Transcriptional control of occludin expression in vascular endothelia: regulation by Sp3 and YY1

Journal Item

Transcriptional control of occludin expression in vascular endothelia: Regulation by Sp3 and YY1

Hadassah Sade, Karen Holloway, Ignacio A. Romero, David Male

PII: S1874-9399(09)00018-2
DOI: doi:10.1016/j.bbagrm.2009.01.006
Reference: BBAGRM 147

To appear in: *BBA - Gene Regulatory Mechanisms*

Received date: 1 May 2008
Revised date: 15 December 2008
Accepted date: 14 January 2009

Please cite this article as: Hadassah Sade, Karen Holloway, Ignacio A. Romero, David Male, Transcriptional control of occludin expression in vascular endothelia: Regulation by Sp3 and YY1, *BBA - Gene Regulatory Mechanisms* (2009), doi:10.1016/j.bbagrm.2009.01.006

Revision-2

Transcriptional control of occludin expression in vascular endothelia: Regulation by Sp3 and YY1.

Hadassah Sade, Karen Holloway, Ignacio A. Romero and David Male*.

Department of Life Sciences, The Open University, Milton Keynes, MK7 6AA, UK.

Running title: Transcription controls in endothelium

Keywords: Endothelium, occludin, tight junctions, differentiation, YY1, Sp3

Abbreviations:

BMEC, brain microvascular endothelial cells

ChIP, Chromatin immunoprecipitation

EMSA, Electrophoretic mobility shift assay

hCMEC/D3, human cerebral microvascular endothelial cell line – D3.

LMVEC, Lung microvascular endothelial cells.

TFR, transferrin receptor

*Address for correspondence:

Professor David K. Male,

Department of Life Sciences,

The Open University,

Milton Keynes, MK7 6AA,

UK.

Tel. +441908659226; Fax. +441908654167; email: D.K.Male@Open.ac.uk

Summary

Endothelium differentiates in response to tissue-specific signals; brain endothelium expresses tight junctions and transporters which are absent from other endothelia. The promoter of the tight junction protein occludin exhibited strong activity in a brain endothelial cell line, hCMEC/D3 but was inactive in lung endothelial cells. Expression of occludin in brain endothelium corresponded with binding of Sp3 to a minimal promoter segment close to the transcription-start site. However, in lung endothelium Sp-transcription factors did not bind to this site although they are present in the cell nucleus. In contrast, repression of occludin in lung endothelium was associated with the binding of YY1 to a remote site in the promoter region, which was functionally inactive in brain endothelium. The work identified a group of transcription factors including Sp3 and YY1, which differentially interact with the occludin promoter to induce expression of occludin in brain endothelium and repression in other endothelia. The mechanism controlling occludin expression is similar to that which controls tissue-specific expression of the transferrin receptor in brain endothelium, leading to a scheme for endothelial differentiation, in which activation or repression of tissue-specific proteins is maintained by a set of transcription factors which include Sp3 and YY1.

Introduction

The properties of vascular endothelium depend on their tissue of origin and position within the vascular tree. Microvascular endothelium in the CNS has continuous tight junctions which confer low permeability to ions and hydrophilic molecules [1]. It also expresses a number of specific transporters which allow selective uptake of nutrients, and it has members of the ATP-binding cassette (ABC) super family, which exclude many toxic molecules and therapeutic drugs from the CNS [2]. These features contribute to the blood brain barrier and are responsible for maintaining brain homeostasis and normal neuronal activity. Brain endothelium differentiates in response to cues from the CNS microenvironment – signals from both astrocytes and neurons have been implicated in the induction of the distinctive properties of these cells [3,4].

Since brain endothelium is induced to express a distinctive set of genes as it differentiates, we hypothesised that these genes could be subject to common transcriptional controls which would allow expression of the tissue-specific genes in brain endothelium and their repression in non-brain endothelium. The hypothesis predicted that transcription factors controlling such genes would be differentially active in brain versus non-brain endothelium. We therefore analysed transcription factor profiles in different endothelia, and found that YY1, TFIID, c-Myb, GATA-1 and Pit-1 vary in activity or expression between brain and non-brain endothelium. These studies identified a role for the Sp family, YY1 and TFIID in the regulation of the human transferrin receptor (TFR) promoter, leading to the proposal that Sp3 was required for TFR expression in brain endothelium, but that control of expression was modulated by YY1, acting either by direct interaction with Sp3 and/or by affecting chromatin organisation [5].

This paper investigates the transcriptional regulation of the tight junction protein occludin in brain and lung endothelium. Occludin was selected because it is characteristic of continuous tight junctions and is strongly expressed in barrier endothelia, including endothelium in the

CNS. It is detectable at low levels or is undetectable in other microvascular endothelia [5], and it has been detected in umbilical vein endothelium [6] which have discontinuous tight junctions [7]. It is strongly expressed in other cell types including epithelium which have continuous tight junctions [8]. Although progress has been made in identifying transcription factors required for the initial growth and differentiation of endothelium, much less is known about the factors that control its terminal differentiation and maintain the differentiated state [9,10].

Our previous work identified an important role for YY1 and the Sp-family of transcription factors in brain endothelium. Four members of the Sp-family have been identified in these cells, of which Sp3 and Sp1 are most abundant – brain endothelium expresses particularly high levels of Sp3 in comparison with lung and dermal endothelium. Sp1 is the prototype of a large family of transcription factors which bind GC-rich segments [11]. Sp1 itself is thought to be a constitutive factor that enhances the transcriptional initiation of numerous genes whereas Sp3 has been reported to act as an activator or repressor depending on the cell type and conditions.

Likewise, Yin Yang 1 (YY1) is a bifunctional protein capable of activating or repressing the transcription of many genes especially during cell growth and differentiation [12]. Moreover, YY1 can have a dual activity even on the same promoter, depending on the cell type or differentiation state [13]. Previous studies have shown that Sp3 and YY1 immuno-coprecipitate in cell extracts from human brain endothelium, suggesting that they could act in conjuction, to control expression of brain-endothelium specific proteins [5].

The aim of this study was to identify transcription factors that are required to drive occludin expression in human brain endothelium, and any factors that may be involved in gene repression in other endothelia. In view of the results on control of transferrin receptor we focussed on YY1, the Sp-family and other transcription factors, which have differential

expression in brain and non-brain endothelium. Other studies have identified two potential transcription start sites for occludin and a minimal promoter, which is active in epithelium and includes the downstream transcription start site [14,15]. In this study we examined a region of ~2000bp, upstream of this minimal promoter, which includes both transcription-start sites and remote evolutionarily conserved segments, of unknown significance.

To gain insight into how human endothelial cells differentiate in different tissues, we compared the activity of the occludin gene in a human brain endothelium cell line (hCMEC/D3) [16] and primary human brain endothelium (BMEC), with lung microvascular endothelial cells (LMVEC). By identifying common mechanisms controlling transcription of genes expressed in brain endothelium, we aim to understand the processes that induce tissue-specific endothelial phenotypes and the mechanisms that maintain them.

Materials and Methods

Cell cultures

The human brain microvascular endothelial cell line, hCMEC3/D3 [16], was grown on collagen-coated plates in EGM-2 MV medium supplemented with 2.5% foetal bovine serum, hydrocortisone, VEGF, epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), human fibroblast growth factor (FGF), ascorbic acid, gentamicin sulphate and amphotericin-B. Lung endothelium was purchased from Clonetics/Biowhittaker (Wokingham, UK) and grown in EGM-2 MV medium according to the manufacturer's recommendations. hCMEC/D3 cells were used at passages 21-30; lung endothelial cells were used at passages 3-7, as previously described [17]. The different endothelial cells were passaged when cultures reached 60-70% confluency using trypsin-EDTA (Invitrogen). Confluent monolayers were rested in EGM-2 MV medium without growth factors but with antibiotics, serum and hydrocortisone for 48 hours before assay, unless otherwise indicated.

Primary human brain endothelium (passage 0-1), was obtained from normal tissue donated by individuals undergoing temporal lobe resection for epilepsy, with informed consent. The method for isolation of primary cells corresponds to that used to isolate the brain endothelial cells which were used to generate the hCMEC3/D3 line.

Immunofluorescence

hCMEC/D3 cells were analysed for expression of tight junction components by immunofluorescence. Cells were washed in HBSS 3-times, detached from the flasks with trypsin/EDTA, fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilized 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 primary antibodies for occludin, claudin-5 or ZO-1 (Zymed) at 1/50. After washing, the cells were incubated with goat anti-rabbit IgG conjugated to FITC (Vector Labs, Burlingame, CA) for 1 hour at RT. Cells were washed 3 times, before resuspending in PBS and analysis by FACS.

Promoter-vectors, transfection and FACS analysis

The occludin promoter and subfragments were prepared by PCR, using primers corresponding to positions in the occludin gene indicated in table 1, and either a full-length template derived by nested-PCR from genomic DNA or from a vector kindly supplied by Dr J. Mankertz. The amplified segments were cloned into pGlowTOPO (Invitrogen) and the sequences of the gene segments and their correct orientation, upstream of the reporter GFP-reporter gene was checked before use in transfection assays.

Human endothelial cells were plated at 2-6 x 10^5 cells per well on 6 well plates in 2 ml of EGM2-MV medium without antibiotics but supplemented with serum and growth factors and cultured until 60% confluent. For each transfection, 5 µg of DNA was diluted in 250 µl of OptiMEM® medium and 10 µl of Lipofectamine™ 2000 was diluted in 250 µl OptiMEM® Medium. DNA-Lipofectamine™ 2000 complexes were produced according to the

manufacturer's protocol (Invitrogen) and 500 µl was added directly to each well and incubated at 37°C for 12 hrs (hCMEC/D3 cells) or for 4 hrs (LMVEC cells). The 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 48-72 hours before analysis for GFP expression by FACS analysis. Transfected cell monolayers were washed in HBSS without Ca^{++}/Mg^{++} and then detached with 0.25% Trypsin-EDTA at 37°C for 5 min. Cells were centrifuged at 300g for 5 min and resuspended in BSS without phenol-red and were then analysed immediately by FACS.

In these conditions transfection efficiency was 40-60%. Transfection of the endothelial cells caused an increase in granularity and a slight decrease in size, such that the transfected cell population could be clearly distinguished from non-transfected cells according to their side-scatter and forward-scatter on the FACS. In the transfection assays, the cells were gated according to their forward and side scatter to show fluorescence of the transfected cell population.

Chromatin Immunoprecipitation (ChIP) assays

The chromatin preparation was based on a kit produced by Active Motif. Cells were grown to confluence and rested in medium without growth factors for 48 hours prior to assay. 4.5×10^6 cells were used for one assay. DNA was cross-linked to nuclear proteins using 1% formaldehyde for 10 min at 37ºC and the reaction stopped by adding 10 ml glycine/PBS for 5 minutes. The cells were washed twice in PBS at 4ºC, containing protease inhibitors, (1mM PMSF, 1µg/ml aprotinin and 1µg/ml pepstatin A) and were then scraped into a tube and centrifuged for 4 min at 2000 rpm at 4ºC. The pellet was resuspended in 1 ml lysis buffer, on ice, to release nuclei and spun at 2500rpm in a microfuge for 10minutes at 4ºC .

The nucleic pellets were resuspended in 1ml digestion buffer with enzyme inhibitors, and the tube warmed at 37°C for 5 min. A working enzymatic shearing cocktail solution was prepared

by diluting 1:100 of the supplied mixture with 50% glycerol (in dH_2O) to make a final stock at 200 U/ml and 50 µl 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 vortexed periodically during the incubation to ensure the chromatin was evenly sheared. The reaction was then stopped by addition of 20 µl ice-cold EDTA (0.5M) and the tube chilled on ice for 10 minutes followed by centrifugation at 15,000 rpm at 4°C in a microfuge for 10 min. The supernatant (1ml) containing the sheared chromatin was used for four ChIP reactions.

The immunoprecipitation stages were based on a kit supplied by Upstate. Each 250µl sheared chromatin lysate was pre-cleared with 40µl of salmon sperm DNA/Protein A Agarose-50% slurry for 1hour at 4°C. The agarose was spun out and the supernatant collected and the immunoprecipitating antibody added; the sample was incubated overnight at 4ºC with rotation. Immunoprecipitation was carried out with 2µg per reaction antibody against Sp1, Sp3 or YY1 (Santa Cruz, Biotechnology). 60µl of Salmon Sperm DNA/Protein A Agarose slurry was then added and further incubated for 1 hour at 4ºC with rotation. The protein A-agarose /antibody/chromatin complex was pelleted and washed successively for 5 minutes on a rotating platform with 1ml of each of: low-salt buffer, high-salt buffer, LiCl complex wash buffer and Tris/EDTA. 250µl of freshly prepared elution buffer (1%SDS, 0.1M $NaHCO_3$) was added to the pelleted protein A agarose complex , vortexed briefly to mix and incubated at room temperature for 15 minutes with rotation. The tubes were centrifuged at 1000rpm for 1 min at room temperatature and the supernatant fraction collected. The elution step was repeated and the eluates were combined (total volume = 500µl).

The protein-DNA cross-links were reversed, by adding 20µl of 5M NaCl to the combined eluates and heating at 65ºC for 4 hours. Then, 10µl of 0.5M EDTA, 20µl 1M Tris-HCl, pH 6.5 and 2µl of 10mg/ml Proteinase K was added to the combined eluates and incubated for one hour at 45ºC. After addition of 3 µl glycogen (2 µg/µl), DNA was recovered by phenol/chloroform extraction and ethanol precipitation and redissolved in Tris/EDTA, pH 8.4

for use in PCR analyses. PCR for ChIP assays used primers to amplify segments indicated in table-1, a Tm of 55ºC, an extension time of 60s and 35 cycles of amplification. Amplified segments were analysed using 2% agarose gels in TAE buffer.

Electrophoretic mobility shift assays (EMSAs)

Nuclear protein extracts were isolated from cells as previously described [18]. EMSA probes, corresponding to occludin promoter fragments (table 1) of <200bp, were prepared by PCR, as outlined above. The fragments were run on 2% agarose gels and fragments migrating at the appropriate size were excised from the gel, extracted and purified (SpinPrep Gel DNA kit, Novagen). The concentration of the DNA was maintained at 1µg/µl for use in EMSA. Additionally, the fragments were ligated into pCR 2.1-TOPO cloning vector (Invitrogen, Paisley, UK). Colonies obtained after transformation of TOP10F` E Coli cells were grown in LB medium with ampicillin and the isolated vectors (Wizard Plus SV Mini-Prep, Promega) were 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. All sequences were verified by automated sequencing.

DNA fragments (1µg) were end-labelled with [γ-^{32}P] (Amersham Biosciences) using T4 polynucleotide kinase (Promega) for 30 min at 37°C and excess unincorporated [γ-^{32}P] was removed using a ProbeQuant G50 microcolumn (Amersham Biosciences). 5µg of nuclear extract from hCMEC/D3 or LMVECs was pre-incubated in 20 mM HEPES, pH 7.5, 4% ficoll, 1µg of polydI-dC (Sigma), 0.1mM $MgCl_2$, 0.1 mM DTT, for 20 min at room temperature on a shaker. For super shift and cold inhibitor assays, 2µl of antibody (200µg/0.1ml) or ds DNA oligonuculeotide (35pMol/µl) were added respectively after this incubation and further incubated for 30 min at 4°C with gentle rocking. Following this incubation, 1µl of the labelled fragment(s) was added to each tube and further incubated at room temperature for 30 min. After addition of loading dye, 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. Gels were removed from plates and were dried on Whatman no 1 filter paper at 70°C under vacuum for 45 min and exposed to Kodak X-Omat film (Amersham) at -80°C.

In experiments where cold-blocking was carried out the the following consensus double-stranded oligonucleotides were used:

AP1, 5' CGC TTG ATG ACT CAG CCG GAA 3';

c/EBP, 5' TGC AGA TTG CGC AAT CTG CA 3';

TFIID, 5' GCA GAG CAT ATA AAA TGA GGT AGG A 3';

YY1, 5' CGC TCC CCG GCC ATC TTG GCG GCT GGT 3';

YY1 mutant, 5' CGC TCC GCG ATT ATC TTG GCG GCT GGT 3'

Sp, 5' ATT CGA TCG GGG CGG GGC GAG C 3';

Sp mutant, 5' ATT CGA TCG GTT CGG GGC GAG C 3';

c-Myb, 5' TAC AGG CAT AAC GGT TCC GTA GTG A 3';

GATA, 5' CAC TTG ATA ACA GAA AGT GAT AAC TCT 3';

NF1, 5' TTT TGG ATT GAA GCC AAT ATG ATA A 3';

NFκB, 5' AGT TGA GGG GAC TTT CCC AGG C 3';

Pit1, 5' TGT CTT CCT GAA TAT GAA TAA GAA ATA 3'.

Supershift, rabbit antibodies against individual transcription factors (X-version) were obtained from Santa Cruz Biotechnology.

Results

Analysis of the human occludin promoter

The occludin promoter has been characterized previously and shown to be active in epithelial cells [15]. This 1853 bp sequence was analysed for potential transcription factor binding sites using TESS®. The positions of potential binding sites for Sp transcription factors and YY1

are shown diagrammatically in figure 1. A cluster of Sp sites is located close to the two reported transcription start sites, while potential YY1 sites are predominantly at 1200-1800 bp upstream.

Activity of the occludin promoter in brain and non brain endothelium

Primers designed according to the published sequence (GenBank accession no: **AF246304**) were used to amplify the full length occludin promoter and sub-fragments, which were cloned into the reporter vector pGlow-TOPO. The CMV promoter cloned similarly served as a control for transfection efficiency and empty vector was used as a negative control. These constructs were transiently transfected into hCMEC/D3 and LMVEC cells. Figure 2 shows expression of GFP driven by the full length occludin promoter in hCMEC/D3 cells. In contrast, GFP expression in transfected lung endothelial cells was similar to the negative control. Transfection efficiency was similar in the two lines, as indicated by expression from the CMV-promoter-reporter. Combined results from 5 separate experiments are shown in supplementary figure-1. These results indicate that differential activity of regulatory elements control expression from the occludin promoter in brain and lung endothelium.

Identification of differential transcription factor binding to the occludin promoter

For use in EMSAs, we generated 15 individual overlapping promoter segments of <200bp (Table 1, Fig. 1). Eight of the probes have potential target sites for Sp-family transcription factors. To determine which regions of the occludin promoter may interact with DNA-binding proteins in different endothelia, all 15 probes were analysed by EMSA using nuclear extracts from hCMEC/D3 and LMVEC. If a band was supershifted using antibody to a specific transcription factor, this was taken as evidence that the named transcription factor was bound to that promoter segment. If a band was removed using a cold oligonucleotide inhibitor, it implies that a transcription factor binds to the target site in the probe, but it does not necessarily identify the transcription factor. The antibodies and oligonucleotides used to investigate each region were selected according to the data from the TESS analysis, of

potential target sites present in each promoter segment. The full data from the EMSAs of hCMEC/D3 is summarised in table 1, right hand column.

Data showing the specificity of the bands produced in the EMSAs is shown in supplementary figure-2. All of the bands described in figures 3-5 (below) are specific, with the exception of band e' produced by lung endothelial nuclear lysate, binding to probe 8.

Two of the probes showed high affinity binding to nuclear proteins and distinctly different binding patterns for brain and lung endothelium. Probe 0.1 contains both an Sp-site and YY1 sites; it produced two complexes with hCMEC/D3, but four with LMVEC (Fig. 3). One of the LMVEC complexes (d') was identified as YY1, since it was supershifted with anti-YY1 antibody (lane 7). The other complex was due to a transcription factor binding to the TFIID-site in the probe (lane 6), however the factor is not TFIID itself, since anti-TFIID antibody did not supershift the band (lane 5). The data indicate that the off-state of the occludin promoter in lung endothelium is associated with the binding of two transcription factorss to the F0.1 region of the promoter, one of which is YY1.

Probe 8 showed distinctive nuclear protein binding in brain and lung endothelium. It produced four complexes with hCMEC/D3 nuclear proteins, but only one band with LMVECs (Fig. 4). The F8 region contains two Sp response elements in addition to a GR and a NF1 site and it includes the transcription start site identified by Mankertz and colleagues [15]. All the complexes were blocked in the presence of either an unlabelled Sp-oligonucleotide (lane 3) or anti-Sp3 (lane 5). DNA binding is also inhibited by 100-fold molar excess of unlabelled NFκB-oligonucleotide (lane 9), which blocks the same complexes as does the Sp-oligo. The effect is specific for NFκB, since it does not occur with other cold oligonucleotides. However, probe 8 does not contain any NFκB target sites, hence the cold inhibitor appears to be acting either by sequestering Sp3 as part of an Sp3/NFκB complex as

indicated previously in epithelial cells [19], or by directly inhibiting Sp3 binding to the labelled probe.

Probe 8 also interacted with nuclear extracts from LMVECs to form a single DNA-protein complex which was not supershifted by antibodies to Sp1 (Fig. 4, lane 3) or Sp3 (lane 4). The complexes formed by brain and non-brain endothelium are totally distinct, implying that lung endothelium does not form an Sp3 complex with region F8, even though Sp3 is present in these cells, albeit at a lower level than in brain endothelium [5]. Furthermore, this complex is blocked in the presence of all of the unlabelled cold competitors (Fig 4, lanes 5-8), implying that the nuclear protein DNA interaction is not sequence-specific. These data imply that one or both of the Sp-sites in F8 bind to Sp3 from brain endothelium, but that these sites do not bind Sp-factors from lung endothelium. In contrast, a single non-specific DNA-binding protein from lung endothelium can occupy this region. It suggests that the on-state of occludin in brain-endothelium is associated with Sp3 binding to region F8 of the promoter.

Identification of other active Sp and YY1 sites in the occludin promoter

Probe 5 has potential binding sites for both YY1 and Sp-transcripition factors; it also showed differential binding patterns for brain and lung endothelium in EMSA and one of the bands in hCMEC/D3 was blocked by a YY1-oligonucleotide and supershifted with YY1 antibody (data not shown), indicating that YY1 can potentially bind to the occludin promoter, but it targets the F5 region rather than F0.1.

Probe 3.1 showed three distinct DNA-protein complexes in the presence of nuclear proteins from hCMEC/D3 cells (Fig. 5). This probe contains Sp-sites, and all of the complexes were competed out by the addition of 100-fold molar excess of unlabelled Sp-oligonucleotide (lane 8). Antibody supershift assays indicated that complexes (a) and (c) were due to Sp3 (lane 6) while complex (b) was due to Sp1 (lane 5). The mobility of these complexes was also affected by antibody to c-Myb (lane 7) implying that the complexes contain both Sp3 and c-

Myb. However, an unlabelled c-Myb-oligo did not block the formation of these complexes (lane 10). This suggests that c-Myb can bind indirectly to F3.1 by interacting with another DNA-binding protein, possibly Sp3. Unlabelled YY1-oligo produced a marginal inhibition affecting all bands equally (lane 9) suggesting some non-specific interference with complex formation. Probe 3.1 did not produce any DNA-protein complexes with nuclear proteins from LMVECs.

Probe 6.1 contains one YY1-site and it interacted with the nuclear proteins from the hCMEC/D3 cells to generate six DNA-protein complexes (Fig. 5, right, lane 2). The YY1-oligonucleotide blocked formation of all complexes (lane 8) but a mutant form of the inhibitor did not affect the complexes confirming the sequence specificity of this interaction (lane 9). However antibody to YY1 only shifted one of the complexes (lane 3, complex (h)). Binding of LMVEC proteins to probe 6.1 was weak and of low specificity (data not shown) These data imply that YY1 from brain endothelium may occupy the single YY1 target site in F6.1 and that the other complexes are produced by other transcription factors bound directly or indirectly to the YY1 target region.

In addition to the Sp and YY1 sites discussed, several regions (F2.1, F2.2, F3.2, F4.1, F4.2, F7.2) also have potential sites for the differentially-expressed transcription factors, Pit-1, GATA-1 and c-Myb. Although the promoter probes 2.1 and 2.2 also interacted with nuclear extracts from hCMEC/D3 to form DNA-protein complexes, they did not correspond to any of these transcription factors when analysed by antibody supershift and/or cold-oligonucleotide blocking experiments (data not shown).

Taken together, these results show that Sp transcription factors from brain endothelium can bind to sites in F3.1, in addition to their principal targets in F8. Although YY1 from brain endothelium does not bind to the site in F0.1, it does bind to a single site in the overlap region of F5 and F6.1. The binding specificity of YY1 from brain endothelium on the occludin

promoter is therefore diametrically opposite to that observed using lung endothelium, where YY1 binds in F0.1 but does not bind to F5.

Interaction of Sp family and YY1 with the endogenous promoter

We investigated whether Sp3, Sp1 and YY1 bind to endogenous human occludin promoter in intact cells by performing chromatin immunoprecipitation assays. Sheared chromatin from LMVECs, hCMEC/D3 and primary BMECs was immunoprecipitated with antibodies to Sp3 (Fig. 6) or YY1 (Fig. 7). Normal rabbit IgG was used as a control. DNA recovered from these immunoprecipitation reactions was amplified using primers for promoter segments, in order to detect whether the antibodies had precipitated the occludin promoter. Note that this technique cannot precisely identify the region in which the transcription factors bind – the sheared chromatin is mostly <1kb, so positive results with a primer-pair indicate that the transcription factor is bound within 1kb of the PCR-amplified segment. Figure 6 shows that Sp3-antibody precipitated the occludin promoter from primary BMECs and the hCMEC/D3 cell line, but not from LMVECs; the PCR reaction was positive with three primer-pairs from the primary BMECs and four pairs from hCMEC/D3. The difference may reflect a genuine variation in the level of Sp3 associated with the occludin promoter in the primary cells and the cell line, but could equally be due to small variations in the efficicency of the chromatin shearing in the two cell types. The results confirm that Sp3 is associated with the occludin promoter in brain endothelium, but not in lung endothelium.

Figure 7 shows that YY1-antibody precipitated the occludin promoter from both LMVECs and BMECs, but implies that YY1 is located at different positions on the promoter in the two cell types. The PCR reaction with BMECs was only positive with primers spanning segments 6.1 – 6.2, indicating that YY1 is located in the downstream region of the promoter in brain endothelium. In contrast, using chromatin from LMVECs the PCR reactions were positive with primer pairs from both upstream and downstream regions of the promoter. There was

some variation in the efficiency of detection in different LMVEC preparations (Fig 7) but primers spanning regions 0.1 – 2.1 were always positive, indicating that YY1 is located in or near this region.

ChIP assays also detected Sp1-associated with the occludin promoter in primary BMEC and hCMEC/D3 cells, but not LMVEC (data not shown). Taken together these data confirm the in vitro EMSA analyses and strongly indicate the differential binding of YY1 and Sp family transcription factors to the occludin promoter in brain and non-brain endothelium.

Requirement for Sp-factors for expression of occludin in brain endothelium

The antibiotic mithramycin blocks the interaction between Sp-transcription factors and their GC-rich target sites [20]. hCMEC/D3 cells were treated with 100 or 200nM mithramycin for 48 hours and were then analysed for occludin expression by immunofluorescence staining and FACS (Fig. 8). Cells treated with the antibiotic showed a decrease in occludin expression. However, expression of other tight junctions proteins claudin-5 and ZO-1 was not affected. To verify if the drug is indeed blocking the interaction of Sp family members with DNA response elements, cells cultured under similar conditions were subjected to EMSA (Fig. 9). In this case 200nM mithramycin blocked the interaction of nuclear protein(s) with the promoter fragment. The action of mithramycin was confirmed in a ChIP assay, in which Sp3 antibody failed to immunoprecipitate the endogenous occludin promoter from cells treated with 100nm or 200nm mithramycin. Finally we found that deletion of Sp-sites in a minimal promoter spanning F7.1, F7.2 and F8 confirmed the requirement for the downstream Sp-site (Sp-a) for full promoter activity (Fig. 10). The combined data from five separate experiments are shown in supplementary figure-3, which indicates that the Sp-a site is of primary importance, whereas deletion of the Sp-b site produces only a marginal reduction in promoter activity in hCMEC/D3 cells. These data confirm that occludin expression by human brain endothelium requires activation of the promoter by Sp-factors, primarily Sp3, acting on a target site close to the previously-reported downstream transcription start site.

Discussion

The broad aim of this work was to identify the mechanisms that allow the expression of specific proteins in brain endothelium, and their repression in non-brain endothelium. This paper focusses on occludin, a protein which is characteristic of the tight junctions in brain endothelium, but which is undetectable in microvascular endothelium from lung or dermis [5]. The results show that the occludin gene is differentially active in brain endothelium and non-brain endothelium and that gene transcription requires binding of Sp-family transcription factors, primarily Sp3, to a single target site close to the 3'-end of the promoter, a position previously identified as a transcription start site in epithelium. In lung endothelium neither Sp1, nor Sp3 occupy this site, even though both transcription factors are present in the cells.

The transcription factors Sp1 and Sp3 are expressed in many different cell types, including different endothelial subtypes and they regulate transcription of a large number of genes. Sp1 is a transcriptional activator, both of widely-expressed and tissue-specific genes [21], whereas Sp3 has been implicated in gene activation or repression, depending on the gene and cell type under investigation. Sp1 appears to have a broader range of activity than Sp3. For example, Sp1 interacts with Ets and GATA as part of a complex which activates genes (eg ICAM-2), which are expressed by most endothelia [22]. Sp3 is reported to occur in three isoforms; the long form acts as an activator whereas the two short isoforms still bind to the Sp-target site, but repress transcriptional activation by Sp1 or full-length Sp3 [23]. We have detected both long and short isoforms of Sp3 in different endothelia. Moreover the long-form predominates in brain endothelial nucleii, whereas the short forms predominate in lung and dermal endothelium, which could partly explain the differential activity of Sp3 in lung and brain endothelium. Clearly, transcription of the occludin gene in brain endothelium requires Sp3, however because Sp3 is present in most cell types, other transcription factors must be involved in switching on occludin expression in brain endothelium and repressing it in others.

We have previously shown that Sp3 interacts with YY1 in brain endothelium and have presented data supporting its role in transcription of the transferrin receptor [5]. The findings with occludin and the transferrin receptor accord with the observations in other systems, that Sp3 activates a more limited range of proteins than Sp1, including genes that are expressed in a tissue-specific pattern.

The differential binding of YY1 to the occludin-promoter F0.1 region, suggests that it could be an important element in the developmental regulation of occludin expression. YY1 is a multi-functional, zinc finger transcription factor, that regulates transcription of many cellular genes, acting as either a repressor or activator. Many promoters contain YY1 binding sequences and YY1 is known to regulate the cell cycle and differentiation both in differentiated cells [24] and during embryonic development [25]. YY1 is subject to complex regulatory mechanisms in different cell types which affect its functional activity [26]. The promoter sequences surrounding YY1 binding sites [27], its relative concentration or post translational modifications [28], can determine whether YY1 acts as a repressor or activator. In addition, since the binding motif of YY1 is present in a large number of genes, the regulation conferred by YY1 is probably modulated by association with other cell-type specific proteins.

A variant form of YY1 is present in brain endothelium, which lacks the four C-terminal DNA-binding domains [5]; ie the 2nd and 3rd zinc fingers which are necessary for nuclear localisation of YY1 [29]. Brain endothelial YY1 interacts with Sp3 and is involved in the differential regulation of the transferrin receptor in brain and non-brain endothelium [5]. The observation that YY1 from lung endothelium binds to the F0.1 region of the occludin promoter, whereas YY1 present in brain nuclear protein does not bind F0.1, but can bind to F5, indicates that YY1 is also involved in the differential expression of occludin.

There are several proposed mechanisms for YY1 mediated repression of transcription. For example, YY1 can directly displace a transcriptional activator by binding to an overlapping DNA segment [30], but the YY1 sites in the occludin promoter are remote from the active Sp-site, which precludes this mechanism of control. Another mechanism proposes direct interaction with transcription factors, including Sp1 [31]. More recent work suggests that YY1 can recruit chromatin-modifying enzymes, so that it could regulate both short-term changes in gene-expression and long-term changes in cell phenotype.

Much recent research has emphasized the importance of epigenetic modifications of DNA/chromatin, particularly CpG methylation and histone H3 acetylation in long-term gene regulation [32] which can prevent access of Sp1/Sp3 to individual genes [33]. Chromatin-level regulation of occludin expression in endothelium could account for the relatively stable phenotypes seen in the different endothelial cell lines, over time. In this context, YY1 can recruit the H4-specific methyl transferase, PRMT1, which acts on H4R3, promoting gene activation [34]. It does so, by permitting a number of histone modifications which maintain active chromatin. YY1 can also recruit the histone deacetylases HDAC-2 and HDAC-1 in humans, which promote gene repression. Hence YY1 is critical as part of the complex which switches chromatin between an active or repressed state, but YY1 has also been shown to provide short-term control over gene activation eg, during the cell cycle [35]. Our ChIP data indicate that YY1 has a long-term role in controlling occludin expression. Additionally, the finding that the occludin promoter-reporter vector was inactive in lung endothelium, implies that differential short-term transcription control mechanisms are in place to prevent occludin promoter transcription in these cells (reporter vectors are unlikely to be subject to chromatin-level regulatory mechanisms). This finding was reinforced by the EMSA data, which showed that the transcription factors present in brain endothelium, promote binding of YY1 to the F5 / F6.1 region, whereas the transcription factors present in lung endothelium promote YY1 interaction with F0.1. These data imply that YY1 is involved in both short-term transcription control of the occludin promoter as well as epigenetic regulation.

We considered the possibility that the transcription factor Snail might repress the occludin promoter in lung endothelium. Snail has been reported to repress occludin expression during the epithelial-mesenchyme transition in development and potentially in tumour development [36]. It acts by binding to an E-box, which is located in the overlap region between F7.2 and F8 (figure 1). However no proteins from lung endothelial lysates bound to fragment F7.2 in EMSA, so this observation excludes the possibility that the DNA-binding protein in lung endothelium is Snail, hence this mechanism cannot account for the repression of occludin in lung endothelium.

Recent findings have shown that the transcription-start site, and hence the functional promoter, for a particular protein often differ between cell types. Previous studies on occludin have focussed on its control in epithelium or fibroblasts, although fibroblasts do not normally express occludin. Our findings indicate that the transcription-start in brain endothelium is likely to be close to that previously identified in epithelium.

Tight junctions are found in many types of epithelium as well as brain endothelium. One can infer that the occludin promoter must have elements which can be activated by transcription factors in all of these cell types. In epithelium, association of occludin with the structural transcription-regulator ZO-1, is one potential route for controlling occludin expression [8]. However tight junctions in epithelium and endothelium can be differentiated according to which of two ZO-1 isoforms they express [35], which suggests that any transcriptional control by ZO-1 could differ in epithelium and endothelium. A variety of short-term transcription controls have been demonstrated for epithelial occludin, including short-term modulation via the Ras pathway [38]. Although we cannot identify how occludin transcription is initially turned on during differentiation of brain endothelium, there are good reasons, to think that it is different from the transcriptional controls in epithelium.

This work indicates that both occludin and the transferrin receptor require Sp3 for activation, and both genes use YY1 as part of their control mechanism. Other genes are currently under investigation. These findings support the view that differential binding of YY1, is a general mechanism that can control gene switching, during endothelial cell differentiation and in maintaining the differentiated state of the cell.

Acknowledgements

This work was partly supported by an overseas research student award from the UK government and the Leverhulme Research Trust (Grant F/00269/D). We are very greatful to Mr Richard Selway, consultant neurosurgeon at KCH, London, who provided tissue for use in this study.

References

[1] H. Wolburg, A. Lippoldt, Tight junctions of the blood-brain barrier: development, composition and regulation, Vasc. Pharm. 38 (2002) 323-337.

[2] D.J. Begley, ABC transporters and the blood-brain barrier, Curr. Pharm. Res. 10 (2004) 1295-1312.

[3] R.C. Janzer, M.C. Raff, Astrocytes induce blood-brain barrier properties in endothelial cells, Nature 325 (1987) 253-257.

[4] W. Risau, R. Hallmann, U. Albrecht, S. Henke-Fahle, Brain induces the expression of an early cell surface marker for blood-brain barrier specific endothelium, EMBO J. 5 (1986) 3179-3183.

[5] K. Holloway, H. Sade, I. Romero, D.K. Male, Action of transcription factors in the control of transferrin receptor expression in human brain endothelium, J. Mol. Biol. 365 (2007) 1271-1284.

[6] M. Wachtel, K. Frei, E. Ehler, A. Fontana, K. Winterhalter, S.M. Gloor, Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition, J. Cell Sci. 112 (1999) 4347-4366.

[7] A.R. Burns, R.A. Bowden, S. MacDonell, D.C.Walker, T. Odebunmi, E.M. Donnachie, S. Simon, M.L. Entman, C. Wayne Smith, Analysis of tight junctions during neutrophil transendothelial migration. J. Cell Sci. 113 (2000) 45-57.

[8] E.E. Schneeberger, R.D. Lynch, The tight junction: a multifunctional complex, Am. J. Cell Physiol. 286 (2004) C1213 – C1228.

[9] W. Risau, S. Esser, B. Engelhardt, Differentiation of blood-brain endothelial cells, Pathol. Biol. 46 (1998) 171-175.

[10] P. Oettgen, Transcriptional regulation of vascular development, Circulation Res. 89 (2001) 380-388

[11] G. Suske, G. The Sp-family of transcription factors, Gene 238 (1999) 291-300.

[12] M. Austen, B. Luscher, J.M. Luscher-Firzlaff, Characterization of the transcriptional regulator YY1, J. Biol. Chem. 272 (1997) 1709-1717.

[13] M.J. Thomas, E. Seto, Unlocking the mechanisms of transcription factor YY1: Are chromatin modifying enzymes the key? Gene 236 (1999) 197-208.

[14] C.M. Van Itallie, J.M. Anderson, Occludin confers adhesiveness when expressed in fibroblasts, J. Cell Sci. 110 (1997) 1113-1121.

[15] J.Mankertz, S. Tavalali, H. Schmitz, A. Mankertz, E.O. Riecken, M. Fromm, J.D. Schulzke, Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma, J. Cell Sci. 113 (2000) 2085-2090.

[16] B.B. Weksler, E.A. Subileau, N. Perriere, P. Charneau, K. Holloway, M. Leveque, H.Tricoire-Leignel, A. Nicotra, S.Bourdoulous, P. Turowski, D.K. Male, F. Roux, J. Greenwood, I.A.Romero, P.O. Couraud, P.O. Blood brain barrier specific properties of a human adult brain endothelial cell line, Faseb J. 19 (2005) 1872-1874.

[17] P. Hillyer, E. Mordelet, G. Flynn, D. Male, Chemokines, chemokine receptors and adhesion molecules on different human endothelia, Clin Exp. Immunol. 134 (2003) 431-441.

[18] F.J. Staal, M. Roederer, L.A. Herzenberg, L.A. Herzenberg, Intracellular thiols regulate activation of nuclear factor-κB and transcription of human immunodeficiency virus, Proc. Natl. Acad. Sci. 87 (1990) 9943-9947.

[19] N-L. Zhu, C. Li, H.H. Huang, M. Sebald, V.A. Londhe, N. Heisterkamp, D. Warburton, S. Ballusci, P. Minoo, TNF-alpha represses transcription of bone morphogenetic protein-4 in lung epithelial cells, Gene 393 (2007) 70-80.

[20] S. W. Blume, R.C. Snyder, R. Ray, S, Thomas, C.A. Koller, D.M.Miller, Mithramycin inhibits Sp1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo, J. Clin. Invest. 88 (1999) 1613–1621.

[21] O.G. Opitz, A.K. Rustgi, Interaction between Sp1 and cell cycle regulatory proteins is important in transactivation of a differentiation-related gene, Cancer Res. 60 (2000) 2825-2830.

[22] P.J. Cowan, D. Tsang, C.M. Pedic, L.R. Abbott, T.A. Shinkel, A.J. d'Apice, M.J. Pearse, The human ICAM-2 promoter is endothelial cell-specific in vitro and in vivo and contains critical Sp1 and GATA binding sites, J.Biol.Chem. 273 (1998) 11737-11744.

[23] S.B. Kennett, A.J. Udvadia, J.M. Horowitz, Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription, Nucleic Acids Res. 25 (1997) 3110-3117.

[24] C.Y. Chen, R.J. Schwartz, Competition between negative acting YY1 versus positive acting serum response factor and tinman homologue Nkx-2,5 regulates cardiac α-actin promoter activity. Mol. Endocrinol. 11 (1997) 812-22.

[25] M.E. Donohoe, X. Zhang, L. McGinnis, J. Biggers, E. Li, Y. Shi, Targeted disruption of mouse Yin Yang 1 transcription factor results in peri-implantation lethality, Mol. Cell. Biol. 19 (1999) 7237-7244.

[26] C. Wang, Y. Liang, Y. Lin, H. Shih, Y. Jou, W.C.Y. Yu, YY1AP, a novel co-activator of YY1, J. Biol.Chem. 279 (2004) 17750-17755.

[27] A. Shrivastava, K. Calame, An analysis of genes regulated by the multi-functional transcriptional regulator Yin Yang-1, Nucleic Acids Res. 22 (1994) 5151-5155.

[28] Y.L. Yao, W.M. Yang, E. Seto, Regulation of transcription factor YY1 by acetylation and deacetylation, Mol. Cell. Biol. 21 (2001) 5979-5991.

[29] S. McNeil, B. Guo, J.L. Stein, J.B. Lian, S. Bushmeyer, B. Seto, M.L. Atchison . S. Penman, A.J. Wijnen, G.S. Stein, G.S. Targetting of the transcription factor YY1 to the nucleolus and the nuclear matrix in situ: The C-terminus is a principal determinant for nuclear trafficking, J. Cell Biochem. 65 (1998) 500-510.

[30] J. Ye, X. Zhang, Z. Dong, Characterization of the human granulocyte-macrophage colony-stimulating factor gene promoter: an AP1 and an Sp1–related complex transactivate the promoter activity that is suppressed by a YY1 complex, Mol. Cell Biol. 16 (1996) 157-167.

[31] E. Seto, B. Lewis, T. Shenk, Interaction between Sp1 and YY1, Nature 365 (1993) 462-464.

[32] B. Rao, Y. Shibata, B.D. Strahl, J.D. Lieb, Dimethylation of histone H3 at lysine 36 demarcates regulatory and non-regulatory chromatin, genome-wide, Mol. Cell Biol. 25 (2005) 9447-9459.

[33] H. Honda, M.J. Pazin, H. Ji, R.P. Wernyj, P.J. Morin, P.J. Crucial roles of Sp1 and epigenetic modifications in the regulation of the *CLDN4* promoter in ovarian cancer cells, J. Biol. Chem. 281 (2006) 21433-21444.

[34] N. Rezai-Zadeh, X. Zhang, F. Namour, G. Fejer, Y-D. Wen, Y-L.Yao, I. Gyory, K. Wright, E. Seto, Targeted recruitment of a histone H4-specific methyltransferase by the transcription factor YY1, Genes and Development 17 (2003) 1019-1029.

[35] L. Palko, H.W. Bass, M.J. Beyrouthy, M.M. Hurt, The yin-yang (YY1) protein undergoes a DNA-associated replication switch in localisation from the nucleus to the cytoplasm at the onset of S phase, J. Cell Sci. 117 (2003) 465-476.

[36] J. Ikenouchi, M. Matsuda, M. Furuse, S. Tsukita, Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail, J. Cell Sci. 116 (2003) 1959-1967.

[37] M.S. Balda, J.M. Anderson, Two classes of tight junctions are revealed by ZO-1 isoforms, Am. J. Physiol. Cell Physiol. 264 (1993) C918-C924.

[38] D. Li, R.J. Mrsny, Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin, J. Cell Biol. 148 (2000) 791-800.

Legends

Figure 1.

Diagram of the occludin promoter. The nucleotide sequence (AF246304) was analysed to identify putative transcription factor sites using TESS (http://www.cbil.upenn.edu/tess). Potential binding sites for Sp-family (squares), and YY1 (triangles) are shown diagrammatically. Previously-identified transcription start sites (in fibroblasts and epithelium) are indicated by arrows. The position of 15 overlapping fragments referred to as F0.1 – F8, are shown above. These fragments were also labelled and used as probes in EMSAs (probe 0.1, probe 0.2 etc).

Figure 2 .

Activity of occludin promoter in brain and non-brain endothelium. The full length occludin promoter in pGlow was transiently transfected into hCMEC/D3 and LMVECs (upper panels). Cells were assayed for expression of GFP by flow cytometry 56 hours (hCMEC/D3) or 72 hours (LMVEC) post transfection. Bold histograms show GFP expression mediated by the promoter compared to expression driven by the empty vector alone. The lower panels show GFP expression when hCMEC/D3 or LMVEC were transfected with a construct containing the CMV promoter.

Figure 3.

EMSA analysis showing the binding of nuclear extracts from hCMEC/D3 or LMVEC to probe 0.1. Numbers indicate lanes, reading from left to right on each gel. Lane 1 contains probe only and lane 2 the probe with nuclear extract. Antibodies and cold-block oligonucleotides were included as indicated. Nuclear extract of hCMEC/D3 produced two complexes (a,b). Nuclear extract of LMVEC produced four complexes (a',b',c',d') Complex c' was blocked by a TFIID-oligo (lane-6) and complex d' was supershifted by anti-YY1 antibody (lane-7).

Figure 4.

EMSA analysis showing the binding of nuclear extracts from hCMEC/D3 or LMVEC to probe 8. Numbers indicate lanes, reading from left to right on each gel. Lane 1 contains probe only and lane 2 the probe with nuclear extract. Antibodies and cold-block oligonucleotides were included as indicated. Nuclear extract of hCMEC/D3 produced four complexes (a,b,c,d). All four complexes were blocked by pretreatment with anti-Sp3 (lane-3); Sp-oligo. (lane-5) and NFκB-oligo. (lane-9). Nuclear extract of LMVECs produced only one complex

(e'), which was not blocked by: anti-Sp1 or anti-Sp3. Complex e' was blocked by all oligonucleotides tested, including: Sp-oligo (lane-5), AP1-oligo (lane-6), NFκB-oligo (lane-7) and mutant Sp-oligo (lane-8).

Figure 5

EMSA analysis of DNA-protein complexes formed between nuclear extracts from hCMEC/D3 cells and probe 3.1 or probe 6.1. Numbers indicate lanes reading left to right on each gel. Lane 1 contains probe only and lane 2 the probe with nuclear extract. Antibodies and cold-block oligonucleotides were included as indicated. Probe 3.1 produced 3 complexes (a,b,c). Probe 6.1. produced 6 complexes with nuclear protein (d-i).

Figure 6.

ChIP analysis of immunoprecipitates from LMVEC, hCMEC/D3 and primary BMEC cells using antibodies to Sp3 or rabbit IgG control (RbIgG). The immunoprecipitates were analysed by PCR, using primer-pairs spanning regions of the promoter detailed in table 1. Arrows indicate the position of a 500bp marker. The LMVEC gel has no amplified products, but the unamplified primers (P) are shown for orientation.

Figure 7.

ChIP analysis of immunoprecipitates from two representative preparations of LMVEC or primary BMEC , using antibodies to YY1 or rabbit IgG control (RbIgG). The immunoprecipitates were analysed by PCR, using primer-pairs spanning regions of the promoter detailed in table 1. Arrows indicate the position of a 500bp marker.

Figure 8.

FACS analysis of hCMEC/D3 cells treated with 0, 100 or 200nM mithramycin for 48hours stained for occludin, claudin-5 or ZO-1. Bars show mean and SEM of the mean fluorescence from 3 independent 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 9.

9A. EMSA analysis of protein/DNA complexes formed between promoter probe 8 and nuclear proteins from hCMEC/D3 cells untreated [lane 2], or treated with 50nM [3] or 200nM [4] mithramycin. Probe only, [lane 1]. 200nM mithramycin removes all four complexes a-d.

9B. CHIP analysis of DNA from hCMEC/D3 cells treated with 50, 100 or 200nm

mithramycin and then immunoprecipitated with normal rabbit IgG or anti-Sp3. The presence of the occludin promoter in the precipitates was detected by PCR using primers spanning region of probe 8. Sp3 is not detected associated with the occludin promoter in cells treated with 100nm or 200nm mithramycin.

Figure 10.

FACS analysis of promoter reporter activity (GFP expression) from hCMEC/D3 transfected with vectors containing promoters spanning F7.1- F8. (filled histograms) compared with control, empty vector (open histograms). Wild-type promoter is compared with two different vectors containing deletion of individual Sp sites: Sp-a , deletion of the Sp-site at position -34: Sp-b, deletion of the Sp-site at position -56. The structure of the inserts is illustrated diagrammatically and relates to positional data in figure 1 and sequences indicated in table 1.

Table 1 Occludin promoter fragments

Fragment	Position on Chr5 NT_006713	Size (bp)	Target sites * Sp	Target sites * YY1	EMSA complexes hCMEC/D3¶
0.1	19380963-19381083	120	X	X	2
0.2	19381054-19381188	128	X	X	-
1	19381165-19381301	136		X	2
2.1	19381275-19381450	175		X	1
2.2	19381428-19381582	154		X	1
3.1	19381561-19381740	179	X	X	3
3.2	19381717-19381827	110			-
4.1	19381805-19381957	152		X	-
4.2	19381935-19382054	119		X	-
5	19382031-19382201	170	X	X	(3)
6.1	19382181-19382362	181		X	6
6.2	19382321-19382487	166	X		(5)
7.1	19382468-19382649	181	X		(4)
7.2	19382631-19382717	86	X		-
8	19382696-19382816	120	X		4

* Fragments containing potential Sp-target sites, YY1, target sites or both, identified by TESS, are indicated.
¶ The number of complexes produced with the fragment using nuclear extract of hCMEC/D3 in EMSA. Figures in brackets indicate low affinity binding.