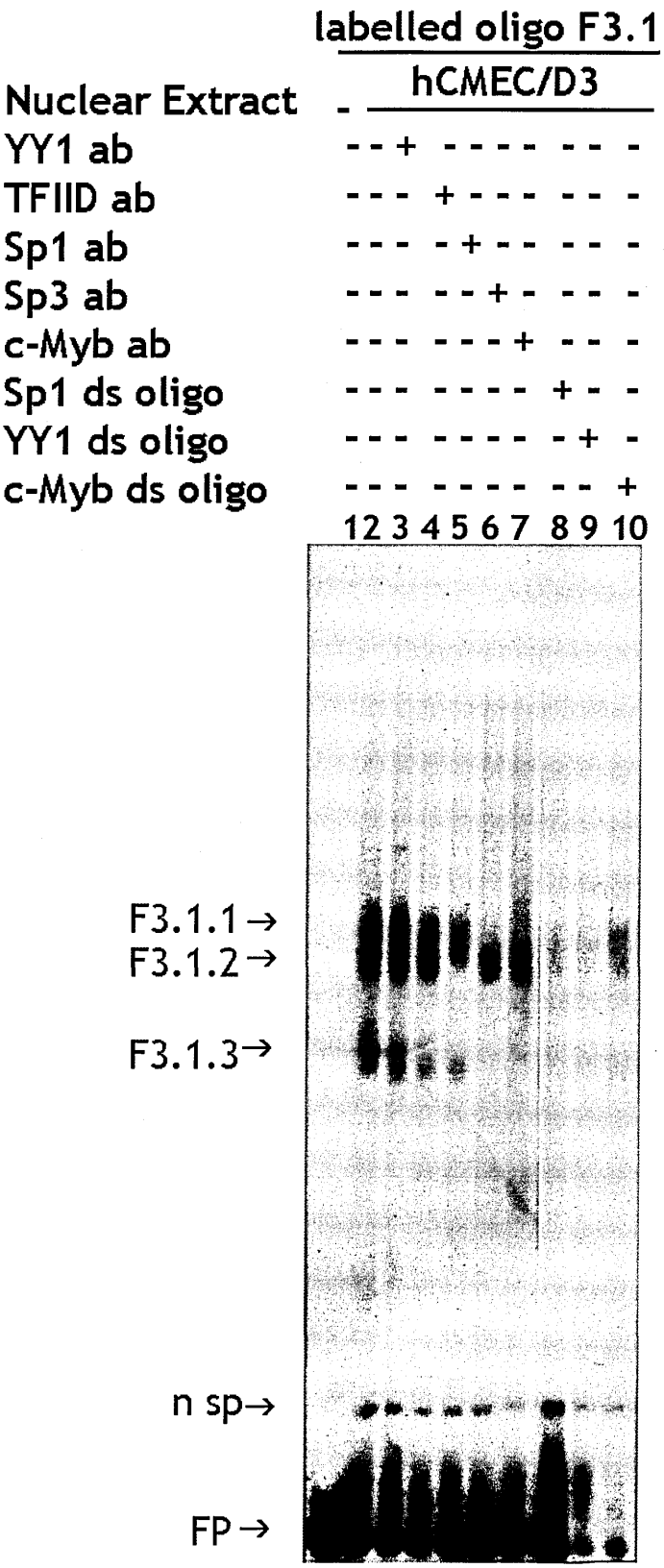
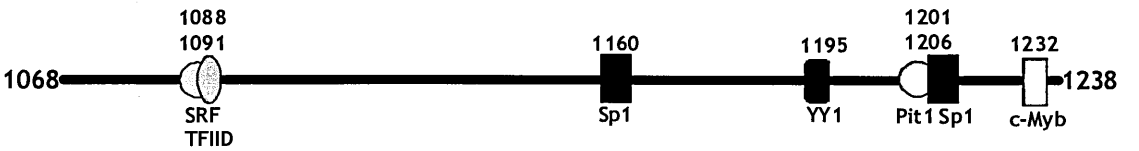


Fig. 4.17: DNA-protein complexes formed between ^{32}P labelled promoter probe F3.1 and nuclear extracts from hCMEC/D3 cells (lane 2) were investigated in the presence of antibodies (3-7) or consensus oligonucleotides (lanes 8-10) against transcription factors predicted to bind to the probe. Antibodies to Sp1 (lane 5) and Sp3 (lane 6) retarded complexes II and III respectively whereas the competitor to Sp1 (lane 8) restricted the formation of all the complexes. Retardation of complex III by the antibody to c-Myb (lane 7) was not specific as the competitor did not affect the formation of the complex (lane 10). Antibodies to YY1 (lane 3) and TFIID (lane 4) did not affect the mobility of the complexes.

4.6.2 Fragment 5



Fragment 5 spans the region 1068 to 1238 of the occludin promoter and includes target binding sites for the TFs SRF (1091), TFIID (1091), Sp1 (1160, 1206), YY1 (1195), Pitl (1201) and c-Myb (1232).

Activity of promoter probe F5 in brain endothelium (hCMEC/D3)

The double stranded probe F5 generates a high mobility DNA-protein complex (5.1) with brain endothelial nuclear proteins (Fig 4.18, lanes 2 and 8) and this interaction is retarded on incubation with an YY1 antibody (lane 6) and is also blocked by the addition of 100 molar excess of unlabelled competitor (lane 12). Addition of 100-fold excess of an unlabelled oligonucleotide containing a mutated YY1 binding site (lane 13) failed to inhibit or retard the complex confirming the sequence specificity of this interaction.

Activity of promoter probe F5 in non-brain endothelium (LMVEC)

The probe interacts with nuclear extracts from lung endothelial cells and generates a single nuclear protein-DNA complex, F5.I (Fig. 4.19, lane 2) which was not affected in the presence of consensus oligonucleotides or antibodies directed against any of the factors predicted to have binding sites on the probe (lanes 3-15).

Table 4-19 Summary of F5 Activity in Brain Endothelium (hCMEC/D3)

Shifted Band	YY1	Sp1	Sp3	TFIID	c-Myb
F5.1	✓	-	-	-	-

Table 4-20 Summary of F5 Activity in Non-Brain Endothelium (LMVEC)

Shifted Band	YY1	Sp1	Sp3	TFIID	c-Myb
F5.I	-	-	-	-	-

Figure 4-18 Activity of Fragment 5 in Brain Endothelium

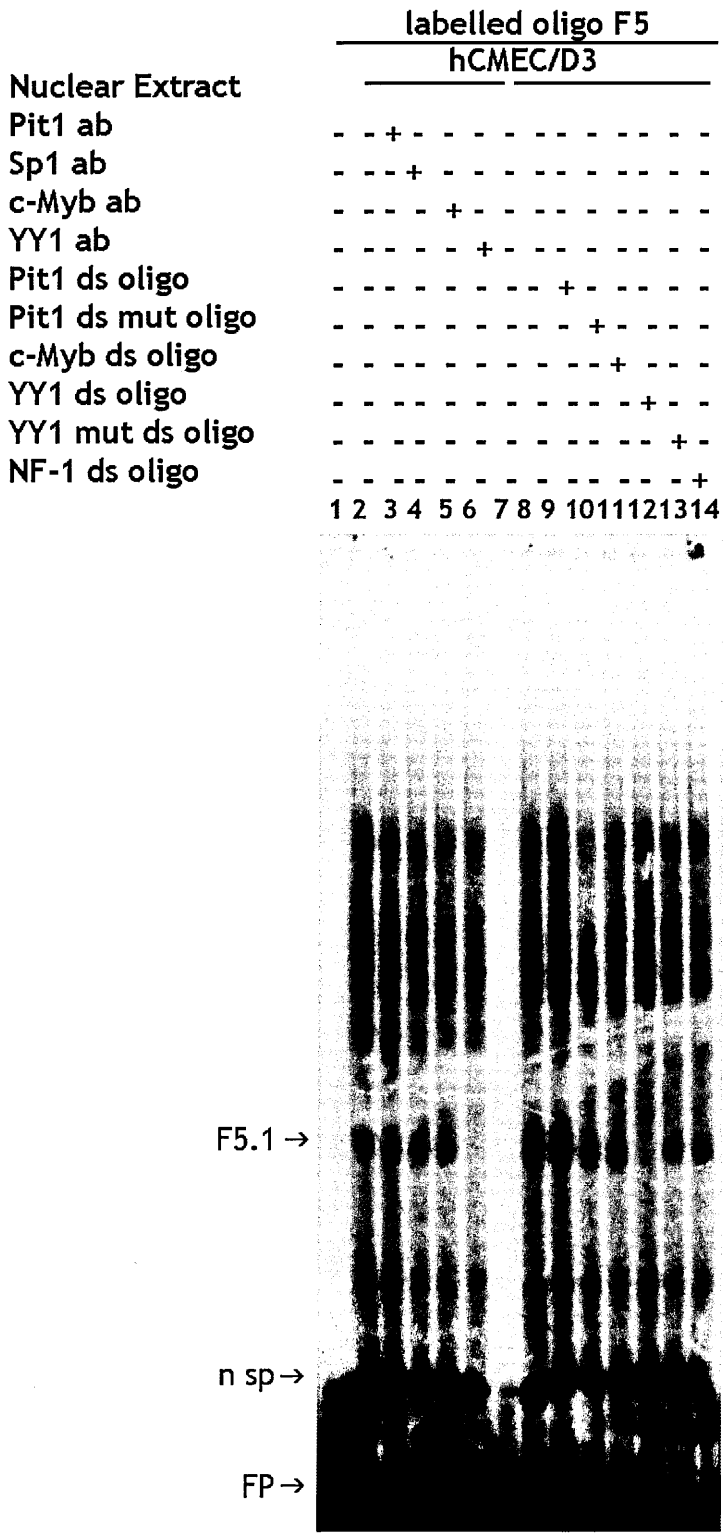


Fig. 4.18: ^{32}P labelled probe F5 was incubated in the absence of (lanes 1, 7) or presence of nuclear extracts from hCMEC/D3 endothelial cells (lanes 2-6; 8-15). DNA-protein complexes formed-F5.1 were characterised by incubating with antibodies, *wild type* or mutant oligonucleotides to transcription factors to Pit1 (lanes 3, *9*); Sp1 (lanes 4, *10*); c-Myb (5, *11*); YY1 (6, *12*, 13) predicted to bind to the sequence.

`Figure 4-19 Activity of Fragment 5 in Lung Endothelium

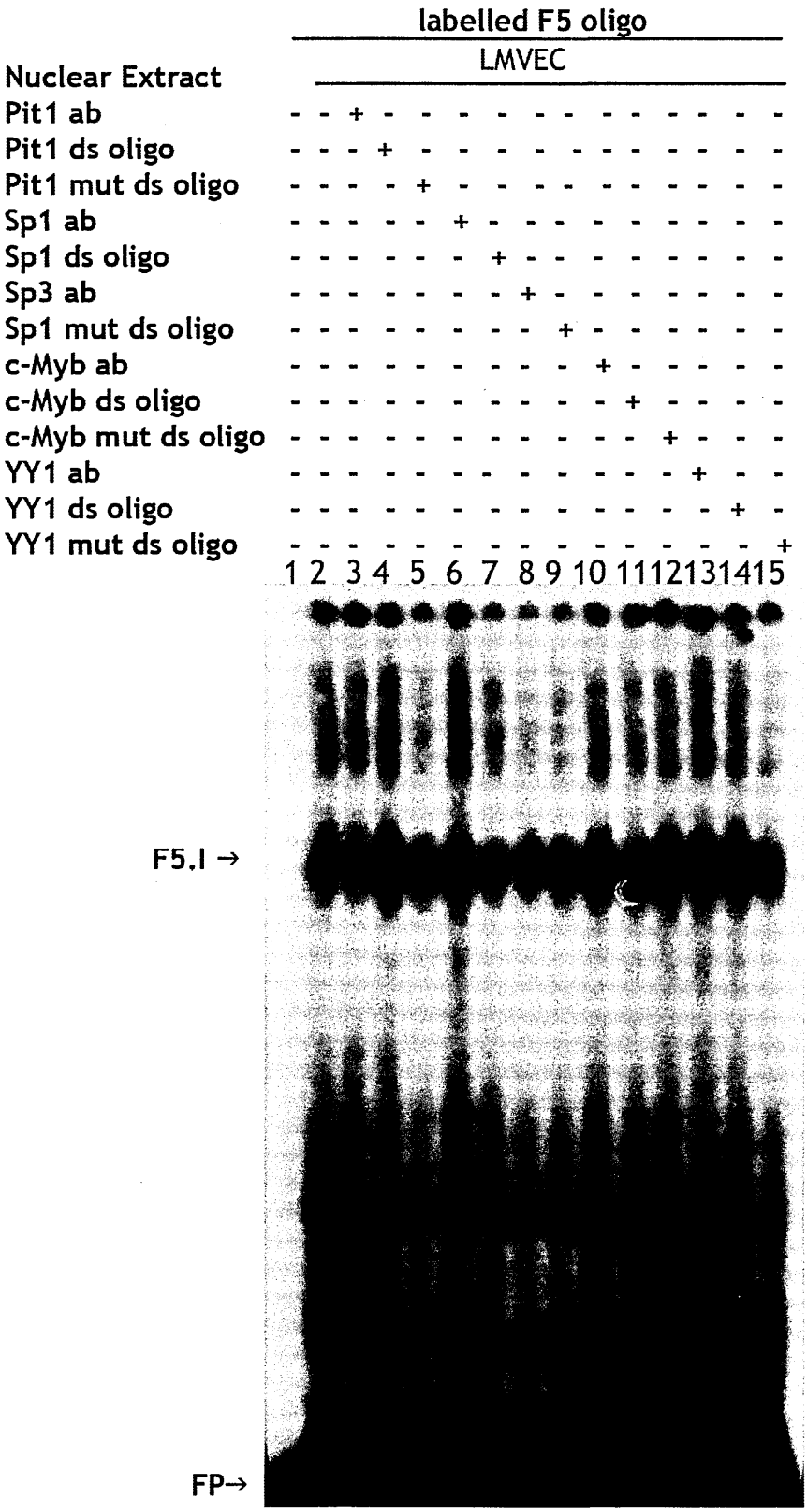


Fig. 4.19: ^{32}P labelled probe F5 was incubated in the absence of (lane 1) or presence of nuclear extracts from LMVEC endothelial cells (lanes 2-15). DNA-protein complexes formed-F5.I were characterised by incubating with antibodies, *wild type* or mutant oligonucleotides to transcription factors to Pit1 (lanes 3, *4,* 5); Sp1 (lanes 6, *8,* 9); Sp3 (7) c-Myb (10, *11*, 12); YY1 (13, *14*, 15) predicted to bind to the sequence.

4.7 Discussion

The occludin protein was identified as a major constituent of brain endothelial tight junctions (Furuse, Hirase et al. 1993; McCarthy, Skare et al. 1996). Apart from its restricted expression in brain endothelium, it is expressed in epithelia where it participates in tight junction formation and regulates paracellular permeability. The regulation of expression from the occludin promoter has been studied in epithelial cells (Mankertz, Tavalali et al. 2000). Information on the mechanism of occludin gene expression is useful in understanding the differential regulation of the protein in different endothelia. Additionally, occludin has been shown to play an important role in pathology of several neurodegenerative disorders and inflammation processes. It is dephosphorylated in experimental autoimmune encephalitis (EAE) (Morgan, Shah et al. 2007). This event has been shown to coincide with inflammation and increased BBB permeability. These data imply occludin is a possible target in the pathological process in EAE. In 2007, (Romanitan, Popescu et al. 2007) showed an increase in occludin expression in Alzheimer's disease (AD), and vascular dementia (VD) brains in comparison to ageing controls. So understanding the regulation of occludin gene expression in normal physiology is helpful to investigate possible alteration of transcriptional regulatory mechanisms in inflammatory or degenerative diseases of the CNS.

In intestinal epithelium, TNF-α has been shown to affect transcription from the promoter. Specific transcription factors have not yet been attributed to the promoter in endothelial cells. We have used the program TESS to screen for potential TFs binding motifs in the promoter. The list of the transcription factors

has been summarised in a table (appendix). In this study, in addition to data from the TESS program, we have particularly focussed our attention on the Sp family and YY1 TFs. Previous work in our group on the regulation of the human transferrin receptor which is also restricted to the brain endothelium has implicated Sp and YY1 TFs to be important for its expression in brain endothelium (Holloway, Sade et al. 2007). Data suggests two important events that lead to the initiation of gene expression from the HTR promoter in brain endothelium:

1. Requirement of the transcription factors TFIID and Sp3.

2. TFIID successfully competes for YY1 binding sites on the HTR promoter DNA in the brain but not non-brain endothelium.

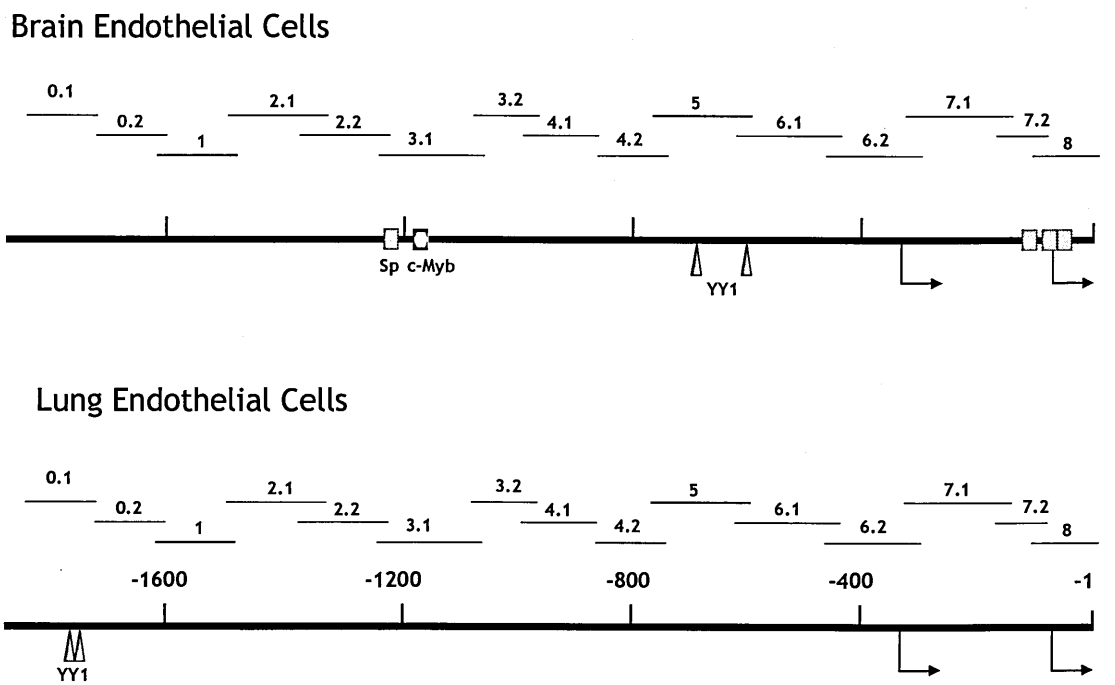
Additional data had shown differences in TF binding profiles between brain endothelial cells and lung, dermal and bone marrow endothelial cells as determined by EMSA. The TFs, Sp1, Sp3, IRF1, CBF, NFAT, SRE, and CDP exhibited similarity in the formation of nuclear protein complexes between the different endothelia. Pit1, GATA, c-Myb were slightly different and YY1 was completely different (Holloway, Sade et al. 2007).

We have used the transformed cell line, hCMEC/D3 and primary lung microvascular endothelium cells (LMVEC) to elucidate the regulation of transcription from the occludin promoter.

Our work identifies TFs Sp3 (Figs. 4.2, 4.3, 4.4, 4.5 and 4.17) and YY1 (Figs. 4.12 and 4.18) present in brain endothelial nuclear lysates to bind their target

elements on the occludin promoter. TFs c-Myb (Fig. 4.17), NFκB (Fig. 4.5) and AP2- α (Fig. 4.5) present in the brain endothelial cells also bind the promoter in EMSA analyses. In the lung endothelium however, only the YY1 TF occupies its target site in F0 (Fig. 4.14) and though Sp1 and Sp3 are expressed in the lung endothelium, they do not appear to bind the target motifs on the occludin promoter probes (Fig. 4.6). Therefore, it would be reasonable to think that these transcription factors might be involved in the expression of occludin in a cell-type-independent manner.

Figure 4-20 Summary of Identified TFs on the Occludin Promoter in Brain and Lung Endothelial Cells.



The transcription factors Sp1 and Sp3 are ubiquitous and play important roles in normal tissue and organ development (Suske 1999). It is to be noted that despite the ubiquitous nature of expression of Sp1 and Sp3, these factors have been

shown to regulate gene transcription in development and these mechanisms differ among cell types (Opitz and Rustgi 2000; Ghayor, Chadjichristos et al. 2001; Yoo, Jeong et al. 2002; Guo, Degnin et al. 2003; Reddy, Vuong et al. 2003; Nakamura, Kawachi et al. 2007). Changes in level of expression and nuclear localisation in cells may occur during different stages of development. And posttranslational modifications like acetylation and sumoylation have been known to affect the activity of these transcription factors whilst preserving their nuclear localisation (Sapetschnig, Rischitor et al. 2002; Stielow, Sapetschnig et al. 2008).

In endothelium, Sp family TFs have been implicated in the regulation of endothelial specific genes, cadherin (Gory, Dalmon et al. 1998), human thrombin receptor (Wu, Ruef et al. 1998), integrin alphaV promoter (Czyz and Cierniewski 1999) and fgl2 (Liu, Leibowitz et al. 2003). Sp1 functions mainly as a transcriptional activator in contrast to Sp3 which is capable of activation or repression depending on cellular context (Hagen, Muller et al. 1994). It is clear from the data obtained from the EMSA analyses that Sp3 competes successfully with Sp1 for the Sp target sites on the occludin promoter in brain endothelial cells. This maybe explained by the Sp3:Sp1 ratio in these cells where Sp3 protein levels are significantly higher than Sp1 (discussed in detail in Chapter 6). Considering that relatively high levels of Sp3 expression are observed in brain endothelial cells, gene expression mediated by Sp3 may be controlled by the expression level of Sp3 (Karantzoulis-Fegaras, Antoniou et al. 1999). And it is well known that promoters containing multiple adjacent Sp-sites form significantly more stable Sp3-DNA complexes than those with single Sp-binding sites. These Sp3-DNA complexes are also more stable than a corresponding Sp1-DNA complex (Yu,

Datta et al. 2003). This observation seems to be true in the case of promoter probe F8 (Figs. 4.4 and 4.5) which has two closely positioned Sp sites (1798 CCCTCCC 1804 and 1820 AGGCGG 1825). Sp1 is excluded from this complex and it is probable that Sp3 occupies both the sites.

There are two possible reasons for the inability of Sp factors present in lung endothelium to bind the occludin promoter fragments:

1. Inaccessibility of the TFs to the occludin promoter due to the presence of other DNA binding proteins that promote chromatin condensation in the occludin promoter region (discussed in chapter 5)

2. Increased level of splice variants of Sp3 which repress Sp1 and Sp3 mediated transcription (discussed in chapter 6)

In addition to Sp3, TF c-Myb binds the promoter probe F3.1 (4.17). However, the binding of c-Myb is not specific because the association is not blocked in the presence of a competitor. Two other proteins bind the occludin promoter on probe F8 and these associations are blocked in the presence of a competitor to NFκB (Fig. 4.5) and AP2-α (Fig. 4.5). We haven't been able to confirm the association of these TFs by super shift assays. Nevertheless, functional interaction between Sp family and AP2-α have been reported in the regulation of human pPro-a1 (I) collagen gene promoter (Vergeer, Sogo et al. 2000). Data from transmission electron microscopy shows Sp1 binding to target sites in an intron resulting in the looping out of DNA. This leads to the interaction of the TF with AP2 bound in the proximal promoter region. Sp1 and AP2 associations have been shown to be

required to drive transcription from the mouse GM3-synthase gene promoter in neuro 2a cells (Xia, Zeng et al. 2005) and from the human fragile-X mental retardation promoter in SK-N-SH neuronal cells (Carrillo, Cisneros et al. 1999). More importantly, associations between AP2 and Sp family have been shown for promoters of genes that are regulated temporally and spatially during development. Sp3, Sp1 and AP2 TFs have been shown to associate in the activation of transcription from the human MIP gene promoter (Ohtaka-Maruyama, Wang et al. 1998).

During neuronal differentiation, the NR1 gene that encodes for the N-methyl-D-aspartate (NMDA) receptor has been shown to be regulated by the binding of Sp1 and Sp3 family members to an NFκB response element (Liu, Hoffman et al. 2004). It is known that Sp family bind NFκB sites and can activate transcription from NFκB sites as seen in the case of the P-selectin promoter (Hirano, Tanaka et al. 1998). Interestingly in the CNS, there appears to be a distinct neuronal-κB-binding factor that is found in a complex of Sp1 and -3 and binds the NFκB consensus (Mao, Moerman et al. 2002). It is possible that this specific binding factor is recruited to the proximal promoter along with AP2-α by virtue of their associations with the Sp family members. Whether this is a functional interaction remains to be elucidated.

We have shown the regulation of the transferrin receptor (Holloway, Sade et al. 2007) by YY1 in brain endothelium. YY1 is multifunctional ZnF TF that can either activate or repress transcription from target promoters (Thomas and Seto 1999). YY1 core consensus binding site is CGCCATnTT (n indicates any base at

that position); other less stringent binding motifs include CCATnTT without the CpG dinucleotide or ACATnTT (Kim and Kim 2008). Since YY1 binding to its CpG-containing binding site (CGCCATnTT) is methylation sensitive it can be controlled by CpG methylation (Kim, Kollhoff et al. 2003) and since it has relatively flexible binding sites, EMSA results may not correspond with YY1-target sequences predicted by the program.

The occludin promoter sequence includes YY1 binding sites (Table 4.1) and we examined if these sites are actively bound by the transcription factor in brain and non-brain endothelial nuclear extracts. YY1 from lung lysates interacted only with the binding motifs in the 5' end of the promoter region (Fig. 4.14). YY1 from brain endothelial lysates interacted with consensus binding sites in the probes from F5 (Fig. 4.18) and F6.1 (Fig. 4.12) though it is likely the YY1 sites in F6.1 are occupied by other transcription factors.

Further analysis is required to reveal the cooperative mechanism between Sp3 and YY1. In vivo analysis of transcription and translation level of occludin in normal brain endothelial cells and cells where the TFs have been silenced is required for further clarification of the involvement of the TFs in regulation.

To conclude, EMSA data clearly indicates there is a difference in the association of transcription factors with the occludin promoter between brain and non-brain endothelium. However, the biochemical technique investigates interaction of transcription factors with naked DNA under *in vitro* conditions and results do not necessarily represent a functional target *in vivo.* It should be remembered that EMSA analyses do not demonstrate all possible interactions between TFs and

binding motifs on target genes and hence we used chromatin immunoprecipitation analysis to look at protein: DNA interactions *in vivo*.

Chapter 5

Interaction of TFs with Human Occludin Promoter

5 Interaction of TFs with Human Occludin Promoter

5.1 Introduction

Protein-DNA interactions influences include DNA replication and repair, transcription and epigenetic silencing of gene expression. Transcriptional regulation of gene expression is required for the maintenance of cellular homeostasis and disruption of this regulation is directly linked to many human diseases, including cancer. The identification of TFs, binding sites within the genome, and regulated genes is necessary to understand signalling in normal physiology and in disease. Hence, investigating interactions between transcription factors and targets *in vivo* is a very important aspect in the field of transcriptional regulation. Techniques such as EMSA, DNase I footprinting, microarrays have been used to study protein–DNA interactions. However, the assays measure TF binding to naked DNA under *in vitro* conditions and results do not necessarily represent a functional target *in vivo* and observed gene expression changes may be due to direct or indirect regulation, making it challenging to detect primary gene targets. In addition, factors like chromatin structure, nucleosome positioning, associations and competitions between different TFs, modifications of histones or DNA control the interaction of a given factor to a target DNA sequence.

Chromatin immunoprecipitation (ChIP) is an experimental method used to determine whether proteins, such as certain transcription factors, are associated

with a specific genomic region in living cells or tissues using specific antibodies that recognize a specific protein or a specific modification of a protein. This cell based technique is often used together with non-cell-based assays to characterize protein: DNA purified and identified by PCR using specific primers to the suspected binding region. This technique is a valuable tool in identifying regulatory regions directly bound by a TF in the context of the native chromatin structure.

Data in the present chapter investigates the role of potential regulatory elements controlling transcription from the occludin promoter in brain and lung endothelial cells.

5.2 Materials and Methods

5.2.1 Chromatin Immunoprecipitation (ChIP) assay

Kits: Active Motif Enzymatic shearing kit and Upstate Chromatin immunoprecipitation kit were used for the assay.

Buffers and solutions

37% Formaldehyde: (Sigma, Cat No: F1635)

Glycine Stop-Fix solution: 3 ml of 10x glycine (supplied in the Active Motif kit), 3ml 10x PBS mixed in 24 ml of deionised water. Made just prior to use and stored at RT.

Treatment with formaldehyde

Cells were grown to confluence in a 175 cm^2 flask and rested for 48 hours prior to assay. 10^7 cells were used for one assay. 37% formaldehyde (Cat no: F1635, Sigma Aldrich) was directly added to the culture medium to achieve a final concentration of 1% (3µl/cm^2) and incubated for 10 min at 37°C. Medium was aspirated and the fixation reaction was stopped by adding 10 ml glycine stop-fix solution and swirling the culture flask to cover the cell monolayer and then rocking at room temperature for 5 minutes. The solution was then discarded and cells washed with ice cold PBS containing protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin and 1µg/ml pepstatin A) for 10 min with two changes of buffer.

Isolation of nuclei

Cells were scraped into a tube and centrifuged for 4 min at 2000 rpm at 4°C. The pellet was resuspended in 1ml chilled lysis buffer and incubated on ice for 40 min to release nuclei. The nuclei were collected by centrifugation at 5000 rpm for 10 min at 4°C.

Shearing of chromatin

The nucleic pellet was resuspended in 1ml digestion buffer (supplemented with 5 µl PIC and 5 µl of 1 mM PMSF) and warmed at 37°C for 5 min. A working enzymatic shearing cocktail solution was prepared by diluting 1:100 of the supplied mixture (Active Motif) with 50% glycerol (in nuclease free water) to make a final stock at 200 U/ml. 50µl of the working stock of enzymatic shearing cocktail was added to the pre-warmed nuclei, vortexed to mix and incubated at 37°C for 40 min. The tube was flicked and/or vortexed periodically during the incubation to ensure the chromatin is evenly sheared. The reaction was then stopped by addition of 20 µl ice-cold 0.5 M EDTA and the tube chilled on ice for 10 min followed by centrifugation at 15000 rpm in a 4°C micro centrifuge for 10 min. The supernatant contains the sheared chromatin and was aliquoted into 4×250 µl; each 250 µl aliquot to be used for one ChIP reaction.

Immunoprecipitation of the Chromatin

The 250 µl sheared chromatin lysate was pre-cleared with 40 µl of Salmon Sperm DNA/Protein A agarose-50% slurry (upstate Biotechnology) for 1hr at 4°C. After a brief centrifugation to pellet agarose, the supernatant fraction was collected.

4 µg of the immunoprecipitating antibody was added per each reaction and incubated overnight at 4°C with rotation. 60 µl of Salmon Sperm DNA/Protein A agarose slurry was then added and further incubated for 60 min at 4°C with rotation to collect the antibody/histone complex. The agarose was pelleted by gentle centrifugation at 1000 rpm at 4°C, ~1min. The supernatant which contains the unbound, non-specific DNA was carefully removed. The protein A agarose/antibody/histone complex was washed for 3-5 min on a rotating platform with 1ml of each of the buffers listed in the order as given below:

Low Salt Immune Complex Wash Buffer, one wash

High Salt Immune Complex Wash Buffer, one wash

LiCl Immune Complex Wash Buffer, one wash

1x TE, two washes

250 µl of freshly prepared elution buffer ((1% SDS, 0.1 M $NaHCO_3$) was added to the pelleted protein A agarose/antibody/histone complex , vortexed briefly to mix and incubated at room temperature for 15 min with rotation. The tubes were centrifuged at 1000 rpm for 1min at RT and the supernatant fraction (eluate) carefully transferred to another tube. The elution step was repeated and the eluates were combined (total volume = ~500 µl).

Reversal of cross links

20 µl of 5 M NaCl was added to the combined eluates (500 µl) and histone-DNA crosslink's reversed by heating at 65°C for 4 hours. Then, 10 µl of 0.5M EDTA,

20 µl 1M Tris-HCl, pH 6.5 and 2 µl of 10 mg/ml Proteinase K(Sigma) was added to the combined eluates and incubated for 60 min at 45°C. After addition of 3 µl of a carrier molecule, glycogen (2 µg/µl), DNA was recovered by phenol/chloroform extraction and ethanol precipitation, resuspended in TE buffer for use in PCR analyses.

5.2.2 Antibodies used in ChIP

All antibodies were purchased from Santa Cruz Biotechnology and 2 µg was used per reaction.

Table 5-1 Antibodies used in ChIP Assays

Antibody	Species of Ab	Immunogen (human)	Clone/PAD*
GATA-2	Mouse	120-235 aa's of Gata-2	CG2-96
c-Myb	Rabbit	500-640 aa's of c-Myb	H-141
Pit1	Goat	Peptide mapping at the N-terminus of Pit1	N-20
Sp1	Rabbit	528-548 aa's within an internal region of Sp1	PEP2
Sp3	Rabbit	Peptide mapping at the C-terminus of Sp3	D-20
TFIID	Rabbit	1-300 aa's representing full length TFIID p36	SI-1
YY1	Rabbit	aa's 1-414 representing full-length YY1	H-414

* PAD: Polyclonal antibody designation

5.3 Results

5.3.1 Interaction of Sp1, Sp3 and YY1 with the occludin promoter

This chapter presents a study addressing the promoter occupancy of occludin by the TFs SP1, Sp3 and YY1 in endothelial cells derived from brain, dermal and lung. EMSA data clearly indicates there is a difference in the association of transcription factors with the occludin promoter between brain and non-brain endothelium. To determine if the TFs Sp1, Sp3 and YY1 are present in the regulatory protein complex on the chromatin of occludin in different endothelia and to elucidate any differences between the cell types, chromatin immunoprecipitation (ChIP) was carried out using specific antibodies directed against the three TFs.

DNA isolated from immunoprecipitation with TF(s) and/or control antibody was subjected to a PCR analyses with primer pairs designed to amplify regions of the promoter DNA that have Sp or YY1 sites and the length of the amplified fragments corresponding to <500 bp.

Chromatin isolated from hCMEC/D3 (Figs. 5.1, 5.4 and 5.8), primary human brain endothelial cells (Figs. 5.2, 5.5 and 5.9), non-brain (Figs. 5.3, 5.6 and 5.10; LMVEC, DMVEC) endothelial cells was immunoprecipitated with antibodies to Sp1 (Figs 5.1, 5.2 and 5.3), Sp3 (Figs. 5.4, 5.5, 5.6) or YY1 (Figs 5.8, 5.9 and 5.10). Results show that varying association of the TFs with the endogenous occludin promoter. Results are summarised in tables 5.2 (Sp1), 5.3 (Sp3) and 5.4 (YY1).

Table 5-2 Summary of ChIP Analysis of Sp1 Association With the Occludin Promoter in Different Endothelia

Promoter region amplified	Sp sites (TESS)	hCMEC/D3	Active sites EMSA – hCMEC/D3	LMVEC	Active sites EMSA-LMVEC	DMVEC	PHBEC
1-487	X	X	-	-	-	X	X
465-994	X	X	X	-	-	X	-
972-1238	X	-	-	-	-	-	-
1218-1524	X	X	-	-	-	-	-
1505-1853	X	-	X	-	-	-	X

Table 5.2: Sp1association with the endogenous occludin promoter in the different endothelia.' X' and '–' denote positive and negative amplification in the PCR. EMSA analyses data has also been included for hCMEC/D3 and lung endothelial cells for comparison.

Table 5-3 Summary of ChIP Analysis of Sp3 Assocation with the Occlduin Promoter in Different Endothelia

Promoter region amplified	Sp sites (TESS)	hCMEC/D3	Active Sp3 sites:EMSA hCMEC/D3	LMVEC	Active Sp3 sites EMSA-LMVEC	DMVEC	PHBEC
1-487	X	X	-	-	-	-	X
465-994	X	-	X	-	-	-	-
972-1238	X	X	-	-	-	-	X
1218-1524	X	-	-	-	-	-	X
1505-1853	X	X	X	-	-	-	X

Table 5.3: Sp3 association with the endogenous occludin promoter in the different endothelia.' X' and '–' denote positive and negative amplification in the PCR. EMSA analyses data has also been included for hCMEC/D3 and lung endothelial cells for comparison.

Table 5-4 ChIP Analyses of YY1 Association with Occludin Promoter in Different Endothelia

Promoter region amplified	YY1 sites (TESS)	hCMEC/D3	Active sites EMSA – hCMEC/D3	LMVEC	Active sites EMSA-LMVEC	DMVEC	PHBEC
1-487	X	X	-	X	X	ND	-
465-994	X	-	-	X	-	ND	-
972-1238	X	X	-	X	-	ND	-
1218-1524	X	X	X	X	-	ND	-
1505-1853	X	-	-	X	-	ND	X

Table 5.4: YY1 association with the endogenous occludin promoter in the different endothelia.' X' and '–' denote positive and negative amplification in the PCR. EMSA analyses data has also been included for hCMEC/D3 and lung endothelial cells for comparison.

5.3.2 Association of the Sp family with the endogenous occludin promoter in brain and non brain endothelium

Sp1 occupied the endogeouns occludin promoter in the transformed brain endothelial cell line, hCMEC/D3 and in the primary brain endothelial cells (Tables 5.2, Figs. 5.3 and 5.4). Sp1 was also seen in association with the occludin promoter in dermal endothelial cells (Fig. 5.6) which do not express the protein (Chapter 2, Fig. 2.2). The TFs did not associate with the endogenous promoter in lung endothelial cells. (Fig. 5.5).

Data shows that Sp3 also occupies target motifs on the occludin promoter but only in brain endothelial cell (Figs. 5.7 and 5.8)s. It interacts with similar regions as the Sp1 TF except that the proximal region in hCMEC/D3 cells was also occupied (Figs. 5.7 and 5.8). Sp1 and Sp3 bind to GC and GT boxes. but with diferent affinities which is dependent on the ratio of their expression in cells. Sp3 levels are higher in brain endothelial cells (Holloway, Sade et al. 2007) and it is possible Sp3 succesfully competes with Sp1 to interact with the consensus binding motifs. Our data clearly shows that Sp3 does not interact with the endogenous occludin promoter.

5.3.3 Association of the YY1 family with the endogenous occludin promoter in brain and non brain endothelium

YY1 interacted with the promoter regions in hCMEC/D3 cells, the primary brain endothelial cells and also in the lung endothelial cells. (Table 5.2, Figs. 5.11, 5.12 and 5.13).

In the lung endothelial cells, YY1 appears to interact at various points through the length of the promoter (Fig. 5.13)

Results clearly demonstrate that the Sp1, Sp3 and YY1 TFs actively associate with the occludin promoter in brain endothelial cells. However, in the lung ECs it appears that the Sp family is not associated with the endogenous occludin promoter. Experiments using higher cell number, increased amount of immunoprecipitating antibody or PCR amplification cycles did not yield a positive PCR amplification. Nevertheless, the TF YY1 associated with the endogenous promoter in the lung endothelial cells.

To our knowledge, this is the first demonstration showing association of the Sp family and YY1 TFs with endogenous occludin promoter in human brain and lung endothelial cells. This is also the first study whereby occludin promoter regulation and hence activity is illustrated by the differential binding of TFs in brain and non-brain endothelium. It was not possible to repeat EMSA analysis undertaken with the transformed cell line in primary human brain endothelial cells as availability of tissue was a limiting factor. However, it was possible to scale down the ChIP protocol for primary human brain endothelial cell cultures and investigate any possible differences in TFs associated with the promoter in transformed and primary cell cultures. The ChIP assays confirm the *in vitro* interaction seen between the three transcription factors present in hCMEC/D3 nuclear extracts and occludin promoter DNA as these complexes were active in cultured live cells. It is clearly apparent that the some of the TF-DNA interactions are also seen in primary cell cultures.

5.3.4 Discussion

Chromatin immunoprecipitation (ChIP) is a powerful technique for studying interactions of DNA with proteins including transcription factors and histones. Data in this chapter represents to the best of our knowledge, the first analyses carried out on transcription factor interactions with the endogenous occludin promoter in primary and transformed brain and lung endothelial cells.

The ChIP assays demonstrate that

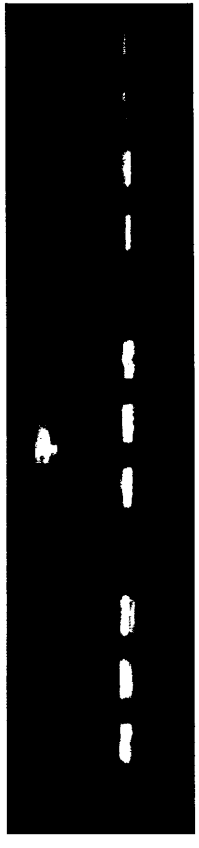
1. Sp family and YY1 interact with target cis-elements in the endogenous human occludin promoter.

2. Sp1 and Sp3 are recruited to the occludin gene promoter in brain endothelial cells in contrast to the lung endothelium, where Sp family TFs are not associated with any of the target sequences and only YY1 association is observed.

3. The TF YY1 binds the promoter at different sites in the brain and lung endothelium raising the possibility that it differentially regulates chromatin structure across different endothelia.

We found that Sp3 in primary and transformed brain endothelium is associated with the proximal GC rich region which has been shown to be a part of the minimal promoter in epithelial cells. It is known that this binding is not exclusive to occludin as expression of a number of other genes involves Sp family binding to similar proximal GC motifs.

Figure 5-1 Input DNA for PCR Reactions



Input DNA hCMEC/D3 cells Fig. 5.3

Input DNA Primary Brain endothelial cells Fig. 5.4

Input DNA lung endothelial cells Fig. 5.5

Input DNA dermal endothelial cells Fig. 5.6

Input DNA hCMEC/D3 cells Fig. 5.7

Input DNA Primary Brain endothelial cells Fig. 5.8, 5.12

Input DNA lung endothelial cells Fig. 5.9

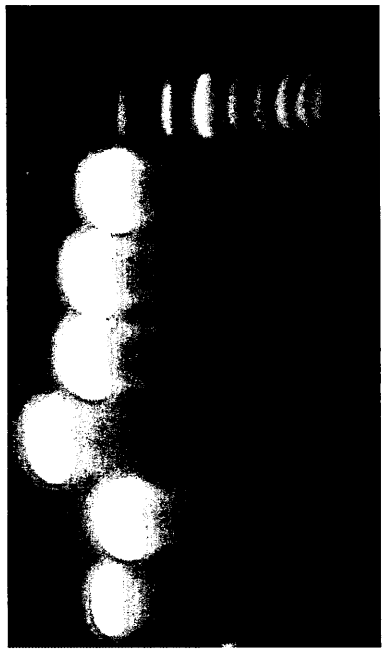
Input DNA hCMEC/D3 cells Fig. 5.11

Input DNA Brain endothelial cells Fig.. 5.12

Input DNA lung endothelial cells Fig. 5.13

Input DNA from hCMEC/D3, primary brain, lung and dermal endothelial cellsfrom the different experiments before it was subjected to immunoprecipitation using antibodies to the SP family, YY1 or an isotype matched control antibody.

Figure 5-2 Amplification of Endogenous Occludin from Input DNA



Marker

Input DNA-hCMEC/D3 cells PCR amplified for occludin promoter fragment F0.1

Input DNA-lung endothelial cells PCR amplified for occludin promoter fragment F0.2

Input DNA-dermal endothelial cells PCR amplified for occludin promoter fragment F3.1

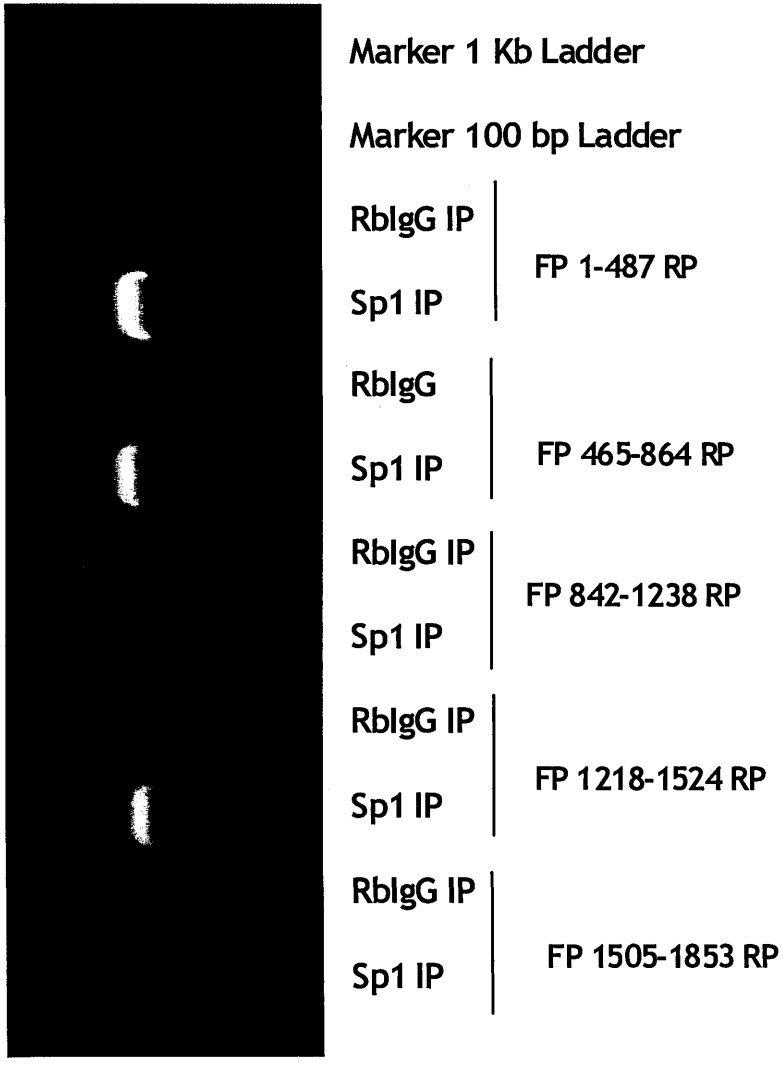
Input DNA-primary brain endothelial cells PCR amplified for occludin promoter fragment F7.2

Input DNA-primary brain endothelial cells PCR amplified for occludin promoter fragment 2.1

Input DNA lung endothelial cells PCR amplified for occludin promoter fragment F1

Sheared chromatin from the different endothelial cells as indicated in the figure were subjected to a PCR using primers (Chapter 4, table 4.1) for different fragments of the occludin promoter.

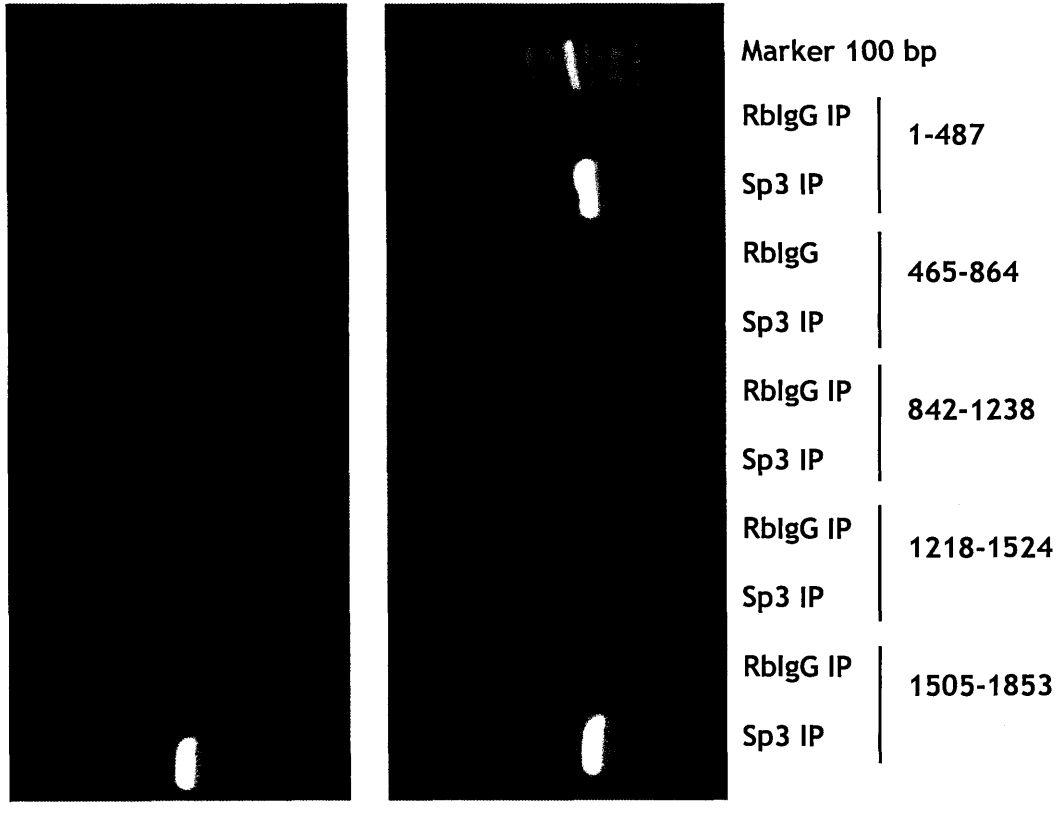
Figure 5-3 Sp1 ChIP in hCMEC/D3 endothelial cells



hCMEC/D3 cells were grown to confluence and ChIP assay was conducted. Antibodies against Sp1 (lanes 4, 6, 8 10, 12) or RbIgG (3, 5, 7, 9, 11) were used to immunoprecipitate chromatin isolated from the cells. Following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Results show positive amplification for three regions 1-487, 465-994 and 1218-1524 in the human occludin promoter.

Figure 5-4 Sp1 ChIP in Primary Human Brain Endothelial Cells

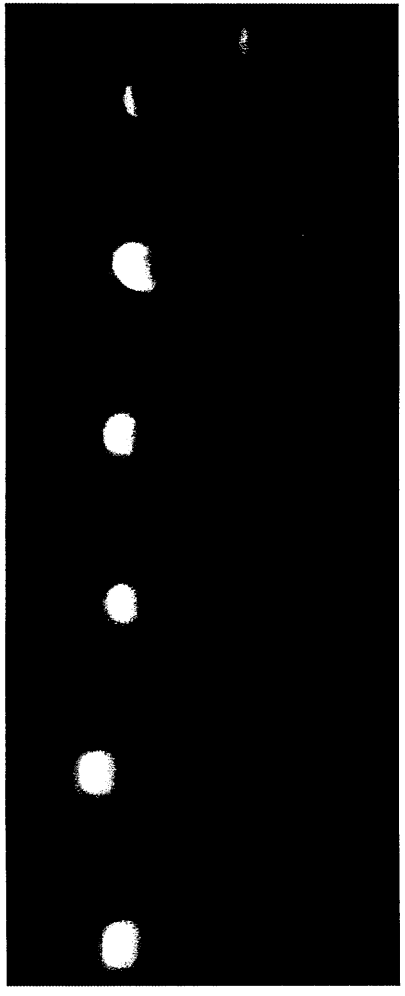


ChIP was performed on primary human brain endothelial cells using antibodies to Sp1 (lanes 3, 5, 7, 9 and 11) or RbIgG (lanes 2, 4, 6, and 10). Following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Results show positive amplification for regions 1-487, and 1505-1853 of the human occludin promoter.

Two representative experiments are shown.

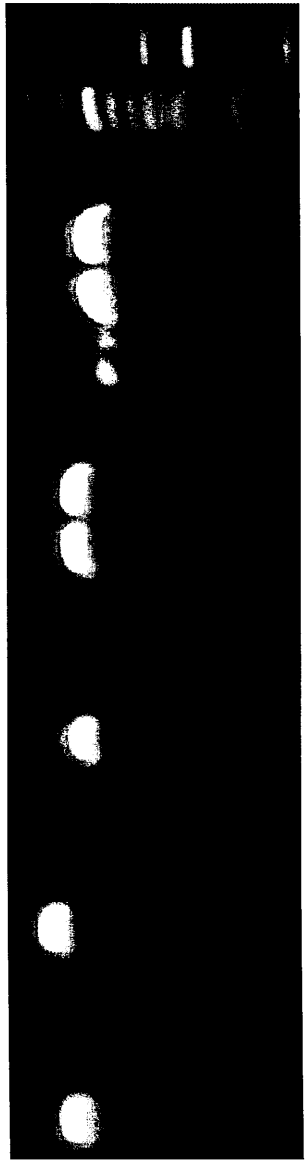
Figure 5-5 Sp1 ChIP in Non-Brain Endothelial Cells-lung endothelial cells



Chromatin isolated from confluent cultures of lung endothelial cells was immunoprecipitated with antibodies to Sp1 (lanes 4, 7, 10, 13 and 16) or an isotype matched control antibody, RbIgG (lanes 3, 6, 9, 12 and 15). Following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

No amplification of any region of the endogenous promoter was observed. Included to indicate working PCR conditions are amplicons of cloned occludin promoter (lanes 5, 8, 11, 14) amplified with the primer pairs used for the ChIP assay.

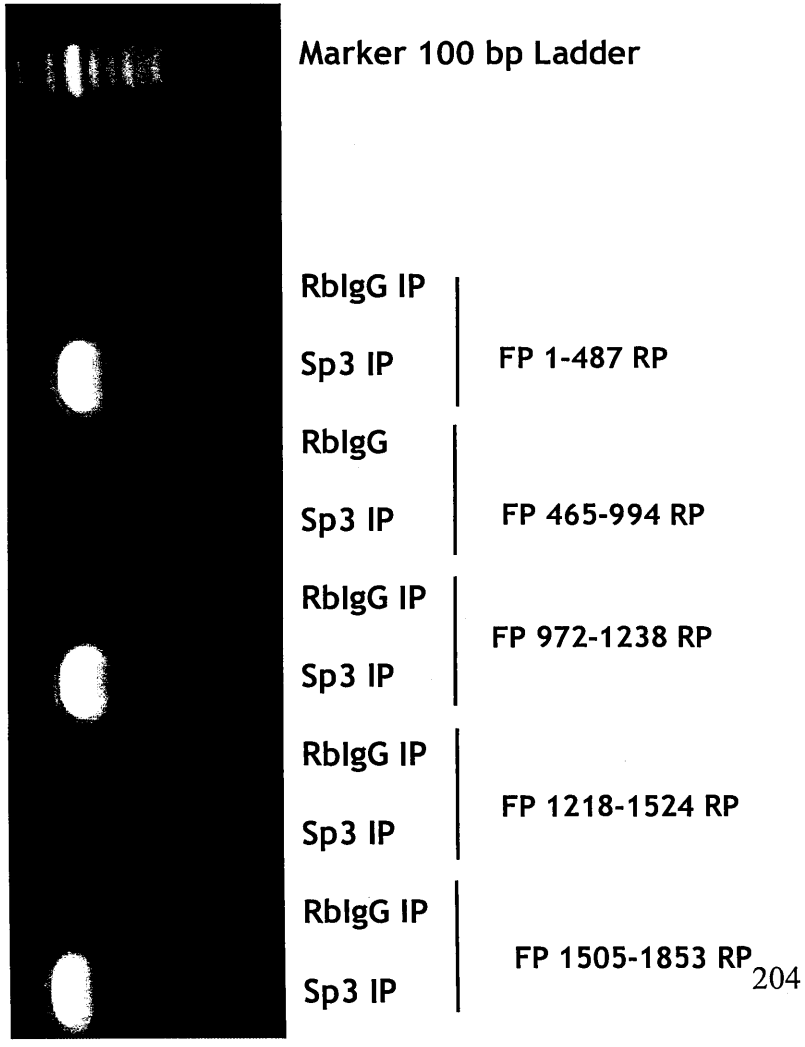
Figure 5-6 Sp1 ChIP in Dermal Endothelial Cells (DMVEC)



Chromatin isolated from confluent cultures of dermal endothelial cells was immunoprecipitated with antibodies to Sp1 (lanes 4, 8, 11, 14 and 17) or an isotype matched control antibody, RbIgG (lanes 3, 7, 10, 13 and 16). Upon reversal of crosslinking and following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Amplification of regions 1-487 and 465-994 of the endogenous promoter were observed. Lanes 5, 9, 12, 15 and 18 are regions of the cloned occludin promoter amplified with the primer pairs used in the ChIP assay.

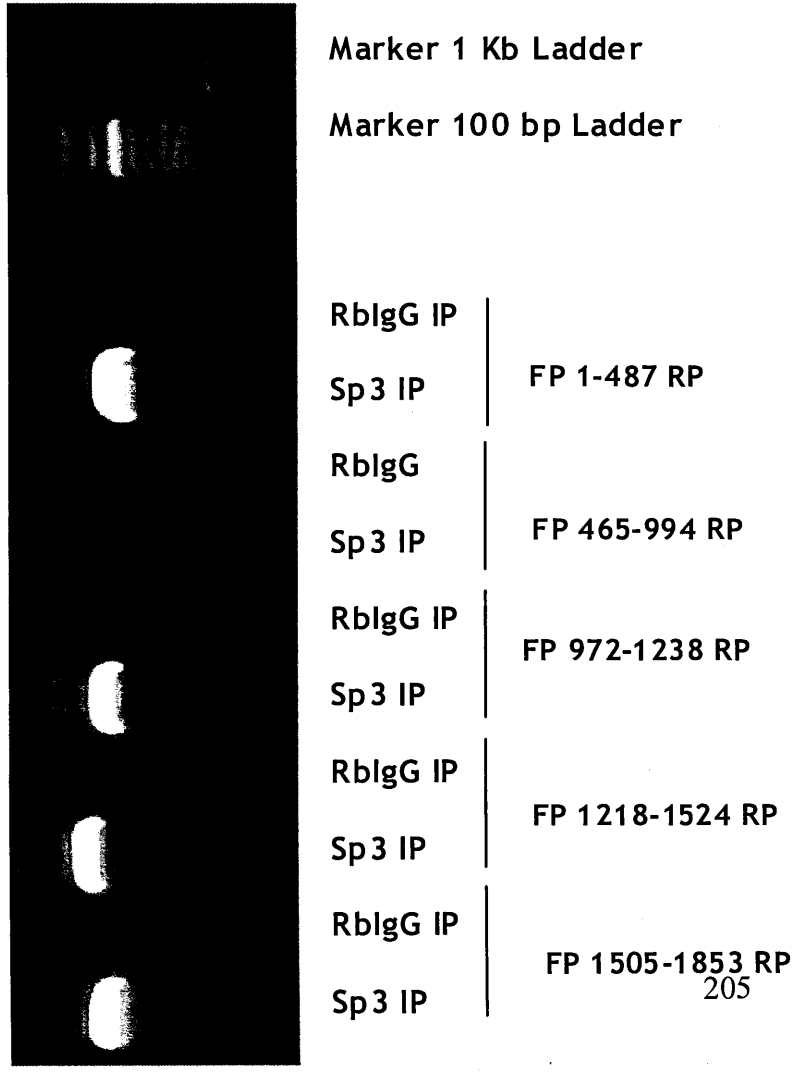
Figure 5-7 Sp3 ChIP in hCMEC/D3 Endothelial Cells



hCMEC/D3 cells were grown to confluence and ChIP assay was conducted. Antibodies against Sp3 (lanes 3, 5, 7, 9 and 11) or RbIgG (2, 4, 6, 8, 10) were used to immunoprecipitate chromatin isolated from the cells. Following reversal of cross linking and DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Results show positive amplification for three regions 1-487, 972-1238 and 1505-1853 in the human occludin promoter.

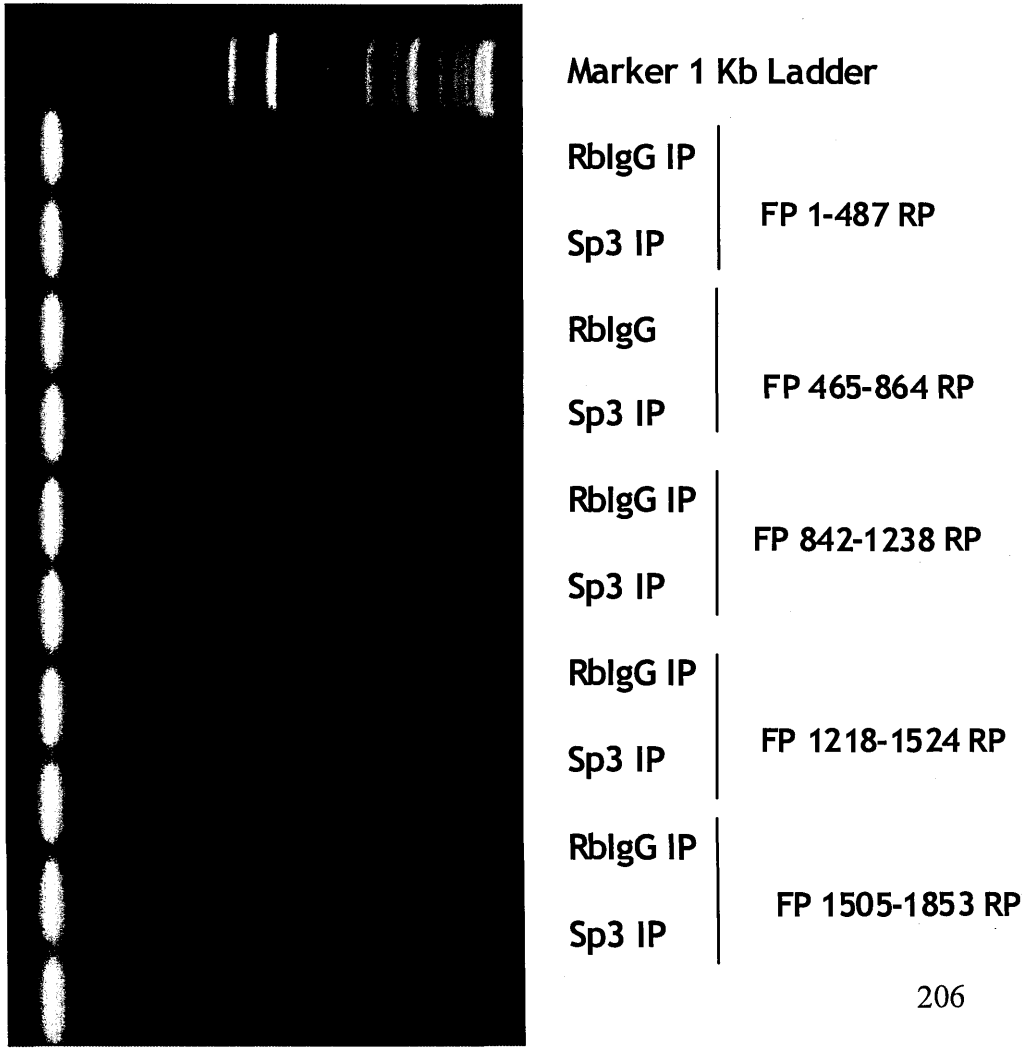
Figure 5-8 Sp3 ChIP in Primarv Human Brain Endothelial Cells



Chromatin isolated from confluent cultures of primary human brain endothelial cells was immunoprecipitated with antibodies against Sp3 (lanes 4, 6, 8, 10 and 12) or an isotype matched antibody control, RbIgG (lanes 3, 5, 7, 9 and 11). Following reversal of crosslinking and DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Results show positive amplification for four regions 1-487, 972-1238, 1218-1524and 1505-1853 in the human endogenous occludin promoter.

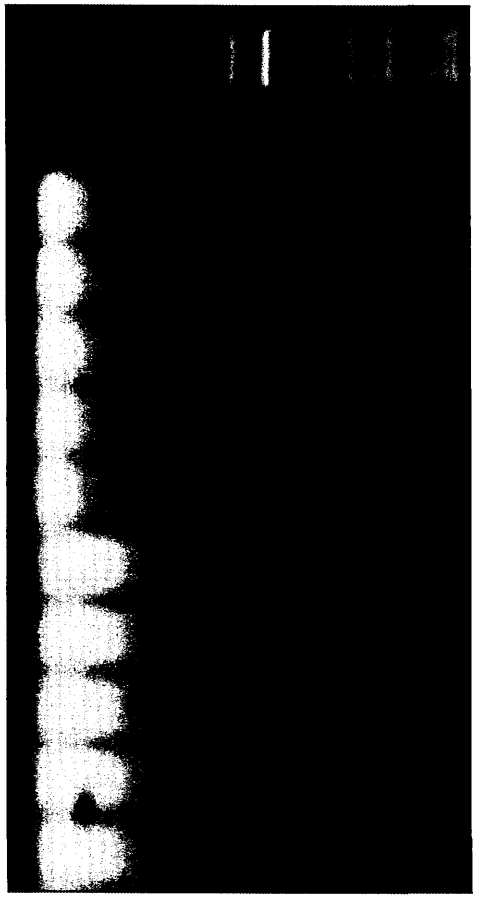
Figure 5-9 Sp3 ChIP in Non-Brain Endothelial Cells LMVEC



Chromatin isolated from confluent cultures of lung endothelial cells was immunoprecipitated with antibodies to Sp3 (lanes 3, 5, 7, 9, 11) or an isotype matched control antibody, RbIgG (lanes 2, 4, 6, 8 10). Following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

No amplification of any region of the endogenous promoter was observed. The PCR reaction was allowed to extend for an additional ten cycles resulting in formation of primer dimers.

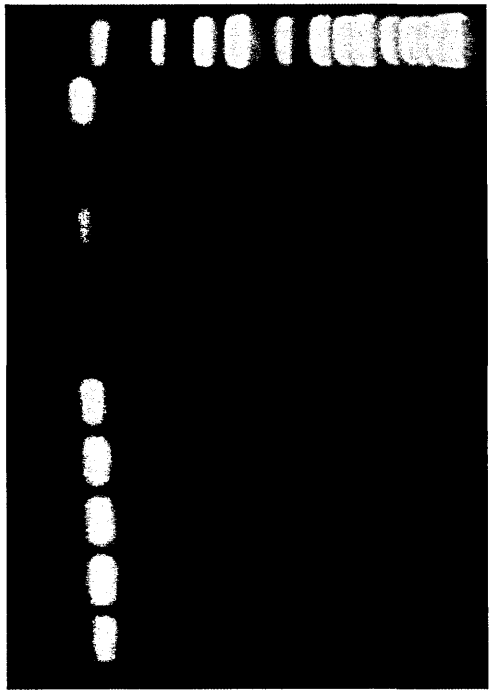
Figure 5-10 Sp3 ChIP in Dermal Endothelial Cells (DMVEC)



Chromatin isolated from confluent cultures of dermal endothelial cells was immunoprecipitated with antibodies to Sp3 (lanes 3, 5, 7, 9, 11) or an isotype matched control antibody, RbIgG (lanes 2, 4, 6, 8 10). Following DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

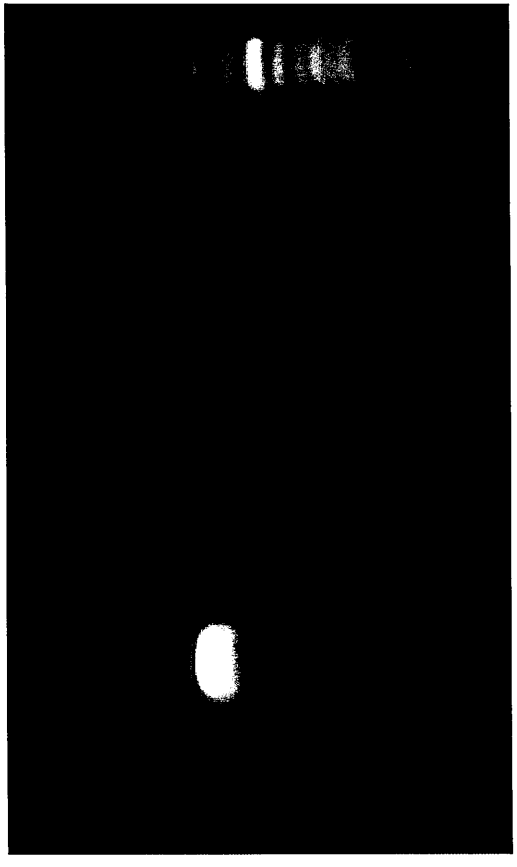
No amplification of any region of the endogenous promoter was observed. As in Fig. 5.7, the PCR reaction was allowed to extend for an additional ten cycles resulting in formation of primer dimers.

Figure 5-11 YY1 ChIP in hCMEC/D3 Endothelial Cells



Chromatin isolated from confluent cultures of hCMEC/D3 cells was immunoprecipitated with antibodies to Sp1 (lanes 2 and 7), YY1 (lanes 3, 5, 6, 9, 11), Sp3 (4, 8 and 10). Following reversal of cross linking and DNA purification, samples were subjected to PCR with primers that have been previously described in Chapter 4 (Fig. 4.1 and Table 4.1). These primers were designed to amplify shorter regions of the occludin promoter. Positive amplification was seen for fragments F0.1-F0.2, F1, F2.1-F2.2, F5 and F6.1. The nomenclature of the fragments is the same as was followed in chapter 4.

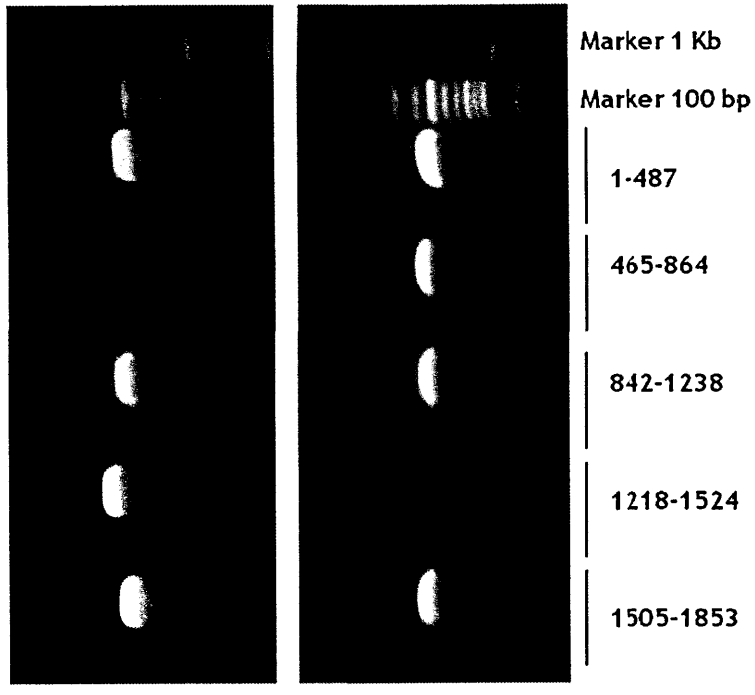
Figure 5-12 YY1 ChIP in Primary Human Brain Endothelial Cells



Chromatin isolated from confluent cultures of primary human brain endothelial cells was immunoprecipitated with antibodies to YY1 (lanes 3, 5, 7, 9, 11) or an isotype matched control antibody, RbIgG (lanes 2, 4, 6, 8, 10). Following reversal of cross linking and DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Positive amplification was seen for the proximal region of the endogenous promoter-1505-1853.

Figure 5-13 YY1 ChIP in Non-Brain Endothelial Cells (LMVEC)



Chromatin isolated from confluent cultures of lung endothelial cells was immunoprecipitated with antibodies to YY1 (lanes 3, 5, 7, 9, 11) or an isotype matched control antibody, RbIgG (lanes 4, 6, 8, 10 and 12). Following reversal of cross linking and DNA purification, samples were subjected to PCR with primers designed to amplify specific regions of the promoter as shown.

Positive amplification was seen for all the regions of the occludin promoter across two different experiments.

5.3.4 Discussion

Chromatin immunoprecipitation (ChIP) is a powerful technique for studying interactions of DNA with proteins including transcription factors and histones. Data in this chapter represents to the best of our knowledge, the first analyses carried out on transcription factor interactions with the endogenous occludin promoter in primary and transformed brain and lung endothelial cells.

The ChIP assays demonstrate that

1. Sp family and YY1 interact with target cis-elements in the endogenous human occludin promoter.

2. Sp1 and Sp3 are recruited to the occludin gene promoter in brain endothelial cells in contrast to the lung endothelium, where Sp family TFs are not associated with any of the target sequences and only YY1 association is observed.

3. The TF YY1 binds the promoter at different sites in the brain and lung endothelium raising the possibility that it differentially regulates chromatin structure across different endothelia.

We found that Sp3 in primary and transformed brain endothelium is associated with the proximal GC rich region which has been shown to be a part of the minimal promoter in epithelial cells. It is known that this binding is not exclusive to occludin as expression of a number of other genes involves Sp family binding to similar proximal GC motifs.

In the lung endothelium, YY1 binds to an upstream 5' cis element (also confirmed by EMSA); this association has not been observed in the brain endothelium. It is possible, YY1 binding to the endogenous promoter may lead to different outcomes in brain and lung endothelia.

YY1 can function as a positive or negative regulator of transcription. The TF positively modulates transcription by inducing bends in DNA (Thomas and Seto 1999) or forming synergistic associations with other proteins. Transcription factors are able to act as activators or repressors by using specific domains. However, certain regulatory proteins are able to exert their control on gene promoters indirectly. These proteins bend DNA resulting in the alteration of promoter structure which can either facilitate or abrogate interactions between co-activators, TFs and other components of the basal transcription machinery (Maher 1998). Examples of DNA-bending proteins include HMGB proteins (Grasser, Launholt et al. 2007), SRY (Harley 2002), YY1 etc. YY1 induced bending of DNA has been reported in the regulation of the c-fos (Natesan and Gilman 1993) and the c-Myc (Austen, Cerni et al. 1998) promoters.

Interactions with other regulatory proteins have been known to alter or modulate the ability of YY1 to interact with its recognition sequence on DNA. A large number of proteins have been reported to interact with YY1 and includes TATA binding protein, Sp1 (Seto, Lewis et al. 1993), c-Myc (Shrivastava, Saleque et al. 1993), C/EBP (Bauknecht, See et al. 1996) etc. YY1 also associates with p300 (Austen, Luscher et al. 1997) and CREB-binding protein (CBP) (Lee, Galvin et al. 1995) which function as histone acetyltransferases. Acetylation of histones

neutralises the charge on their N-terminal tails and reduces the affinity of histones for DNA. This leads to a decondensation of chromatin resulting in a relaxed structure that can be accessed by other TFs and co-activators. YY1 also interacts with histone deacetylases (HDACs) (Yang, Inouye et al. 1996; Yang, Yao et al. 1997) which deacetylate the repressor domain(s) and hence determine the ability of YY1 to function as a transcriptional repressor. The binding of YY1 to the F0.1 (Fig. 4.14) in the lung endothelium could lead to changes in chromatin structure that impairs the access of Sp family members to the occludin promoter.

In the brain endothelium, however, it is possible that synergistic association between the Sp family and YY1 transcription factors contribute to activating transcription from the occludin promoter. ChIP analysis from primary brain endothelium shows the association of YY1 in the region which has been identified to be a minimal promoter in epithelial cells. The Sp family also interact with the promoter in this region. Sp and YY1 TFs interactions have been observed in the activation of other gene promoters. This could be the case for occludin gene expression as well. Beyond directly regulating occludin basal promoter activity, they could also potentially contribute to remodelling the chromatin structure. Recent work identified the ability of Sp family to interact with p300 a histone acetyltransferase (Hung, Wang et al. 2006).

Further chromatin structure modifications could be undertaken by the recruitment of a TF that binds to a NFκB-like site in the proximal promoter (Fig. 4.5). As discussed before, there appears to be a distinct neuronal-κB-binding factor that is found in a complex of Sp1 and -3 and binds the NFκB consensus (Mao, Moerman

et al. 2002). And if this factor, can like NFκB recruit histone acetyl transferases could result in increased acetylation of histones in the occludin promoter region leading to relaxation of the chromatin structure and allowing easy access for Sp3 and YY1 TFs to the binding regions. It is likely that the chromatin structure of the occludin promoter is predominantly in the relaxed form in brain endothelial cells.

Detailed study of chromatin remodelling in the occludin promoter region in brain endothelial cells could thus potentially contribute to an understanding of how transcriptional regulation is differentially controlled in the brain and non brain endothelium.

Chapter 6

Sp family and YY1 Transcription Factors in Endothelium

6 Sp family and YY1 Transcription Factors in Endothelium

6.1 Introduction

Sp1, Sp3 and YY1 are ubiquitously expressed ZnF transcription factors. Sp1 is a known activator and has been implicated in the activation of diverse genes regulating tissue specificity, cell-cycle and is required to prevent methylation of CpG islands (Suske 1999). Sp3 apart from its role as a transctivator is known to repress Sp1 activity (Hagen, Muller et al. 1994). Sp1 and Sp3 when expressed in the same cell compete for the binding sites on the DNA and hence the levels of expression of the individual proteins determine the binding of either Sp1 or Sp3 to bind target motifs (Sjottem, Anderssen et al. 1996). The structure and arrangement of the recognition sites and the ratio of the factors in a cell appear to determine activity or repression. It has been established that promoters containing a single binding site are activated, whereas promoters containing multiple binding sites often are not activated (Dennig, Beato et al. 1996).

YY1 is a bi-functional protein that serves as a repressor or activator (Thomas and Seto 1999). YY1 represses transcription from the adeno-associated virus promoter and activates transcription of the c-myc and ribosomal proteins L30 and L32 (Lee, Galvin et al. 1995; Yang, Inouye et al. 1995; Austen, Cerni et al. 1998). YY1 activity is further complemented by its ability to induce DNA-bending which affects position and interaction of other proteins present on the promoter or

enhancer regions (Thomas and Seto 1999).

Aims

In the present study, the occludin promoter interacts with the Sp and YY1 family of transcription factors in brain endothelial cells. However, in lung endothelial cells, the association of occludin promoter fragments with Sp TFs is absent and YY1-DNA complexes are seen in different regions of the promoter.

1. To establish the expression and localisation of YY1 and Sp family in brain and lung endothelial cells
2. Investigate if the transcription factors interact at the level of the protein
3. To determine the effect of inhibition of transcription factor-DNA complexes on the expression of the occludin protein

6.2 Material and Methods

6.2.1 Immunofluorescence

Cells were grown to confluence on collagen-coated cover slips and rested for 2 days in EGM-2 medium with serum but lacking growth factors. The monolayers were washed with chilled PBS for 15 minutes with three changes of the buffer. For staining for transcription factors, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at RT. Cells were then washed with PBS for 15 min with three changes of buffer were permeabilised with 0.2% Triton-X-100 in PBS for 10 min. After 15 min incubation with blocking buffer (0.5% BSA in PBS), cells were incubated with the primary antibody for 2 hours at RT and washed in PBS for 15 min with three changes of the buffer. This was followed by incubation with rabbit anti-mouse or goat anti-rabbit IgG conjugated FITC (1:250, Vector Labs, Burlingame, CA) for 1 hour at RT. Cells were washed for 40 min with four changes of buffer and the coverslips were mounted on slides with DakoCytomation fluorescent medium (Carpinteria, CA) and analysed by confocal or fluorescent microscopy.

6.2.2 Immunoprecipitation analysis

Buffers

RIPA buffer: 50mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4 and 1 mM NaF. 1mg/ml aliquots of the protease inhibitors were stored at -20°C and were made up in water (leupeptin and

aprotinin) or methanol (pepstatin). All other components were stored at 4°C

40 µl of Protein G slurry (Pierce, Chester, UK) was used per immunoprecipitation reaction. The slurry was spun down, washed once and then resuspended in 250 µl of chilled RIPA buffer and 2 µg of the specific antibody was added and incubated for 1 h at 4°C on a cell mixer. Isotype matched IgG was used to control for specificity in all immunoprecipitation assays.

10^6 D3, DMVEC or LMVEC endothelial cell monolayers were washed twice with chilled PBS and then scraped into 1ml of ice-cold RIPA lysis buffer. The samples were incubated at 4°C for 15 min on a cell mixer. The lysates were then centrifuged at 14000 rpm for 15 min at 4°C to remove cell debris. The supernatant was carefully removed and immunoprecipitated overnight at 4°C with the protein G beads previously incubated for 1 h at 4°C with the specific antibody. After three washes with chilled RIPA buffer to remove non-specific protein, beads were resuspended in SDS lysis buffer and boiled at 100°C for 10min and samples spun briefly at 14000 rpm. The supernatants containing the immune complexes were separated on 10% SDS-PAGE gels and subunit composition of the complexes was detected by western blotting as described in below.

6.2.3 Western blot analysis

Buffers

Tris/EDTA: 6.057g Tris, 0.93g EDTA, pH adjusted to 7.4 with HCl

Lysis Buffer: 1%TNE, 1% Triton-X-100, 1 mM PMSF and 1µg/ml each of

leupeptin, aprotinin, pepstatin and made up in water.

Laemelli's reducing buffer: 62.5 mM Tris HCl pH 6.8, 2% SDS, 10% (v/v) glycerol, 50 mM DTT, 0.5% bromophenol blue made up in distilled water.

10x Running Buffer-10x Tris/Glycine/SDS: 0.25 M Tris, 1.92 M Glycine and 1% SDS (Cat No: EC-870, National Diagnostics)

10x Transfer Buffer-10x Tris/Glycine: 0.25 M Tris, 1.92 M Glycine (Cat No: EC-880, National Diagnostics)

Blocking buffer: 5% non-fat dry milk (Blotto®, Cat No: sc-2325, Santa Cruz Biotechnology) in 0.1% Tween 20 in PBS

ProtoGel 30%: (Cat No: EC890, National Diagnostics)

PBS-Tween 20 (PBS-T): 1x PBS (pH 7.4) with 0.1% Tween 20

Cells were harvested and washed twice by centrifugation in chilled PBS. Cells were lysed in 100 µl of lysis buffer, vortexed and then sonicated on ice for 10 sec to reduce sample viscosity. Protein concentration was measured using the DC Protein assay kit according to the manufacturer's instructions (Cat no: 500-0116, Bio-Rad Laboratories). Samples were made up in 2x or 4x Laemelli's buffer depending on the protein concentrations and boiled at 100°C for 5 min. Samples were centrifuged at ~14000 rpm for a min before loading on 10-15% SDS-PAGE gels. 5 µl of pre-stained protein molecular weight marker (Cat No: P7708S, NEB) was loaded on the gels to visualise and determine the molecular weights of the target proteins. Proteins were transferred onto Hybond-ECL nitrocellulose

membrane (Cat No: RPN303D, Amersham) for 2 hours at 0.45 mA using the wet transfer technique. Protein transfer was validated by using Ponceau Red (Cat No: 09189, Sigma) staining. Membranes were washed in PBS constituted with 0.1% Tween-20 for 5 min to rid of excess dye and then incubated in blocking buffer for 1 h at RT. The membrane was incubated overnight with primary antibody in blocking buffer at 4°C, with gentle rocking. Membranes were washed for 30 min with three changes of buffer and then incubated for 1 h at RT with goat anti-rabbit HRP or with rabbit anti-mouse HRP (1:5000, Pierce Biotechnology), diluted in blocking buffer. After another three washes, proteins were detected by chemiluminescence, according to the manufacturer's instructions (Amersham)

6.2.4 Treatment with mithramycin

Mithramycin: cat no: M6891 (Sigma). Stock solutions were made up in DMSO and stored at -20°C.

EMSA: Double stranded ^{32}P-labeled fragment 8 containing two Sp sites was used as a probe. To block Sp1/3 binding to DNA, DNA probes were preincubated for 1 h at 4°C with mithramycin (50 nM, 100 nM, 200 nM) or DMSO (control) before being used in binding reactions with nuclear extracts derived from hCMEC/D3 ECs.

Immunostaining: For analysis of tight junctions in mithramycin treated cells, confluent hCMEC/D3 cells were cultured with 100 nM and 200 nM mithramycin for 48 hours. Cell monolayers were washed in HBSS without Ca^{2+} and Mg^{2+} and then detached from the matrix by incubation with 0.25% Trypsin-EDTA at 37°C

for 5 min. Cells were then centrifuged at 1500 rpm for 5 min and gently resuspended in PBS containing 0.4% paraformaldehyde and incubated for 20 min at room temperature followed by centrifugation. Supernatants were discarded and cell pellets were washed twice with PBS and then resuspended in PBS containing 0.01% triton-X-100 (permeabilisation buffer, PB) and incubated on ice for 20 min. Samples were microfuged at 4000 rpm for 5 min and pellets resuspended in 100µl of primary or isotype antibody made up in the PB and incubated on ice for 40 min. Samples were microfuged at 4000 rpm for 5 min and cell pellets washed twice with excess chilled PBS to rid of unbound antibody. 100 µl of secondary antibody (1:200, goat anti rabbit Alexa Fluor 488, Cat No: A11094) was added to the cell pellets and incubated on ice for 40 min following which samples were microfuged and cell pellets washed in excess chilled PBS. Finally, the cells were resuspended in chilled PBS for analysis on FACS.

Trypan blue exclusion assay: cells were incubated at RT for 10 min with 0.1% trypan blue and the cells were counted in a hematocytometer for dye uptake. At least 300 cells were counted for each data point.

6.3 Results

6.3.1 Immunoreactivity and localisation of Sp family and YY1 in Brain and Non-brain Endothelium

hCMEC/D3 (Fig. 6.1), LMVEC (Fig 6.2), BMEC (Fig. 6.3) and primary human brain endothelial cells (Fig. 6.4) were assayed for the expression and localisation of the regulatory proteins. The transcription factors were detectable on all three endothelia and show a predominantly nuclear localisation, though cytosolic localisation is observed following Sp3 and YY1 immunostaining in lung and dermal endothelial cells respectively. Levels of the transcription factors were similar between cells in various stages of cell cycle. However, the levels and profiles of the transcription factors are dependent on the endothelial cell type.

6.3.1.1 Sp 1 and Sp3

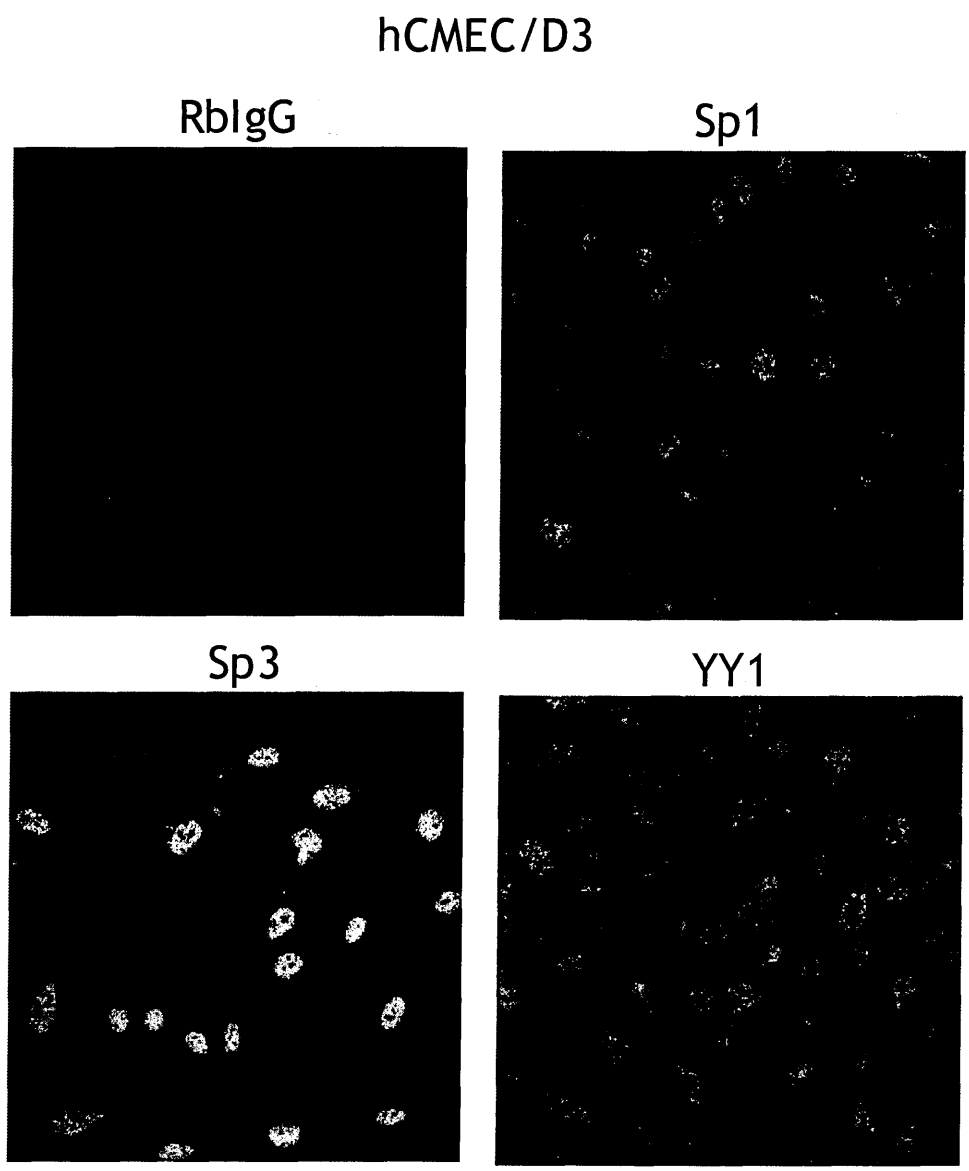
The Sp3:Sp1 ratio is highest in brain endothelium, in both the primary as well as the transformed line (Figs. 6.1 and 6.4). Similar Sp3:Sp1 ratio was maintained in lung (Fig. 6.2) but not in dermal ECs (Fig. 6.3) where the expression of Sp1 was favoured over Sp3. Sp1 expression in hCMEC/D3 and lung endothelial cells was predominantly nuclear but speckled. In the primary brain and dermal endothelial cells, Sp1 expression was seen in the nucleus and excluded from the nucleolus. Additionally, in the primary endothelial cells, traces of Sp1 protein were also seen in the cytoplasm. Sp3 protein was predominantly nuclear localised in hCMEC/D3, dermal and primary brain endothelial cells.

However, in lung endothelial cells, the Sp3 protein was also observed in the cytoplasm. It should be noted that the antibody to Sp3 recognises in addition to the full length protein, the shorter isoforms (N-terminal truncations) which are also nuclear localised.

6.3.1.2 YY1

The expression of YY1 was low in hCMEC/D3 cells and a distinctive punctuate nuclear localisation was observed (Fig. 6.1). Lung (Fig. 6.2) and dermal (Fig. 6.3) ECs expressed higher levels of YY1 though the cytosolic localisation was restricted to the latter cell line. YY1 expression was excluded from the nucleolus in cells of both the lines. It must be noted that YY1 expression and, especially, localisation in the primary endothelial cells is similar to that visualised in primary lung and dermal endothelium. It is indeed possible that some of the characteristics of the hCMEC/D3 cell line could be contributed by virtue of the SV-40 large T transformation. We investigated whether a bone marrow endothelial cell line which has been transformed by SV-40 large T showed similar pattern of staining to hCMEC/D3 cells (Fig. 6.5). Bone marrow endothelial cells showed strong expression of YY1 in the nucleus and did not show the nuclear punctate staining seen in the brain endothelial cell line. These data suggests that the YY1 expression and localisation of YY1 in hCMEC/D3 is a characteristic of the cell line which is not dependent on the transformation.

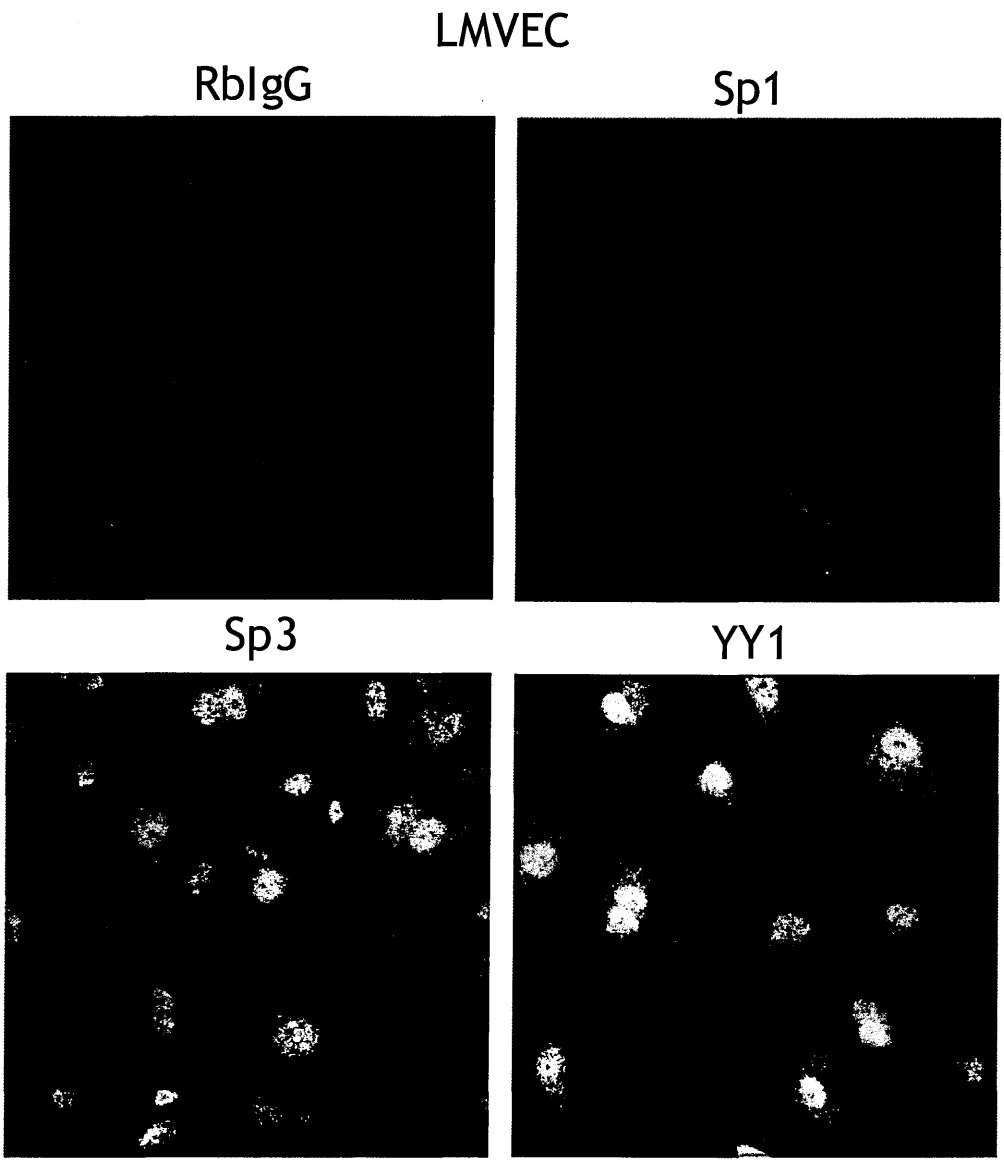
Figure 6-1 Expression of Sp Family and YY1 in hCMEC/D3 Cells by Immunofluorescence Confocal Microscopy



Cells were rested for two days after reaching confluence and were then stained with antibodies to Sp1, Sp3 or full length YY1 (H414). Normal rabbit IgG served as a negative control. All TFs are predominantly nuclear localised. Sp1 and YY1

exhibit a speckled nuclear profile. Data shown is representative of 3 independent experiments.

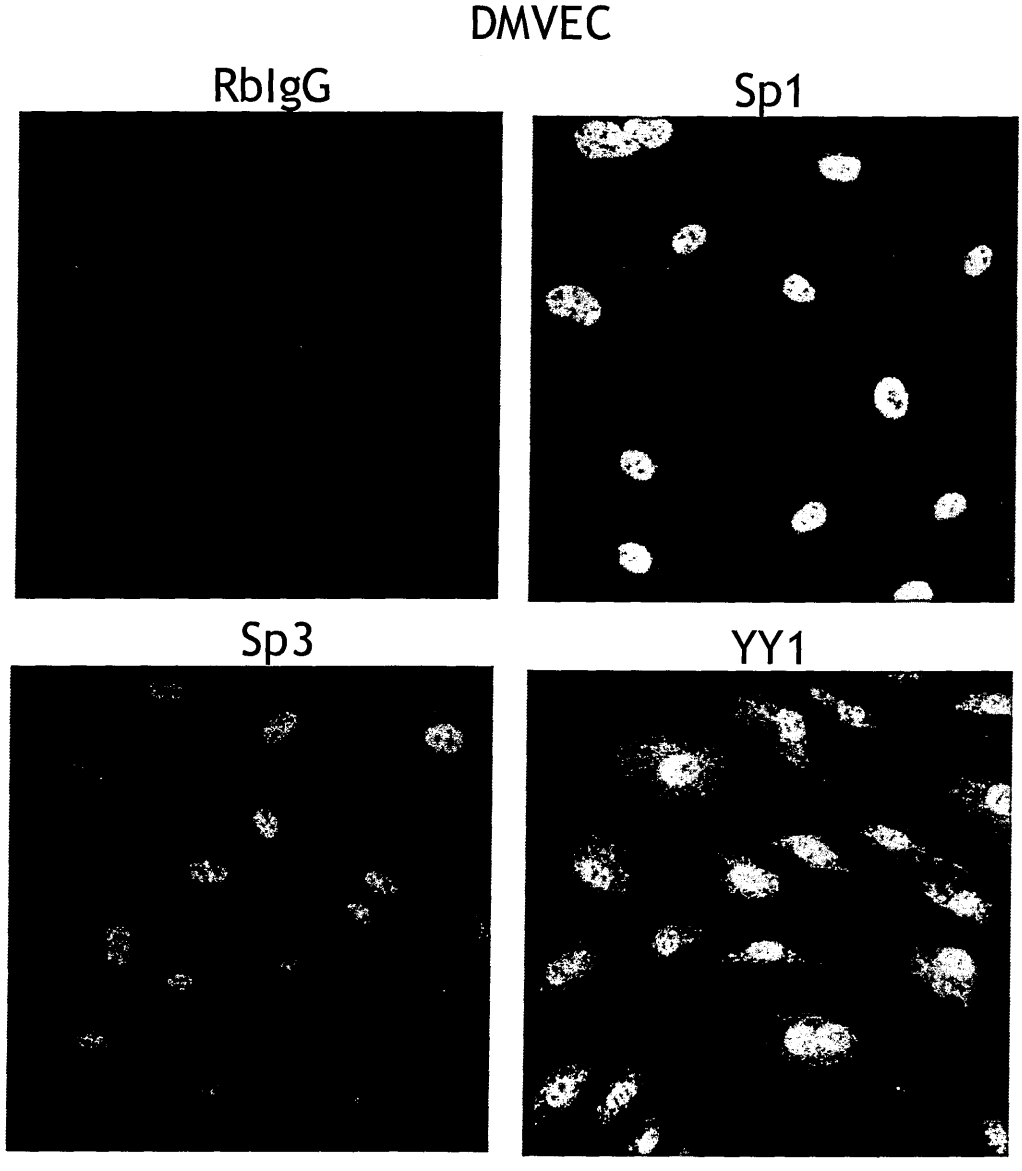
Figure 6-2 Expression of Sp Family and YY1 in Lung Endothelial (LMVEC) Cells by Immunofluorescence Confocal Microscopy.



Cells were rested for two days after reaching confluence and were then stained with antibodies to Sp1, Sp3 or full length YY1 (H414). Normal rabbit IgG served

as a negative control. Sp1 expression in very low but is nuclear localised. Sp3 and YY1 are strongly expressed but expression also seen in the cytosol. Data shown is representative of 3 independent experiments.

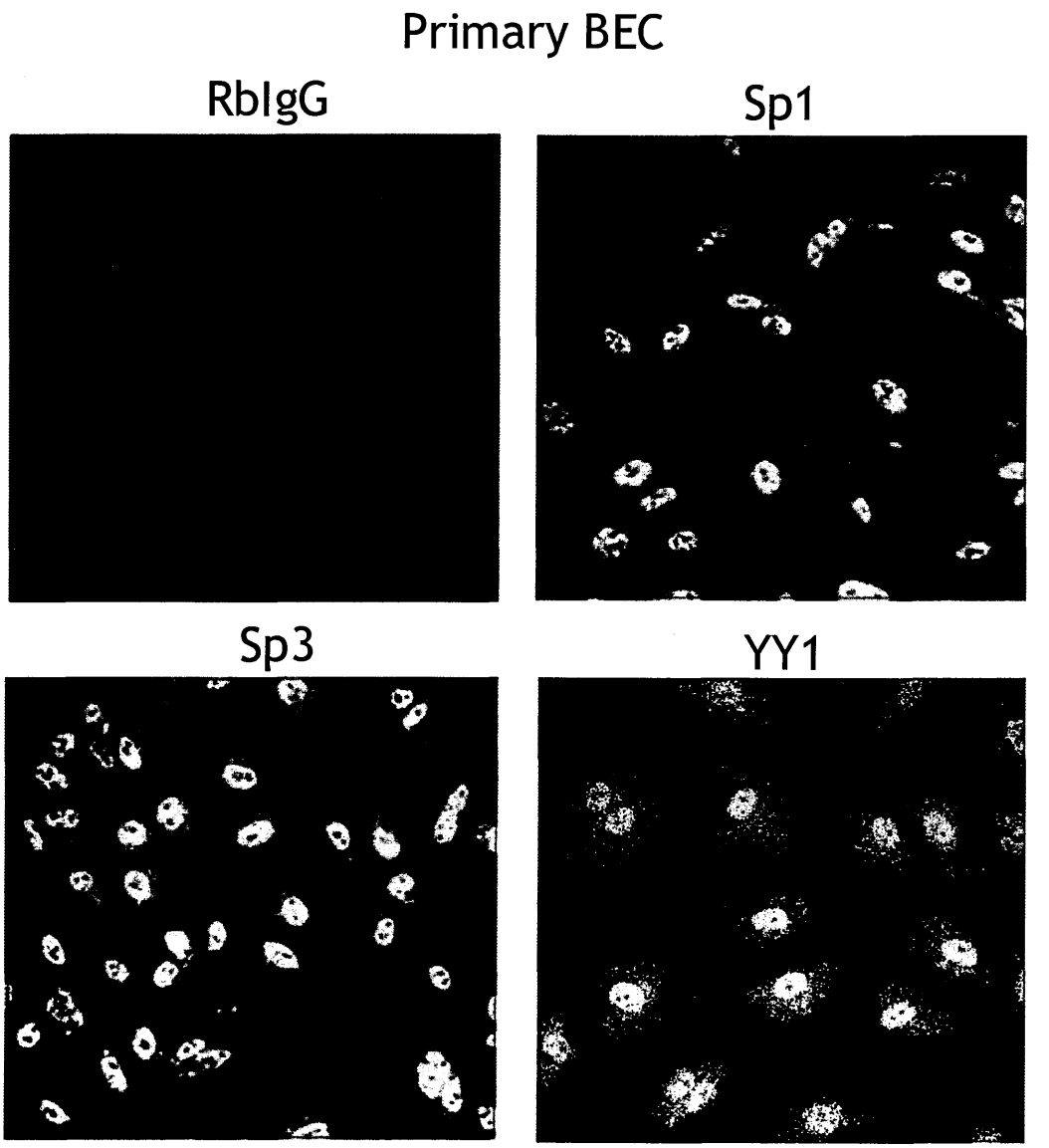
Figure 6-3 Expression of Sp Family and YY1 in Dermal Endothelial (DMVEC) Cells by Immunofluorescence Confocal Microscopy



Cells were rested for two days after reaching confluence and were then stained with antibodies to Sp1, Sp3 or full length YY1 (H414). Normal rabbit IgG served

as a negative control. Sp1 and YY1 are strongly expressed; Sp3 levels are low with some localisation in the cytosol. YY1 levels in the cytosol are the highest in among the endothelia.

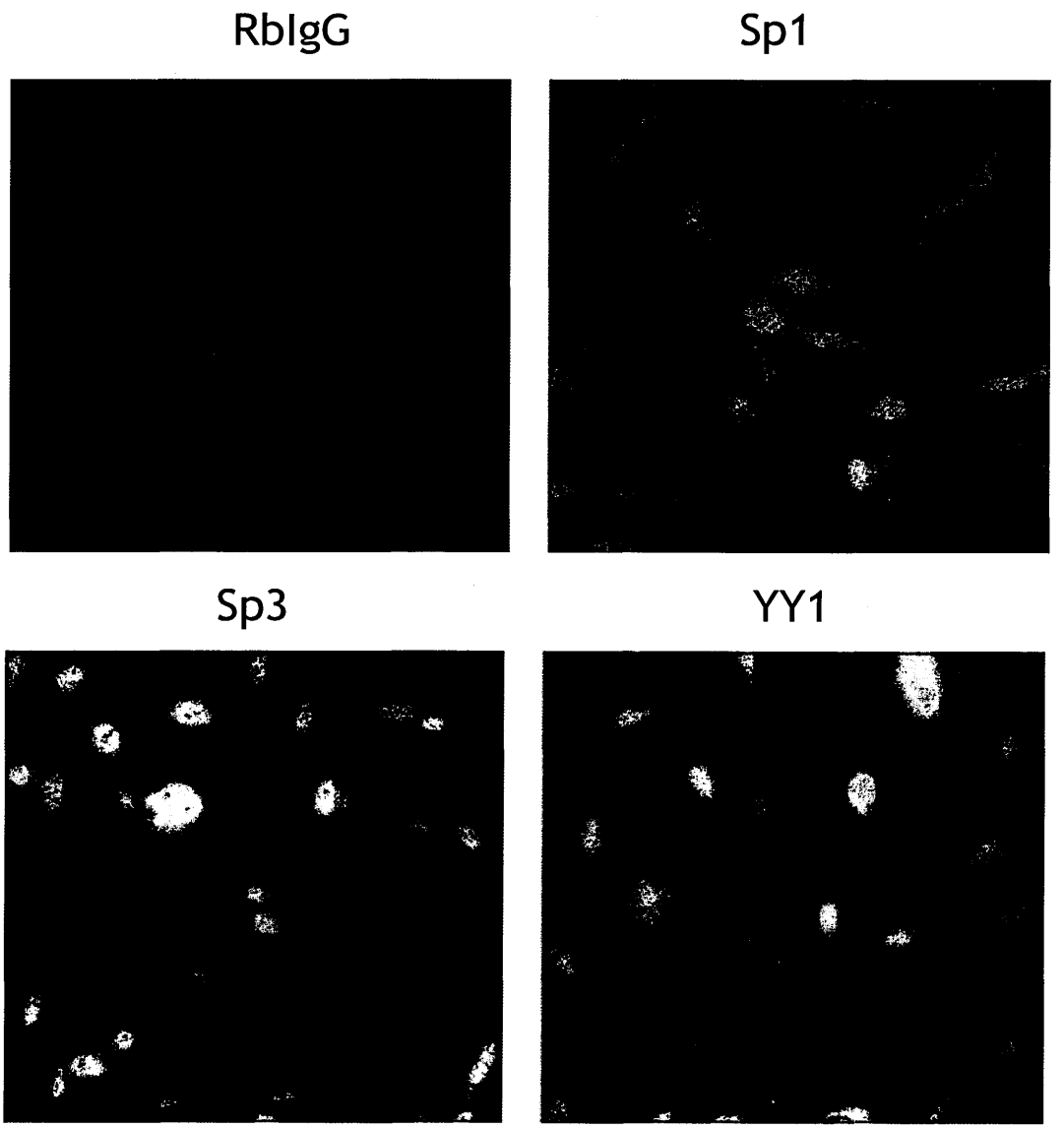
Figure 6-4 Expression of Sp family and YY1 in Primary Human Brain Endothelial Cells by Immunofluorescence Confocal Microscopy



Cells were rested for two days after reaching confluence and were then stained with antibodies to Sp1, Sp3 or full length YY1 (H414). Normal rabbit IgG served

as a negative control. Sp family and YY1 are strongly expressed, the latter shows punctate cytoplasmic localisation.

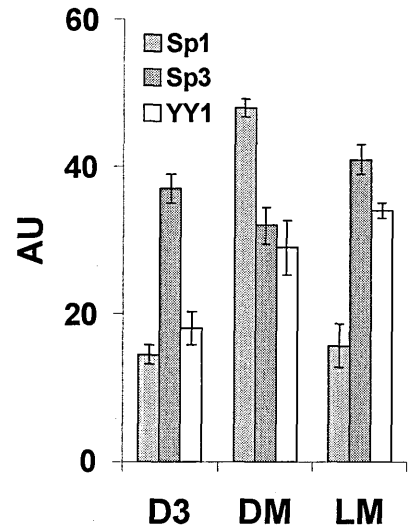
Figure 6-5 Expression of Sp Family and YY1 in Bone Marrow Cells by Immunofluorescence Confocal Microscopy



Cells were rested for two days after reaching confluence and were then stained with antibodies to Sp1, Sp3 or full length YY1 (H414). Normal rabbit IgG served as a negative control. Sp 3 family and YY1 are strongly expressed, Sp1 exhibits

speckled localisation in the cytosol and nucleus.

Figure 6-6 Quantification of differences in Sp family and YY1 TFs in hCMEC/D3 (D3), LMVEC (LM) and DMVEC (DM) cells



After correction for background intensity which was estimated from several intensity measurements in regions of the coverslip that did not contain any cells, boxes were drawn around the fluorescent structure in each cell and the pixel intensity was determined. This measurement was repeated for ~40 cells each from three experiments done on different days.

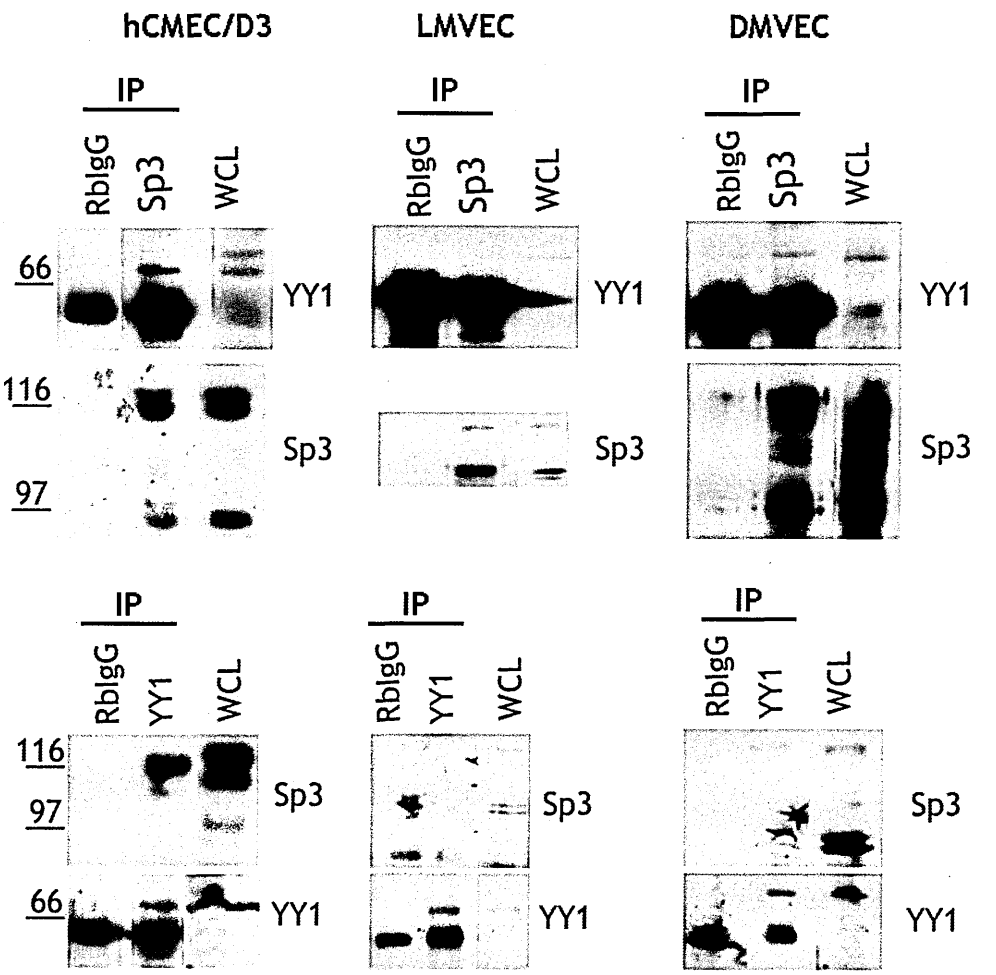
6.3.2 Interaction of Sp3 and YY1 in Brain Endothelium

Whole cell lysates from different endothelia were investigated by immunoprecipitation analyses to determine if the TFs interact *in vitro* (Fig. 6.6). Sp3 (top panel) or YY1 (lower panel) protein were immunoprecipitated with their respective antibodies and the immunoprecipitates from each condition were analysed by western blotting for the presence of the precipitated protein as well as for the other transcription factors as evidence of interaction.

The results show that in hCMEC/D3 cells, Sp3 immunoprecipitation causes the co-precipitation primarily of the smaller YY1 variants. We detected some co-immunoprecipitation of the full length YY1 (70 kDa) by anti-Sp3 in dermal endothelium, but not in lung endothelium. The result was confirmed when the analyses was carried out by immunoprecipitating YY1 and investigating for the presence of Sp1 and Sp3 in the complexes. YY1 interacts with the larger variant of Sp3 (116 kDa) in the brain endothelium, but not from lung endothelium. Co-precipitation of Sp3 with anti-YY1 from dermal lysates was barely detectable.

The results indicate a strong association of YY1 and Sp3 in brain endothelium which is absent in non-brain endothelium.

Figure 6-7 Interaction of Sp3 and YY1 Proteins in Endothelium



IP: Immunoprecipitate
WCL: Whole Cell Lysate

Cell lysates of hCMEC/D3 cells, lung (LMVEC) and dermal (DMVEC) endothelia were immunoprecipitated with protein-G beads in the presence of antibodies to transcription factors (Sp3 or YY1) or RbIgG as negative control. Whole cell lysates (WCL) were used as positive control to confirm the size/presence of the transcription factor in each cell type. The precipitates were examined by western blotting for the presence of Sp3 or YY1. The upper 6 blocks

show experiments in which immunoprecipitating antibody was Sp3, to detect co precipitation of YY1. The lower 6 blocks show an experiment in which immunoprecipitation was carried out with anti-YY1 antibody to detect co precipitation of Sp3.

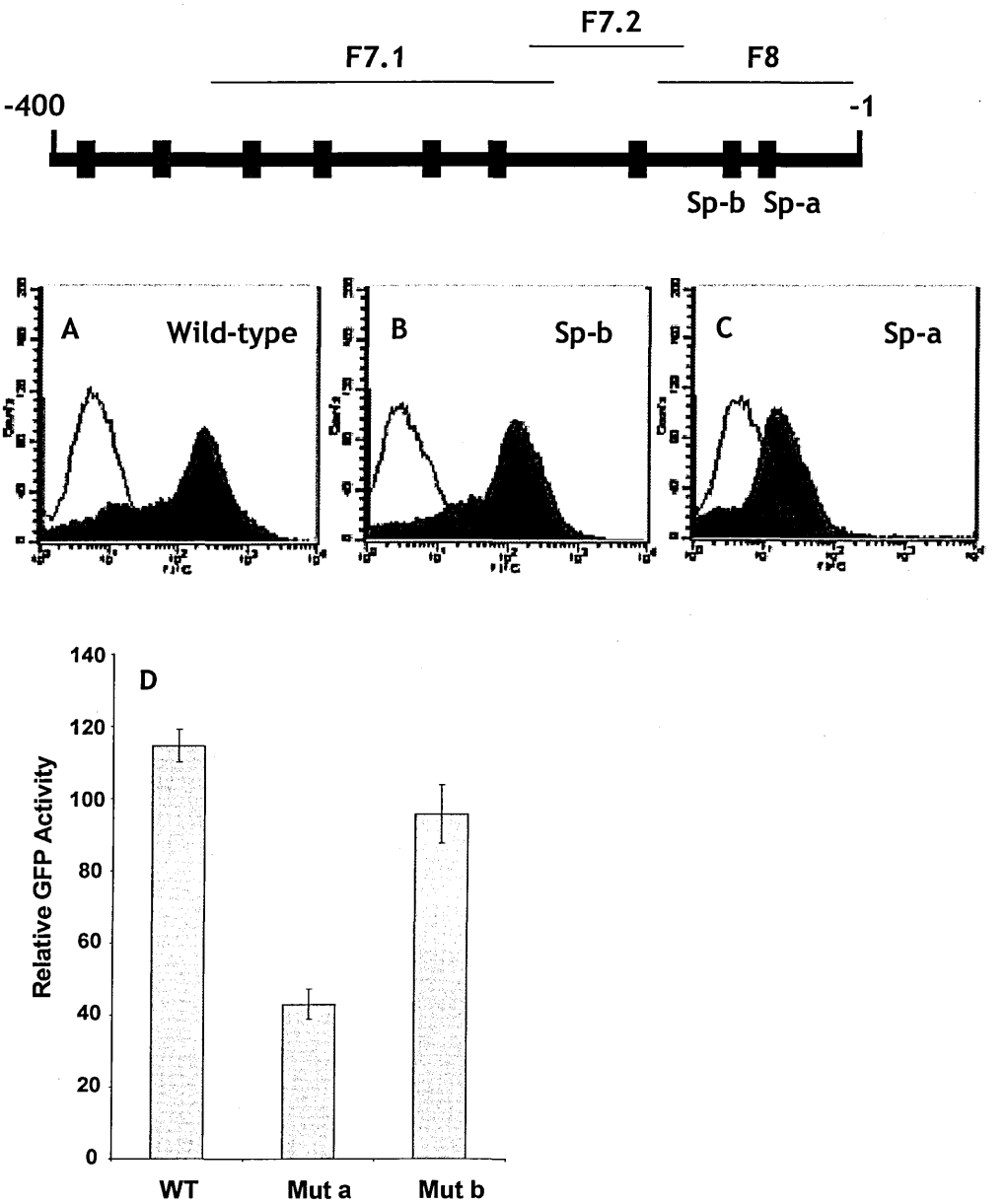
6.3.3 Promoter Activity of Fragment 8 with Sp Site Deletions

A single Sp site has been shown to be sufficient to drive transcription in promoters of diverse genes. Most promoters contain multiple GC boxes to which Sp family members can bind but it is possible that only one or two sites are important in driving transcription. The human H-ras promoter has six Sp1 sites of which only the most proximal one located around -45 is required for expression (Pintzas and Spandidos 1991). DNase I footprinting experiments using a TATA-less promoter containing Sp1 binding sites have shown that binding of Sp1 stabilizes interaction of TFIID with the transcription start site region (Emami, Burke et al. 1998).

Hence we hypothesised that the two Sp sites on fragment 8 could be important for the regulation of the occludin promoter by the Sp TFs. The two Sp sites on fragment 8; 1798 CCCTCCC 1804 and 1820 AGGCGG 1825 were targeted for deletion and two single deletion mutants were generated. EMSA were carried out with the wild type and the mutants but unfortunately the results were not clear as the mutant probes generated a lot of background (not shown). However, the double stranded DNA probes were cloned into promoter deficient vectors and transient transfections were carried out in hCMEC/D3 cells (Fig. 6.3)

Results show strong mediation of GFP expression in cells transfected with the wild type fragment 8. In cells transfected with the promoter fragment lacking the first Sp1 binding sequence-CCCTCCC (1798-1804), the GFP expression was comparable to WT albeit slightly decreased. However, deletion of the second Sp site AGGCGG (1820-1825) dramatically decreased the ability of the promoter fragment to drive GFP expression. The results imply that the downstream Sp site is important for the expression of the occludin protein in brain endothelium. Sp3/Sp1 interacts with two putative target binding sites close to the transcription start to initiate or maintain expression. However, these sites are inactive in lung endothelium.

Figure 6-8 Contribution of Sp Sites to the Activity of Fragment 8



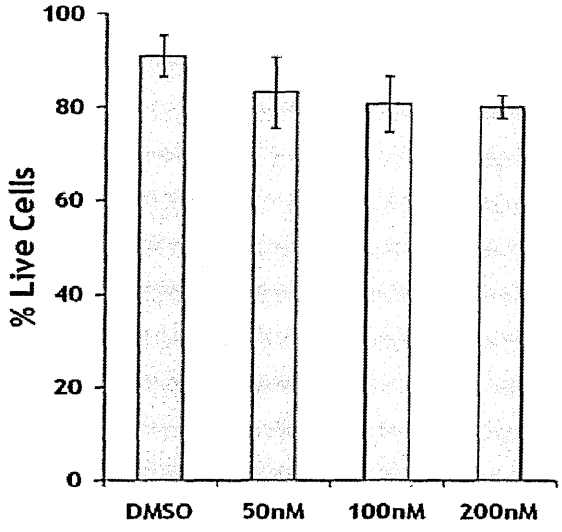
Promoter activity implicated by GFP expression in hCMEC/D3 transfected with vector containing wild-type F8 (A), or F8 with deletion of Sp target sites (B and C) (filled histograms) compared with control vector (open histograms). (B) Mutant represents deletion of the Sp-site at position -34 and (C) denotes deletion of the Sp-site at position -56. (D) represents relative GFP fluorescence of the three

vectors from 5 different experiments.

6.3.4 Inhibition of Sp Transcription Factors by Mithramycin

Mithramycin is an aureolic acid antibiotic that specifically binds to GC sequences on the DNA and selectively inhibits transcription. Mithramycin A is a potent inhibitor of Sp1 binding to GC boxes in DNA, and is capable of interfering with Sp1-mediated gene transcription (Blume, Snyder et al. 1991). Mithramycin has been shown to block binding of Sp factors consensus sites in the human c-myc, the SV40 early promoter and in the human dhfr promoter (Ray, Snyder et al. 1989; Blume, Snyder et al. 1991; Snyder, Ray et al. 1991). Mithramycin has been used as a specific inhibitor of Sp1, although the inhibition of the binding of other transcription factors that recognize GC-containing sequences in the minor groove of the DNA cannot be excluded (Miller, Polansky et al. 1987). For example, other ZnF transcription factors, including Egr-1, bind to GC-rich sequences in DNA (Krikun, Schatz et al. 2000). A lack of specificity would lead to primary mithramycin cytotoxicity. With this possibility in mind we used concentrations of mithramycin that did not affect cell viability. Mithramycin used at 50, 100 and 200 nM did not affect cell survival 72 hours post incubation as assayed by trypan blue exclusion (Fig. 6.8).

Figure 6-9 Cytotoxicity of Mithramycin in hCMEC/D3 Cells



6.3.4.1 Inhibition of Sp1/3 Binding to F8 Probe

We first tested the effectiveness of mithramycin in reducing the binding of Sp1/Sp3 to the occludin promoter in a gel-shift assay (Fig. 6.9). The DNA probe F8 was used as it functions as minimal promoter in hCMEC/D3 cells. Labelled ds DNA probe was incubated with three different concentrations of mithramycin before the binding reaction with brain endothelial nuclear extracts. In chapter 3, I discussed the activity of the promoter probe in brain endothelial cells and concluded that Sp3 binds to the probe and is responsible for nuclear protein-DNA complexes F8.1, F8.2 and F8.3. Nuclear protein-DNA complex F8.4 was not abrogated in the presence of an antibody to Sp3 but was inhibited in the presence of a Sp competitor. Sp1 did not bind to the probe as evident by supershift assays. In the present experiment, Mithramycin reduced the binding of Sp3 to its

recognition sequence in a dose-dependent manner (Fig. 6.9, lanes 5, 6 and 7). In the presence of 50 nM mithramycin, complex F8.1 was partially abrogated (lane 5). Incubation with 100 nM concentration of the drug abrogated complexes F8.1 and F8.2. Higher concentration (200 nM) of the drug inhibited the same complexes as the condition in which Sp3 antibody was present (lanes 4 and 6).

Figure 6-10 Mithramycin Blocks Binding of Sp TFs to Target DNA Motifs

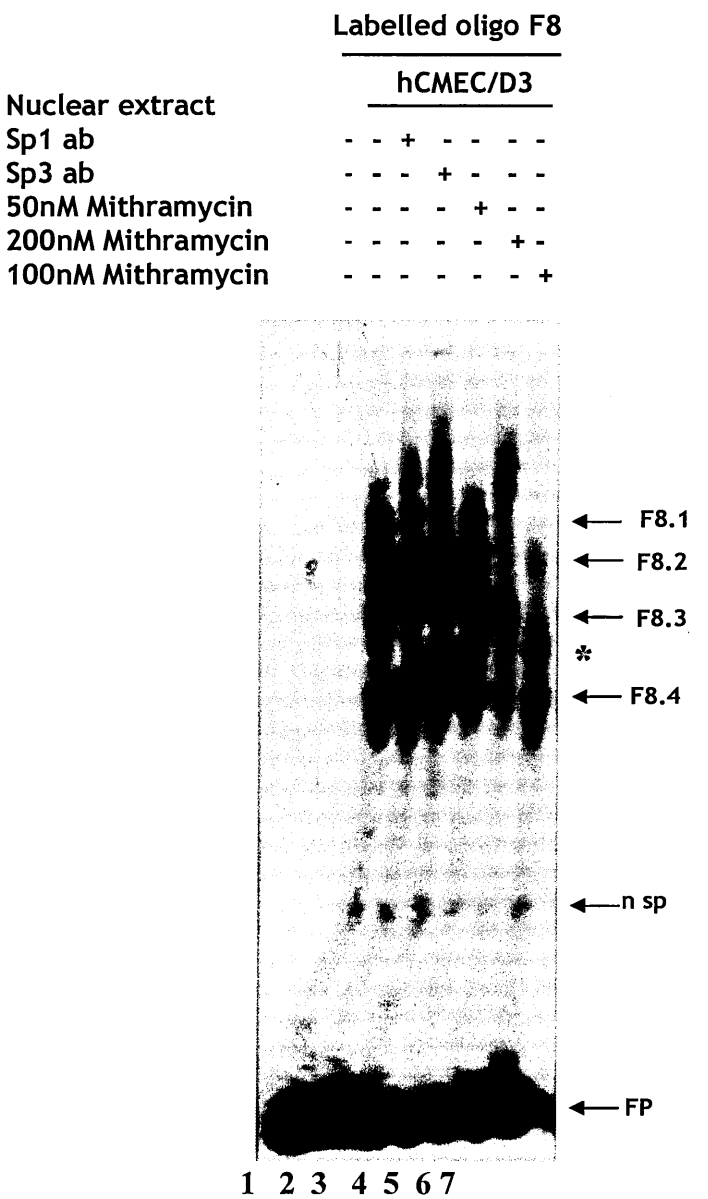
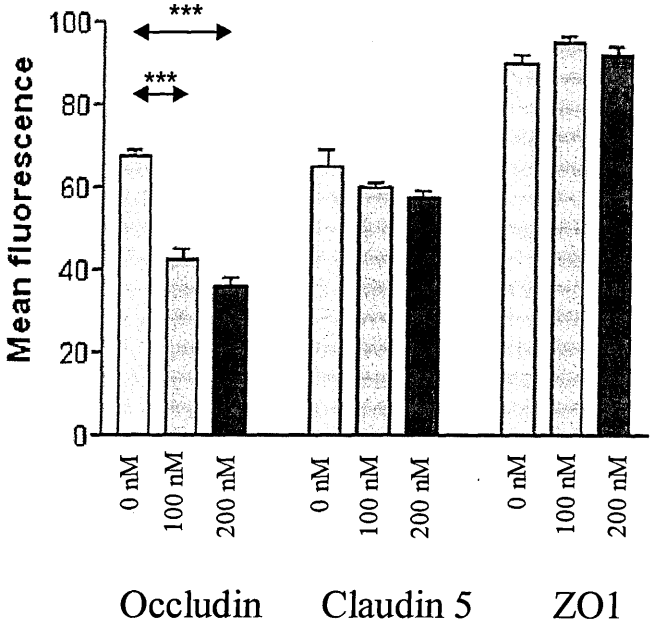


Fig. 6.10: EMSA analysis of complexes (8.1, 8.2, 8.3 and 8.4) formed with promoter probe F8 and hCMEC/D3 nuclear extracts cultured in the absence (lanes 2) or presence of different concentrations of the drug, mithramycin (lanes 5, 6 and 7). Lane 1 represents mobility of the probe in the absence of nuclear protein. Presence of 100nM (lane 7) or 200nM (lane 6) mithramycin inhibited the formation of nuclear protein complexes identical to those inhibited in the presence of Sp3 supershift antibody.

6.3.4.2 Occludin Protein in Mithramycin Treated hCMEC/D3 Cells

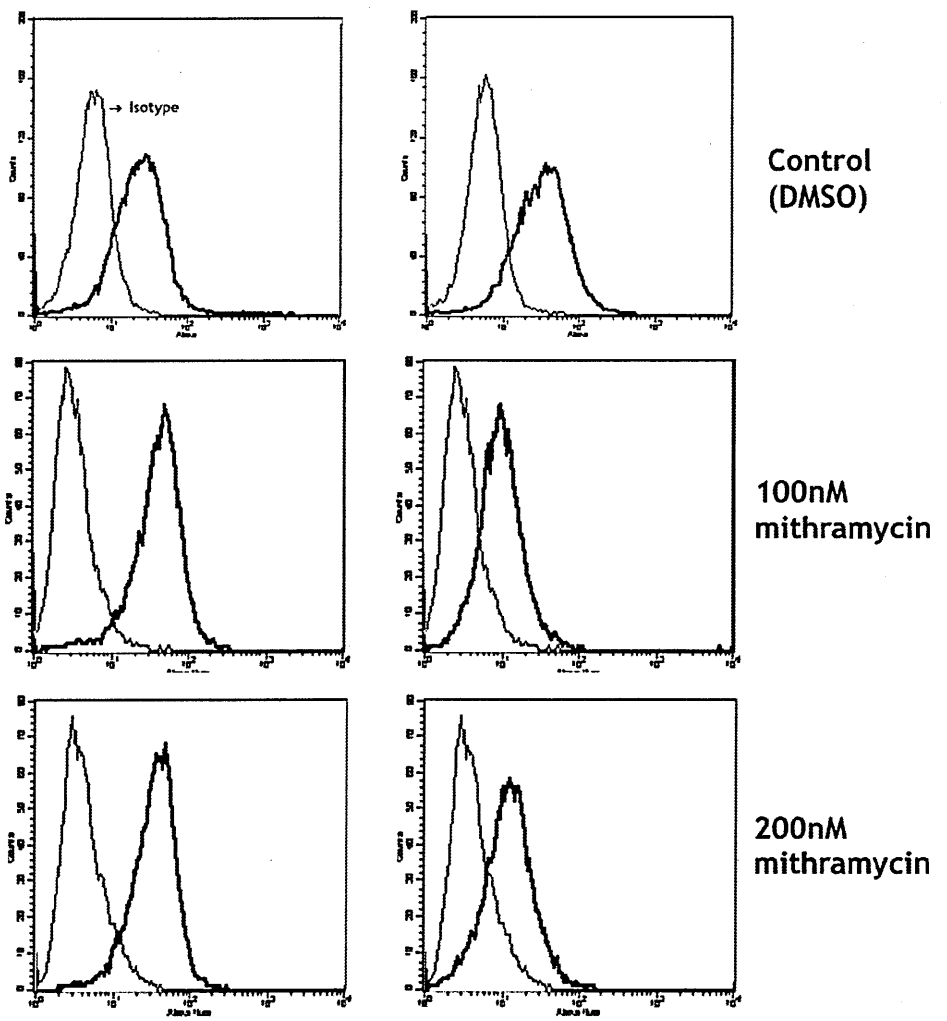
To further demonstrate the role of Sp transcription factors in the expression of occludin, cells treated in the presence or absence of 100 nM or 200 nM mithramycin were immunostained with an antibody to occludin (Fig. 6.12). Total occludin protein levels were down regulated in the presence of the pharmacological inhibitor (Figs. 6.11 and 6.12). This appears to be specific as expression of other tight junction proteins-claudin 5 and ZO1 was unaffected.

Figure 6-11 Expression of TJ Proteins in Mithramycin Treated Brain Endothelial Cells



Graph represents percentage of hCMEC/D3 cells expressing claudin 5 or occludin when cultured in the presence or absence of mithramycin. Data is obtained from 3 experiments. Each protein was analysed by ANOVA followed, if $p<0.05$, by Dunnett's multiple comparison test. *** indicates expression is significantly different from untreated cells, $p<0.001$.

Figure 6-12 Loss of Occludin Protein in Mithramycin Treated Brain Endothelial Cells



Fully confluent hCMEC/D3 cells were cultured in the presence of DMSO (control), 100 nM mithramycin or 200 nM Mithramycin for 48 hours following which the cells were immunostained for the tight junction proteins-claudin 5 (left hand panel) and occludin (right hand panel) and assayed on FACS. Isotype matched IgG was used as a control.

6.4 Discussion

Data so far shows that nuclear factors belonging to the Sp family and YY1 present in brain endothelial cells bind the occludin promoter *in vitro* and *in vivo* and the key question is investigating whether one or both of these proteins are involved in tissue-specific expression of occludin in CNS endothelium.

Occludin mRNA levels increase with increasing confluence in brain endothelial cells cultures. The levels of occludin protein at the tight junction increase as cell-cell contacts and junctions are established in fully confluent and resting culture. In this study we found that one of the two Sp elements namely the one located between 1820-1825 (AGGCGG) of the occludin promoter is responsible for a major part of occludin promoter activity in hCMEC/D3 cells (Fig. 6.8C) and Sp3 bound to this element (Figs. 4.4 and 6.10). It was slightly unexpected that promoter activity could be determined by a single Sp element especially as the data with the reporter vector constructs indicated activity in regions of the promoter which lack this site. A single Sp site has been shown to be sufficient to drive transcription in promoters of diverse genes. Most promoters contain multiple GC boxes to which Sp family members can bind but it is possible that only one or two sites are important in driving transcription. The TATA-less human H-ras promoter has six Sp1 sites of which only the most proximal one located around -45 is required for expression (Pintzas and Spandidos 1991). Based on these results, it was suggested that Sp1 is required for functional assembly of the basal transcription machinery on the H-ras promoter. DNase I footprinting experiments using a TATA-less promoter containing Sp1 binding sites have

shown that binding of Sp1 stabilizes interaction of TFIID with the transcription start site region (Emami, Burke et al. 1998). Another reason could be the fact that occludin expression is restricted and its regulation is different from genes that are commonly expressed. One of the main differences in the regulation of promoters from housekeeping and tissue specific genes is the presence of extensive redundancy in the house keeping gene promoters. Both kinds of promoters display numerous transcription factor binding sites but large deletions or removal of TF binding sites have relatively little effect on promoter activity on the promoters of housekeeping genes. Examples of such housekeeping promoters are the mouse DHFR promoter, the mouse hprt promoter and several promoters of ribosomal protein genes. It has been proposed that different cell types exhibit different sets of transcription factors and hence multiple regulatory elements are required for gene expression in each cellular background. Another proposal contends that activity of housekeeping genes is not sensitive to singular or transient cues from the environment and altering promoter activity in these promoters requires manifold changes in the cell environment and recruitment of multiple regulatory elements.

We also confirmed the importance of the Sp elements by using mithramycin, a drug that modifies GC-rich regions of the DNA and blocks Sp1/Sp3 binding (Blume, Snyder et al. 1991). Mithramycin treatment inhibited Sp3 binding to the Sp element (Fig. 6.10) and the protein levels of occludin also decreased in mithramycin treated cells (Fig. 6.12) which strongly suggests that Sp3 binding is essential for occludin expression. Other TJ proteins like claudin 5 and ZO1 were not affected. All of these results clearly suggest that induction of occludin gene

expression is dependent on activity of Sp3.

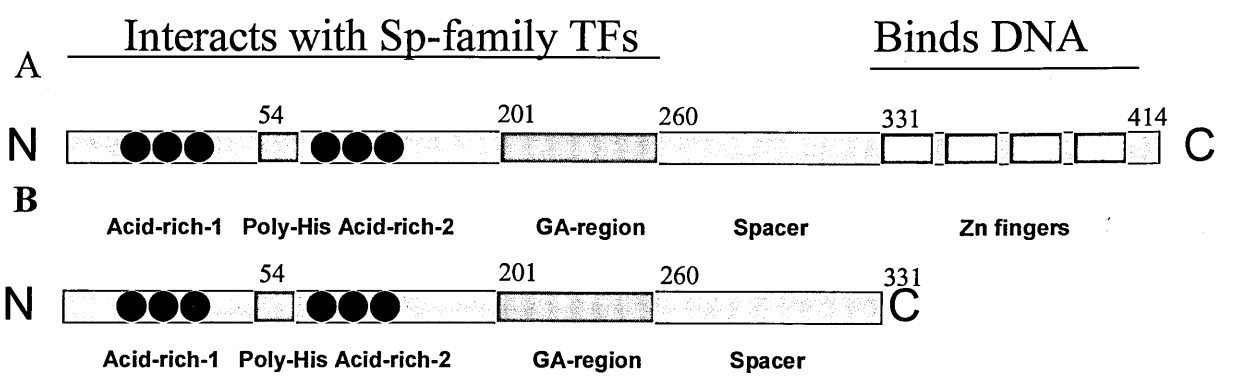
It is unlikely that Sp3 acts alone to activate expression from the occludin promoter. Sp3 is not a strong transctivator like Sp1 (Hagen, Muller et al. 1994) and usually interacts with other co-activators to drive transcription. We have investigated if the Sp and YY1 family members interact in brain endothelium. Sp3 interacts with YY1 in the brain but not in lung endothelium (Fig. 6.7).

The lung and dermal endothelial cells express the full length form of YY1-70kDa. The full length form of YY1 in the brain and non-brain endothelium is subject to posttranslational modification like phosphorylation and acetylation that regulate the DNA-binding affinity and the capacity to form complexes with other nuclear proteins (Thomas and Seto 1999). In the brain endothelium there is a shorter isoform of YY1 that migrates at 45 kDa in addition to the full length form (Holloway, Sade et al. 2007). Ths shorter isoform lacks the C-terminus which contains the DNA binding region but retains the ability to interact with TFs. This isoform migrates at 45kDa and is only present in the brain endothelium and absent from lung and dermal endothelia. This isoform could in essential act like a dominant negative or constitutively active form of YY1 by being able to bind other TFs that if were allowed to interact with full length YY1 could result in activation or inhibition of transcription from a responsive promoter. In the brain, this isoform is as strongly expressed as the full length form (Holloway, Sade et al. 2007).

In the brain, it is clear that the shorter isoform of YY1 interacts specifically with functional form of Sp3-118 kDa and not the shorter form of 97 kDa that has been

shown to negatively regulate Sp3 activity. Despite interaction of Sp3 and YY1 protein, data from the YY1 antibody super shift (lane 5, Fig 4.4) indicates that Sp3 and YY1 do not interact in this region. Could it be possible that YY1 and Sp3 interact only at the level of proteins but not as a complex with the promoter DNA? It is possible because the shorter isoform lacks the DNA binding region but is able to bind TFs. It has to be remembered that YY1 has the dual nature of activating or inhibiting transcription. It is possible in the brain endothelium; the shorter isoform binds Sp3 and acts to initiate transcription. Sp3 is a weak transactivator but in the presence of YY1 and Sp1 is able to initiate transcription.

Figure 6-13 YY1 isoforms in Brain Endothelium



A: Full length YY1 protein

B: Brain specific shorter isoform

However, it is also possible that yet another single or groups of transcription factors are involved. Promoter probe F8 forms four nuclear protein complexes with hCMEC/D3 nuclear extract. Formation of all complexes is completely and partially abolished in the presence of a Sp competitor and an antibody to Sp3

respectively. In addition, Sp1 is not a component of these complexes as presence of an antibody to the protein does not affect the formation of these complexes. Hence, all the DNA-nuclear protein interactions in this probe are Sp3 dependent. There are however, only two Sp sites on this probe. Several possibilities can explain the mismatch between the number of binding sites and number of nuclear protein-DNA complexes:

1. There is a low molecular weight isoforms of Sp3 in addition to the full length protein. The faster migrating bands on the EMSA gel may be attributed to these variants which would migrate differently in EMSA but not in western blots.

2. Presence of post translational variants of Sp3 generated via sumoylation, phosphorylation or acetylation. F8.3 appears to have a higher affnity than F8.1 or F8.2 as it is harder to block. Hence it is possible these two complexes are formed by a post translational variant of Sp3.

Chapter 7

Conclusion and Future Work

7 Conclusion

Understanding the mechanism of tissue-specific gene expression remains a fundamental question in genome biology. Despite major advances in developmental biology and bioinformatics, unravelling the seemingly simple concept of different tissue types being determined by the same DNA template still remains a major challenge. The human genome has more than 25000 genes which display remarkable diversity in patterns and levels of expression in different tissues. However these vast changes in spatial and temporal gene expression are controlled by only ~1850 TFs. Computational approaches have provided important tools in understanding transcriptional regulation in being able to predict identification of TF binding sites, regulatory elements of combinatorial pathways and identification of gene targets for TFs in addition to promoter regions, but these predictions remain to be validated *in vitro*. Research which incorporates ChIP and microarray analyses in concert with cis-regulatory element analysis is proving a popular choice for researchers in this field.

In the present study, we investigated the transcriptional regulation of the tight junction protein occludin whose expression is restricted to the brain endothelium. Endothelial cells from different tissues differ widely in the expression of junctional proteins and transporters. The expression of proteins like occludin, p-glycoprotein and transferrin receptor is restricted to brain endothelial cells in contrast to claudin-5 which is strongly expressed in the brain and to a lesser extent in dermal and lung endothelia (Holloway, Sade et al. 2007). The mechanism(s) responsible for the differential expression of these proteins is not known.

Previously, we have shown that human brain, dermal and lung endothelial cells have distinct expression profiles of transcription factors and have demonstrated that a select few play important roles in the regulation of a brain endothelial specific protein, the transferrin receptor promoter. Our study represents the one of but many investigations where prior knowledge of TFs expression was used to understand tissue specific gene expression patterns.

A brief summary of key findings from the work in the regulation of occludin in brain and lung endothelial cells are as follows:

1. Analysis of the *in vitro* activity of the promoter by using a reporter vector transfection assay system concluded that the promoter in brain endothelial cells is capable of driving gene expression in contrast to the lung endothelium where no activity was observed.

2. Sp1, Sp3 and YY1 present in nuclear extracts of hCMEC/D3 cells associate with the occludin promoter under *in vitro* and *in vivo* conditions.

3. Sp3 interacts with YY1 protein in brain endothelium and this is absent in the lung endothelium. The association is absent in lung in the case of the Sp family.

4. Blocking Sp3 binding to target sites by the antibiotic mithramycin lead to the down regulation of the occludin protein but not other tight junction proteins such as claudin 5 and ZO1.

5. Deletion of one of the proximal Sp sites on the promoter lead to a decrease in its activity on transient over expression within a reporter vector in brain

endothelium.

From the data, it is clear that Sp family positively regulates the expression from the occludin promoter in brain endothelium and YY1 augments this function and these TFs have important roles in maintaining the phenotype of the differentiated brain endothelial cells.

It has been established in literature that Sp and YY1 TFs regulate differentiation in various cell types. Sp1 has been shown to regulate promoters of genes associated with differentiation and is also involved in transactivation of promoters of housekeeping genes. Sp1 interacts with MyoD to positively up regulate the expression of many muscle-specific genes during muscle cell differentiation (Guo, Degnin et al. 2003). In corneal epithelial cells, the promoter of keratin 4 which is important in maintaining the differentiated phenotype is transactivated by Sp1 (Okano, Opitz et al. 2000). In F9 cells the induction of tissue plasminogen activator (t-PA) in the presence of retinoic acid is mediated by Sp1 binding to proximal GC boxes in the t-PA promoter (Darrow, Rickles et al. 1990). Sp1 binds to a proximal GC box in the promoter of the DNA topoisomerase IIa gene promoter in proliferating rat cells and activates transcription of the gene (Yoon, Kim et al. 1999). Other promoters include dihydrofolate reductase (DHFR) (Dynan, Sazer et al. 1986), thymidine kinase (Dou, Fridovich-Keil et al. 1991), p21waf1/cip1 (Pardali, Kurisaki et al. 2000), GM-CSF (Brettingham-Moore, Rao et al. 2005), EGFR (Liu, Innocenti et al. 2005). Sp1 physically interacts with other DNA binding proteins like PU.1 (Feng, Teitelbaum et al. 2000), E2F-1 (Lin, Black et al. 1996), and p53 (Schavinsky-Khrapunsky, Huleihel et al. 2003). Rb

protein is known to stimulate Sp1 and Sp3 mediated transcription (Chen, Nishinaka et al. 1994; Udvadia, Templeton et al. 1995).

Importantly, the Sp family have also been implicated in regulation of genes that are specific to endothelial cells such as those coding for the platelet-derived growth factor B-chain, the platelet-derived growth factor A-chain, the endothelial nitric oxide synthase, the vascular cell adhesion molecule-1, and KDR/flk-1.

In Chapter 1, I have discussed the upregulation of SSeCKS (Src-suppressed C kinase substrate) in response to oxygen tension in astrocytes. Supernatants from SSeCKS-expressing cells increase the protein levels of ZO1 and claudin 1, constituents of the TJs of the BBB (Lee, Kim et al. 2003). An interesting observation is the the presence of a GC box between –106 and –49 in the proximal promoter of SSeCKS which is bound by Sp1/Sp3. In v-*Src*-transformed NIH3T3 cells, this region is negatively regulated by HDAC1 which is recruited into this complex (Yahao Bu and Irwin H. Gelman 2007).

The proximal promoter of GDNF (Glial cell line-derived neurotrophic factor) which is also involved in the development of blood brain barrier properties exhibits binding sites for Sp, AP2 and NFκB TFs (Woodbury, et al., 1998). GDNF is a weak promoter which is thought to be due to the presence of an upstream YY1 binding site and target motifs for other repressors namely epidermal growth factor receptor (ETF) and GC factor (GCF) (Baecker, Walter et al., 1999).

Sp1 along with a member of the Ets family of TFs, GABP has been implicated in

the transcription of Robo4, which is expressed exclusively in endothelial cells (Okada, Yano et al. 2007). In this study, GABP and Sp1 associate with the endogenous promoter in endothelial cells and transfection of these cells with small interfering RNA against GABP and Sp1 resulted in a significant decrease in endogenous Robo4 mRNA expression.

Vascular endothelial cadherin another junction protein that is specific to endothelial cells is also regulated by the Sp family (Gory, Dalmon et al. 1998). Sp1 and Sp3 have been shown to associate with two GT boxes at positions −48 and −40 in the proximal VE cadherin promoter and in conjunction with Ets TFs drive transcription.

Sp3 was initially described to inhibit Sp1 mediated transcription (Hagen, Muller et al. 1994; Dennig, Hagen et al. 1995) but later studies have identified it as a transcriptional activator (Liang, Robinson et al. 1996; Ihn and Trojanowska 1997; Prowse, Bolgan et al. 1997). This property depends on cellular context, levels of Sp1 protein, number of Sp elements on the promoter (Yu, Datta et al. 2003) and levels of the shorter isoforms of Sp3 itself (Kennett, Udvadia et al. 1997). There are three isoforms of Sp3; the full length isoform acts as an activator and the two shorter isoforms retain DNA binding activity and associate with target sequences to repress transcriptional activation by full length Sp3 or Sp1(Kennett, Udvadia et al. 1997). The full length form predominates in brain endothelial nuclei, whereas the short isoforms predominate in non-brain endothelium, which could partly explain the differential activity of Sp3 in lung and brain endothelium.

Sp3 competes successfully with Sp1 for the Sp target sites on the occludin

promoter. Data indicates that it is possible the Sp3 protein singularly or via associations with other TFs acts to initiate transcription as Sp3 has been reported to be a poor activator in certain cellular systems. Our data on protein interactions indicate that Sp3 interacts with the transcription factor YY1 in brain endothelial cells and this specific interaction is absent in lung endothelial cells.

The Sp proteins present in nuclear extracts derived from the lung endothelial cells do not bind to the endogenous promoter or to the naked DNA in EMSA. Since lung endothelial cells express Sp1 and Sp3 it is possible that the binding motifs are occupied by other DNA modifying proteins restricting access to the promoter as seen with F8 in lung endothelial cells or there is a transcription factor that represses activation of occludin transcription in the lung endothelium. Data suggests the possible candidate is the TF YY1 which like the Sp family proteins is widely expressed and acts as a repressor or as an activator dependent on cellular contexts.

YY1, a member of the Polycomb Group protein family is a ubiquitous and multifunctional zinc-finger transcription that has critical roles in hematopoiesis and cell cycle control (Thomas and Seto 1999). Promoters that have been established to be under YY1 control include endothelial nitric-oxide synthase promoter (Karantzoulis-Fegaras, Antoniou et al. 1999), c-Myc (Austen, Cerni et al. 1998), c-Fos (Zhou, Gedrich et al. 1995), p53 (Sui, Affar el et al. 2004), α-actin (Wu and Lee 2001), E6 and E7 of HPV (O'Connor, Tan et al. 1996) and a number of other viral LTRs (Shrivastava and Calame 1994). YY1 present in the lung endothelial nuclear extracts associates with the promoter region up stream of

the transcription start site and this specific binding is not seen in the brain endothelial cells. However, YY1 in the brain endothelial nuclear extract binds to other target motifs on the occludin promoter namely F5 and F6.1 raising the possibility that the preferential binding on the F0.1 in the lung endothelium is important in repression of transcription. We have shown the interaction of YY1 with Sp3 in the brain endothelium where it is likely that the association is synergistic.

Interestingly, the Sp and YY1 family have been shown to associate with pathways that modulate expression and localisation of the occludin protein at the tight junctions. I have discussed the regulation of occludin protein in section 1.4.1.1.6 by cytokines, phosphatases, Rho, PI3K and MAPK signalling. It is possible the regulation is mediated at the transcriptional level i.e. the decrease in protein levels may be correlated to the disruption of transcription factor(s) involved in occludin promoter regulation.

Glucocorticoids such as hydrocortisone and dexamethasone have been shown to upregulate protein levels of occludin and increase localisation at the tight junction in brain endothelial cells. In brain endothelial cells, the activated glucocorticoid receptor has been shown to bind glucocorticoid-responsive elements, GREs in the occludin promoter (Forster, Silwedel et al. 2005). It will be interesting to know whether YY1 or Sp family of TFs are recruited to the promoter in this context. YY1 functions as a repressor in the regulation of β casein promoter and in mouse mammary epithelial cells activation of the glucocorticoid receptor can lead to disassembly of YY1 at the β casein proximal promoter and enhancer elements

thus allowing for transcription (Meier and Groner 1994). It would be interesting to investigate if the converse is true in the brain endothelium. Does activation of GR induce increasesd recruitment of YY1 to the oclcudin promoter? ChIP analysis of hCMEC/D3 cells treated with dexamethasone and investigated for YY1 association with the endogenous promoter would provide information.

Treatment of a multiple myeloma cell line, LP1 with dexamethasone induced the proteasomal degradation of c-maf (Mao, Zhu et al. 2008). Mao and colleagues investigated the mechanism by which GCs were able to upregulate ubiquitin mRNA without direct association with the promoter. The authors showed increased association of the TF Sp1 with the ubiquitin promoter in dexamethasone treated cells. Since the protein levels or localisation patterns of Sp1 were not modulated it is speculated the increased affinity of Sp1 for the ubiquitin promoter may be due to increased phosphorylation. In rat muscle cells, increased binding of Sp1 to the ubiquitin promoter has been attributed to MEK1 dependent Sp1 phosphorylation (Marinovic, Zheng et al. 2002). GR induced phosphorlatyion of Sp1 is specific as other Sp1 regulated genes such as Glut1 are not increased in these cells. The mechanism behind the selective activation of Sp1 in the regulation of specific genes is is unknown. Again, treatment of hCMEC/D3 and lung endothelial cells with dexamethasone and investigating any possible effects on the phosphorylation status of the Sp1 and Sp3 protein would provide additional insights.

Activation of signalling through the protein kinase C pathway induces phosphorylation of occludin and its redistribution at cellular contacts. Activation

of the PKC pathway in primary human nasal epithelial cells induces transcription from the GATA-3 and -6 gene promoters and these TFs have been shown to be responsible for upregulating levels of ZO-1 and-2 and claudin-1 in these cells (Koizumi, Kojima et al. 2008). In T cells, PKC isoforms transactivate the p21WAF1 gene promoter by mediating the binding of Sp1-p53 complex to binding sites on the promoter (Schavinsky-Khrapunsky, Huleihel et al. 2003). Synergistic associations between PKC and Sp1 drive the transcription of the human serum paraoxonase 1 (PON1) in hepG2 cells (Osaki, Ikeda et al. 2004). It remains to be seen if transfection of small interfering RNA to the respective PKC isoform in our system would disrupt occludin protein expression via decreased association of Sp family with the occludin promoter.

However, the role of Sp family and YY1 is not restricted to signalling pathways that upregulate occludin protein. The Sp family have been shown to play an important role in signalling pathways that lead to the down regulation of occludin protein and increase permeability. The cytokine TNF-α, VEGF A, reactive oxygen species are known to activate signalling pathways that downregulate the occludin protein (discussed in chapter 1). Sp family have been implicated in the activation of transcription of the VEGF A promoter in many systems (Hasegawa, Wakino et al. 2006; Bermudez, Yang et al. 2007; Pages 2007; Santra, Santra et al. 2008). In ovarian cancer cells, treatment with VEGF leads to an increase in telomerase activity which is highly dependent on Sp and the AP family of TFs (Bermudez, Yang et al. 2007). Some of these mentioned pathways also synergise to mediate their effects. Presence of reactive oxygen species enhances VEGF A gene expression and analysis of the VEGF-A promoter mapped the

oxidative stress response element to a minimal sequence that contains two target sequences for Sp1 and Sp3 (Schafer, Cramer et al. 2003).

When human brain microvascular endothelial cells are exposed to methamphetamine, depletion of glutathione and corresponding increase in reactive oxygen species have been shown (Lee, Hennig et al. 2001). Activation of TFs, AP-1 and NFκB was central to these effects. These TFs are regulated by cellular redox status and are known activators of TNF-α the cytokine that downregulates occludin expression. It is interesting to note the proximal promoter in occludin contains two sites that are bound by TFs with similar motifs to AP2-α and NFκB. Shono and collagues in a different study have also shown the activation of AP-1 and NFkB in brain endothelial cells and have further investigated the involvement of Sp family. Data indicates the Sp family are not activated in response to oxidative stress (Shono, Ono et al. 1996).

The work identifies the importance of Sp family and YY1 in the regulation of occludin promoter in brain and non-brain endothelium. The occludin gene is actively transcribed in brain endothelium which involves the association of the Sp family, particularly Sp3 which occupies the Sp binding motif close to the 3'-end of the promoter. In lung endothelium the Sp family of TFs do not associate with their target motifs due to a possible change in chromatin structure induced by the binding of YY1 to an upstream cis-element and this is associated with the lack of occludin gene transcription.

Figure 7-1 Proposed model for occludin gene transcription in brain and non brain endothelium

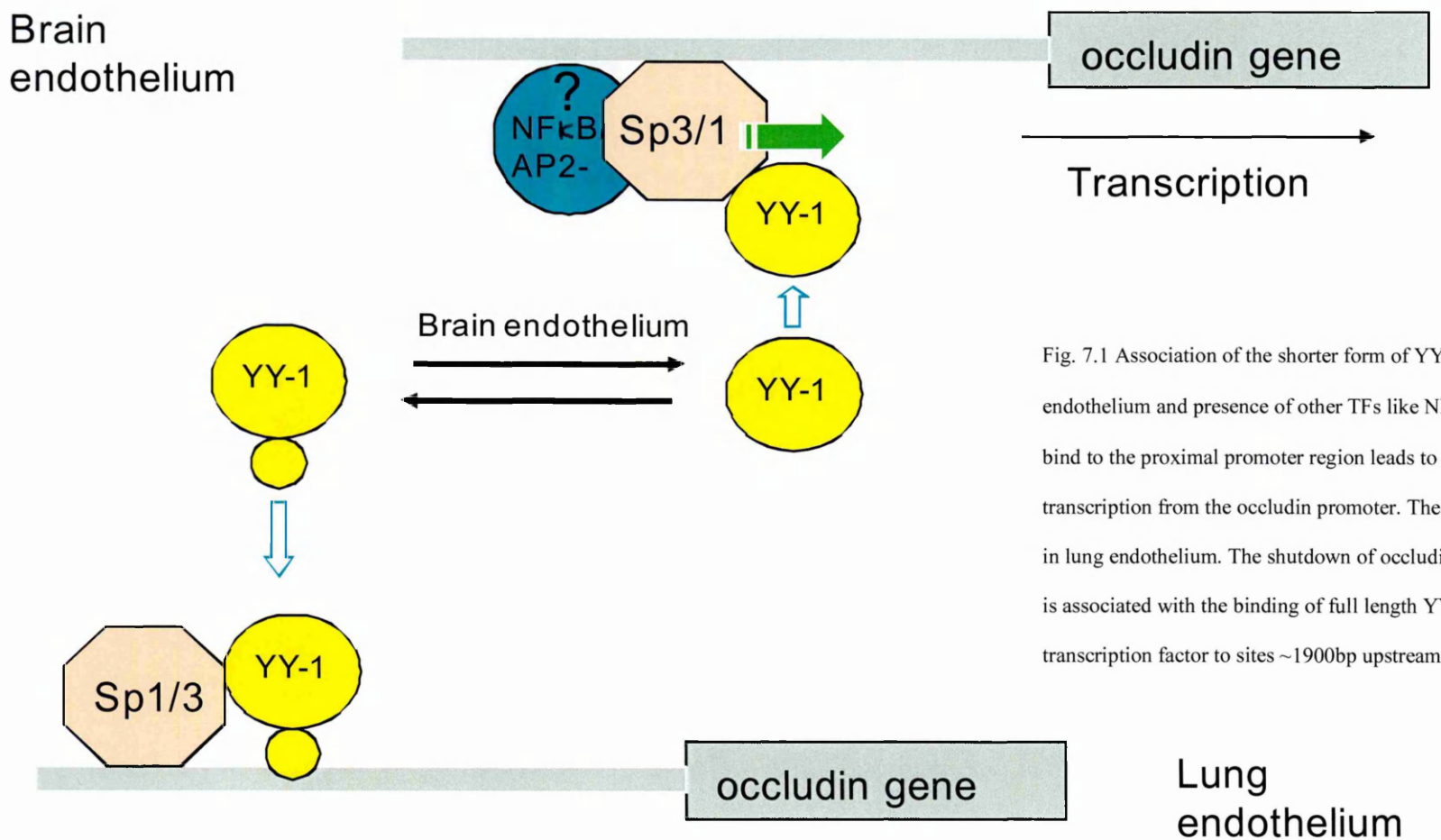


Fig. 7.1 Association of the shorter form of YY1 with Sp3/1 in brain endothelium and presence of other TFs like NFκB and AP2α that bind to the proximal promoter region leads to activation of transcription from the occludin promoter. These Sp-sites are inactive in lung endothelium. The shutdown of occludin in lung endothelium is associated with the binding of full length YY1 and another transcription factor to sites ~1900bp upstream of the transcription

7.1 Future Work

1. The data from reporter vector assays is not conclusive in the brain endothelium. We were not successful in identifying a minimal promoter region and in addition all the fragments exhibited fairly strong promoter abilities. Deletion analysis resulting in the differing lengths of the promoter from the proximal end of the promoter would probably be more useful and provide data as to the region of the promoter that is most indispensable for function.

2. Chromatin immunoprecipitations in healthy and pathological brain tissue where occludin expression is disrupted. If the decrease in expression of occludin is regulated at the transcription level rather than posttranslational mechanisms, this work would provide valuable data on possible disruption in TF networks.

3. Deletion or mutation of the upstream YY1 target motif (position 100 in F0.1) in the occludin promoter which is actively bound by the TF only in the lung endothelium. We hypothesise that YY1 interaction at this site is responsible for changing chromatin structure and impairing the accessibility of the Sp family to the target sites on the promoter region. Hence, deletion or mutation of this site should relieve the structural repression of YY1 and this occludin promoter construct in a reporter vector should be able to mediate GFP/luciferase expression.

4. Analysis of other gene promoters of other proteins similarly restricted in expression to the brain endothelium.

Chapter 8

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8 References

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9 Appendix

Table 9-1 TESS analysis of the human occludin promoter

TF	Beg	Sense	Length	Sequence
IRF-1, 2	9	N	6	AAGTGA
PRDI-BF1	9	N	10	AAGTGAAAGt
GR	16	R	6	AGAAGA
GR	24	N	6	GACACA
Sp1	32	N	5	GGGCA
GR	33	R	6	GGCACA
Sp1	34	R	5	GCACA
NF1	45	N	10	ATTWNNNATK
Pit-1	46	N	8	TTATCCAT
Pit-1a	48	R	10	ATtCATTCAT
Pit-1a	51	N	7	CATTCAT
SRF	56	R	5	ATATA
TFIID	57	N	6	TATAAA
TBP	57	N	7	TATAAAA
YY1	100	N	6	AAATGG
Sp1	121	R	11	GAGGGGAGgGG
Sp1	124	N	10	GGGgGAGGGG
YY1	124	N	6	GGGgGA
Sp1	125	N	10	GGgGAGGGGA
YY1	134	R	6	AAATGG
AP-2α	137	N	6	TGGGGA
Sp1	138	N	10	GGGGAGTGgC
p300	139	N	7	GGGAGTG
AP1	144	N	4	TGAC
N-Oct-3	149	R	7	ATTWATK
Sp1	155	N	5	GGGCA
AP2	156	R	8	SSSNKGGG
GR	171	R	9	TTGGGGGCG
N-Oct-3	180	R	9	ATTWNNATK
F2F	182	N	6	TAAAAT
YY1	184	R	6	AAATGG
Pit-1a	185	R	8	AATGGAAT
GR	210	N	6	TGTACA
GR	210	R	6	TGTACA
GATA-1	214	R	6	CAATCT

c-Myb	220	N	6	GGTGAG
c-Myb	239	N	6	ATTGAA
Sp1	252	R	5	TGCCC
F2F	270	R	6	ATTTTA
GR	283	N	6	TGAACT
YY1	284	N	5	GAACT
TBP	292	R	7	TATAAAA
TFIID	295	N	6	TATAAA
NF-ATp	302	R	8	TGGAAAAA
NF-ATc	303	N	6	GGAAAA
NF-ATp	303	R	6	GGAAAA
GR	328	N	6	AGAACA
GR	328	R	6	AGAACA
AP1	335	R	4	TGAC
YY1	338	N	11	CCWTNTTNNNW
c/EBP-β	345	R	10	ATTAcAAAAT
TFIID	346	R	5	TTATA
TFIID	347	N	6	TATAAA
F2F	349	N	6	TAAAAT
YY1	357	N	6	ANATGG
Pit-1a	364	R	7	GTGTTTA
c/EBP	384	N	7	ATTAGGA
c/EBP-α	385	N	9	TTAGGAAAT
Pit-1a	386	N	10	TAGGAAATcT
PEA3	387	N	6	AGGAAA
YY1	398	N	11	CCWTNTTNNNW
C/EBP-β	399	N	10	CTTGTTTAAT
Pit-1a	400	R	10	TTGaTTAATT
TBP	404	R	7	TTAATTA
SRF	405	N	8	TAWWWWTA
SRF	405	R	8	TAWWWWTA
TFIID	408	R	5	TTATA
TBP	409	N	6	TATAAA
TFIID	409	N	6	TATAAA
YY1	417	R	6	AAATGG
Pit-1a	418	R	8	AATGGAAT
H4TF-1	433	N	6	GATTTC
c/EBP-α	436	N	8	TTNNGTAA
c/EBP-α	436	R	9	TTNNGTAAT

AP1	446	N	4	TGAC
c-Myb	468	N	4	TAAC
AP1	473	N	4	TGAC
c-Ets-2	476	R	6	CTTCCC
AP-2-α	478	R	6	TCCCCA
AP-2	478	N	10	TCCCCAGTGg
YY1	481	N	6	CATTT
c-Myb	486	R	4	GTTA
SRF	496	N	5	CCAAT
TBP	498	R	7	AATTTAA
SRF	513	N	5	CCAAT
TFIID	514	N	6	TTCAAA
AP1	529	R	4	GTAC
YY1	531	N	6	CATTT
AP1	553	N	4	TGAC
YY1	562	N	6	CATTT
TBP	577	N	7	TTAAATT
N-Oct-3	580	N	7	MATWAAT
N-Oct-3	581	R	7	ATTWATK
TBP	582	R	7	TTAATTA
N-Oct-3	585	R	10	ATTWNNNATK
YY1	598	N	9	GCTGCCATC
N-Oct-3	606	N	10	MATNNNWAAT
SRF	622	R	8	TAWWWWTA
SRF	622	N	8	TAWWWWTA
TBP	622	R	10	TATATATATA
TBP	622	N	10	TATaTATATA
TFIID	624	R	6	TTTATA
TBP	624	R	8	TTTATATA
TFIID	626	N	6	TATATA
TFIID	626	R	6	TATATA
YY1	640	N	6	CATTT
GR	658	N	6	TGTGCC
Sp1	658	N	5	TGTGC
AP-1	683	N	7	AGAGTCA
AP-1	686	R	4	GTAC
c-Myb	696	N	4	TAAC
YY1	699	N	6	CCATTT
TBP	703	R	7	TTAATTA

TBP	704	N	7	TAATTAA
CACCC-binding factor	723	N	6	GGGTGG
Sp1	723	R	6	GGGTGG
GATA-1	723	R	7	GGGTGGG
Sp1	724	N	6	GGTGGG
CCAAT-binding factor	729	N	6	GATTGG
SRF	730	R	5	ATTGG
GATA-1	733	N	6	GGATAG
GATA-1	734	R	6	GATAGA
N-Oct-3	741	R	7	ATTWATK
AP1	756	N	4	GTAC
IL-6 RE-BP	761	R	6	TTCCAG
GATA-1	765	N	6	AGATTG
CCAAT-binding factor	766	N	6	GATTGG
SRF	767	R	5	ATTGG
PEA3	786	N	8	CAGGATGT
c-Myb	805	R	6	AAGTTC
c-Myb	814	R	6	TTCAAT
GATA-1	822	R	6	AGATAG
GATA-1	822	N	6	AGATAG
GATA-1	822	N	6	AGATAG
c-Myb	827	R	4	GTTA
NF-1	833	R	6	TGCCAA
GR	838	R	6	AGAACT
TFIID	843	N	5	TATAA
PEA3	853	R	8	ACATCCTG
c/EBP-β	868	R	10	ATTAcAAAAT
TBP	870	N	7	TAAAAAA
SRF	907	R	5	ATATA
c-Myb	918	R	4	GTTA
Sp1	923	R	5	GTGCA
NF-κB	933	N	10	gGAAATTTCC
NF-κB	933	R	10	gGAAATTTCC
YY1	976	N	6	CATTT
TBP	1008	R	7	TCTTAAA
TBP	1011	N	7	TAAAAAA
TBP	1034	R	7	TTTATTT
GR	1058	R	6	TGTTCT
GR	1058	N	6	TGTTCT

GR	1058	R	6	TGTTCT
GR	1058	N	6	TGTTCT
TBP	1061	R	7	TCTTAAA
SRF	1088	R	10	cCGTATAAGG
TFIID	1091	N	5	TATAA
TBP	1130	N	7	TTAAATT
AP2	1158	R	8	SSSNKGGG
CACCC-binding factor	1159	N	6	GGGTGG
Sp1	1159	R	6	GGGTGG
GATA-1	1159	R	7	GGGTGGG
Sp1	1160	N	6	GGTGGG
Sp1	1163	N	6	GGGCAG
AP-2-α	1176	R	7	CCCTGGG
CACCC-binding factor	1181	N	6	GGGTGG
Sp1	1181	R	6	GGGTGG
YY1	1195	N	6	CATTT
Pit-1a	1201	N	10	TATTATtCAC
Sp1	1206	N	5	TGCAC
c-Myb	1232	N	4	TAAC
GR	1245	R	6	AGTTCA
F2F	1256	R	6	ATTTTA
AP-3	1257	R	10	TTTaAACCCC
c-Myb	1260	N	4	TAAC
c-Myc	1279	N	7	TCTCTTA
CBF	1313	N	6	GCCAAT
CTF	1313	N	7	GCCAATG
SRF	1314	N	5	CCAAT
TFIID	1333	R	6	TTTGAA
C/EBPα	1338	N	9	AATTTTCCC
NF-ATp	1340	R	6	TTTTCC
NF-ATc	1340	N	6	TTTTCC
C/EBPβ	1342	R	7	TTYCCAG
AP-2	1342	N	10	TcCCCAGGAG
IL-6 RE-BP	1343	R	6	TCCCAG
c/EBP-α	1360	R	9	TGGAGCAAT
Pit-1a	1377	R	10	ATGCaTTTTT
NF-ATp	1382	N	8	TTTTTCCA
NF-ATc	1383	R	6	TTTTCC

c/EBP-β	1384	R	7	TTYCCAG
IL-6 RE-BP	1385	R	6	TTCCAG
AP-1	1398	N	7	TTAATCA
GATA-1	1399	R	6	TAATCA
IL-6 RE-BP	1409	N	6	CTGGAA
GR	1424	N	6	TGTCCT
NF-1	1442	N	6	CTTTCC
NF-ATp	1442	N	7	CTTTCCT
PEA3	1443	R	6	TTTCCT
GATA-1	1447	R	6	CTATCA
GATA-1	1447	N	6	CTATCA
NF-1	1475	N	6	CTTTCC
NFAT-1	1482	R	7	GAGGAAA
PEA3	1483	N	6	AGGAAA
NF-ATp	1483	R	8	AGGAAACA
Sp1	1491	N		AGGCG
GR	1496	R	5	CTCTG
Sp1	1550	R	10	gAGGCGGAGC
Sp1	1551	N	6	AGGCGG
CAC-binding protein	1568	R	6	CACCCC
AP1	1570	R	4	GTCA
AP2	1570	R	8	SSSNKGGG
Sp1	1572	N	10	tCGGGGTGGT
CAC-binding protein	1574	N	6	GGGGTG
CBF	1575	N	6	GGGTGG
Sp1	1575	R	6	GGGTGG
AP1	1580	R	4	GTCA
AP2	1590	N	8	CCCMNSSS
GR	1601	N	6	TGTCCC
GR	1601	R	6	TGTCCC
NF-ATp	1610	N	6	GGAGCC
AP-2	1614	N	7	CCCGCGC
Sp1	1614	R	10	tCCGCGCCTC
GR	1626	R	5	CTCTG
Sp1	1627	R	10	TCTcCGCCCC
Sp1	1632	N	10	GCCCCGCCcC
Sp1	1632	R	15	GCCCCGCCcC
c-Ets-2	1663	R	6	TTCCTT
c-Ets-2	1663	N	6	TTCCTT

c-Myb	1669	N	4	TAAC
Sp1	1675	N	5	TGCGC
CBF	1683	R	10	CAGGGTGgGG
c-Ets-2	1691	N	6	GGGAAG
Sp1	1709	N	10	CcCCCGCCCC
Sp1	1711	R	8	CCCGCC
Sp1	1711	N	9	CCCGCCCCC
EGR2	1712	N	10	CCGCCCCCgC
AP-1	1712	N	8	CCGCCCCC
Sp1	1714	N	11	GCCCCCTCCCc
SRF	1714	R	5	ATTGG
AP2	1716	N	8	CCCMNSSS
AP-1	1729	N	7	AGTTTCA
AP-1	1745	R	4	GTAC
GR	1764	N	6	GACACA
Sp1	1767	R	7	ACACCAC
NF-1	1767	R	7	ACACCAC
p300	1779	R	7	CACTCCC
Sp1	1790	R	15	CCACCNNNNCCaCCC
Sp1	1798	N	7	CCCTCCC
PEA3	1806	R	8	RCWTCCKS
EGR2	1816	R	10	GCGGgGGCGG
Sp1	1820	N	6	AGGCGG
NF-1	1837	N	6	AGCCAG
GR	1846	N	5	CAGAG