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**ACTION OF TRANSCRIPTION FACTORS IN THE CONTROL OF TRANSFERRIN
RECEPTOR EXPRESSION IN HUMAN BRAIN ENDOTHELIUM**

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Key Words: Brain endothelium, transcription factors, transferrin receptor, YY1, Sp3.

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Summary

Brain endothelium has a distinctive phenotype, including high expression of transferrin receptor, p-glycoprotein, claudin-5 and occludin. Dermal endothelium expresses lower levels of the transferrin receptor and it is absent from lung endothelium. All three endothelia were screened for transcription factors that bind the transferrin receptor promoter and show different patterns of binding between the endothelia. The transcription factor YY1 has distinct DNA-binding activities in brain endothelium and non-brain endothelium. The target-sites on the transferrin receptor promoter for YY1 lie in close proximity to those of the transcription initiation complex containing TFIID, so the two transcription factors potentially compete or interfere. Notably, the DNA-binding activity of TFIID was the converse of YY1, in different endothelia. YY1 knockdown reduced transferrin receptor expression in brain endothelium, but not in dermal endothelium implying that YY1 is involved in tissue-specific regulation of the transferrin receptor. Moreover a distinct YY1 variant is present in brain endothelium and it associates with Sp3. A model is presented, in which expression from the transferrin receptor gene in endothelium requires the activity of both TFIID and Sp3, but whether the gene is transcribed in different endothelia, is related to the balance between activating and suppressive forms of YY1.

Running Title: Transcription factors in endothelium

Abbreviations used: BMEC, bone marrow endothelial cells: DMVEC, dermal microvascular endothelial cells: EMSA, electrophoretic mobility shift assay: hCMEC/D3, human cerebral microvascular endothelial cell-D3: HTR, human transferrin receptor: LMVEC, lung microvascular endothelial cells: pgp-1, p-glycoprotein-1: Sp1, Specific protein-1: TF, transcription factor: YY1, Yin Yang 1.

Introduction

Endothelial cells have both barrier properties and selective transport functions. Although all endothelial cells share some common properties, they are heterogeneous with respect to their structure, protein expression, surface phenotype and secreted molecules depending on their tissue of origin and position in the vascular tree.¹⁻³ Brain endothelial cells which form the blood brain barrier (BBB) are coupled by continuous tight junctions of extremely low permeability to hydrophilic substances, which are more like those of epithelial barriers. In contrast endothelial cells in non-neural tissues have discontinuous tight junctions.⁴ Central nervous system endothelia display a unique pattern of receptors and transporters including transferrin receptor (TR) and p-glycoprotein-1 (pgp-1) in addition to the structural proteins occludin and claudin-5. The molecular mechanism controlling expression of the different junctional phenotypes and the associated transporters in endothelia is not yet clear.

During development, multipotent progenitors differentiate via specific lineages into several specialised cell types. Progress has been made in identifying transcription factors required for the initial growth and differentiation of endothelium, but much less is known about the factors that control the terminal differentiation in different tissues including the brain.⁵ The 5' promoter region of genes selectively expressed in brain endothelium, including the transferrin receptor, claudin-5, occludin and pgp-1, all contain GC-rich segments with common sequence motifs despite their diverse functions and locations within the genome. Hence we hypothesised that these genes are under common transcriptional control and common transcription factors could act on all these genes to induce their expression in brain endothelium. The approach taken in this programme has been to identify transcription factors that are present/active in one type of endothelium but absent or functionally inactive in another. In this study we have focussed on the transcriptional control of the transferrin receptor, a potential therapeutic target for delivery of macromolecules across the blood-brain barrier.⁶

Iron transport across the blood brain barrier involves the transferrin receptor, a cell membrane associated glycoprotein that serves as a transporter of iron.⁷ These receptors are expressed on other cell types but levels vary greatly,⁸ and expression is generally low or undetectable on non-brain endothelium. Expression of the transferrin receptor may be controlled by a post-translational

mechanism in response to iron demand.⁹ However control of expression in different cell types appears to be at the transcriptional level,¹⁰⁻¹⁴ although little is known about the regulatory mechanisms that influence the selective expression of the TR gene in brain endothelial cells. The aim of this study was therefore to identify transcription factors which regulate the expression of the transferrin receptor in human brain and non-brain endothelium. The human transferrin receptor (HTR) promoter region contains potential binding sites for more than 12 transcription factors identified by TRANSFAC. In an initial screening we identified specific protein-1 (Sp1), Yin Yang 1 (YY1) and TFIID as potential candidates in controlling the tissue-specific expression of HTR, - binding sites for each of these factors are present in the HTR promoter, and the DNA-binding activity of these factors varied greatly between the endothelia.

YY1 is a bifunctional protein capable of activating or repressing the transcription of many genes especially during cell growth and differentiation.¹⁵ YY1 can have a dual activity even on the same promoter, depending on the cell type or differentiation state. Sp1 is the prototype of a large family of transcription factors. Sp1 itself is generally thought to be a constitutive factor that enhances the transcriptional initiation of numerous genes. Saffer *et al.*,¹⁶ found that there are large variations in Sp1 levels during development and between various tissues, suggesting a regulatory role for Sp1 in growth and development.

To understand how human endothelial cells vary in transcription factor profile, we examined human brain endothelium (hCMEC/D3),¹⁷ primary human dermal microvascular endothelial cells (DMVEC), lung microvascular endothelial cells (LMVEC) and a human bone marrow endothelial cell line (BMEC).¹⁸ The aim of this work was to identify differences in the expression of transcription factors between differentiated endothelia, that could determine the tissue-specific phenotypes of endothelial cells and in particular, brain endothelium. Since the blood brain barrier limits the access of many potential therapeutic agents into the central nervous system, the identification of transcription factors that regulate the phenotype of brain endothelium, could be the basis for modulating their expression *in vivo*, which will allow more effective drug delivery to the central nervous system.

Results

Phenotyping of endothelial cells

HTR expression in brain endothelial cells (hCMEC/D3) was examined by Western blotting and the expression levels were compared with that in endothelial cells from non-neural tissues (DMVEC, LMVEC). Figure 1 shows that brain endothelial cells express HTR, detected as a band migrating at 95 kDa. DMVEC had lower expression and very low expression was seen in LMVEC. The results confirm that HTR expression differs greatly in the three different endothelial cell types. To confirm that the hCMEC/D3 cells maintain the phenotype of brain endothelium, we also examined the expression of pgp-1, occludin and claudin-5 by Western blotting. P-glycoprotein-1 was detected at 170 kDa, claudin-5 at 20-22 kDa and occludin at 65 kDa in hCMEC/D3 cells (Figure 1). Lower expression of claudin-5 was seen in DMVEC and LMVEC, and a trace of occludin was detected in lung endothelium.

Identification of DNA-binding proteins in different endothelia

EMSAs were used to detect DNA binding proteins in the nuclear extracts of the three types of endothelium, using the 220 bp and 330 bp HTR-promoter gene segments as targets. Figure 2 shows that nuclear extracts from hCMEC/D3 cells bind to both gene segments, producing a distinctive high molecular weight complex on both segments. Nuclear extracts of DMVEC and LMVEC also bind the 330 bp fragment (Figure 2 (a)), and to a lesser extent the 220 bp fragment (Figure 2 (b)), but the DNA binding proteins are clearly different in the non-brain endothelium.

The number of transcription factors in the hCMEC/D3 lysates that bind to the DNA fragments was estimated by incubating the nuclear proteins with radiolabelled gene segments in the binding conditions as normal, and then UV cross-linking the complexes prior to SDS PAGE. Figure 2 (c) shows that brain endothelium has a single additional band at Mr ~100 kDa which was not present in other endothelia. In addition a specific band at Mr ~30 kDa was seen in the LMVEC nuclear extracts binding to the 330 bp fragment. It was not possible to obtain accurate molecular weight values of bound TFs from these experiments, since the 330bp radiolabelled target DNA is covalently cross-

linked to the TFs to allow their detection. The presence of distinct bands in the different endothelia shows that different transcription factors are active on the HTR promoter in each endothelial cell type, and it suggests that a distinctive high molecular weight TF is active in brain endothelium.

To identify which proteins present in the hCMEC/D3 cells could bind to the HTR promoter, a transcription factor consensus sequence analysis database, TRANSFAC, was used to search for potential transcription factor binding sites in the 330 and 220 base pair fragments. Other studies have indicated the role of these segments in transcriptional control of HTR.¹⁰ Sequence analysis indicated several potential binding sites for known transcription factors, including TFIID, YY1, Sp1, IRF-1, CBF, NFAT, SRF, CDP, Pit-1, GATA and c-myb sites. The promoter sequence of HTR and the location of potential transcription factor binding sites in the 330 bp fragment is shown in Figure 3. Note that the Sp-family consists of over 20 members, of 95-105 kDa in size, each being able to bind the same consensus sequence, since they have greater than 90% DNA binding sequence homology.

To test whether the different endothelia did in fact contain transcription factors that were capable of binding to these regions, consensus oligonucleotide probes containing putative transcription factor binding sites were used in EMSAs with nuclear extracts prepared from the different endothelia. For the initial characterization we used a panel of oligonucleotides corresponding to the target sequences that had been identified in the HTR promoter (Figure 3). The oligonucleotide probes were end labeled and used in EMSAs with nuclear proteins from hCMEC/D3, DMVEC, LMVEC and BMEC. We were particularly interested to identify probes that showed different EMSA patterns with extracts from different endothelia. The probes fell into 3 groups:

Same for all endothelia: Sp1, IRF-1, CBF, NFAT, SRF, CDP.

Slight differences: Pit-1, GATA, c-myb

Major differences: YY1, TFIID (See Figures 5 and 6)

The following analysis focuses on YY1 and TFIID, since the initial EMSA screen had shown that they were very different in their DNA-binding activity between the endothelia. In addition, we also examined the Sp-family of transcription factors, since they appear to be particularly important in endothelia, they have a high molecular mass appropriate for the HTR-promoter binding proteins

demonstrated by SDS PAGE (Figure 2(c)) and because TFIID interacts with both YY1 and Sp-family transcription factors.¹⁹⁻²¹

Functional activity of Sp-family in endothelia

We first examined the Sp-family transcription factors. Figure 4(a) shows EMSA analysis of the interaction between endothelial nuclear proteins and an Sp-consensus dsDNA probe. The migration on the EMSA of a complex containing purified, recombinant Sp1 protein (lane 1) was similar to the migration of one band that was observed with the nuclear extracts, which suggests that the slowest migrating band is Sp1. A similar pattern of nuclear protein/DNA complexes was produced by all three cell types, each showing four distinct major Sp protein/DNA complexes. This indicates that four members of the Sp-family of transcription factors are present in all three endothelial cell types studied. All complexes were eliminated by the addition of an unlabeled, competitive Sp oligonucleotide, but not by an unlabeled, unrelated NFκB oligonucleotide (Figure 4(c)).

To identify specific proteins binding to the Sp-consensus oligonucleotide, supershift assays were performed with the labeled probe, endothelial cell nuclear proteins, and specific antibodies to Sp1, Sp2 and Sp3. Figure 4(b) shows that the slowest mobility DNA/protein complex was supershifted by the addition of an anti-Sp1 specific antibody, which had no effect on any of the other complexes. We noted that the complex that is supershifted by the anti-Sp1 antibody corresponds to the complex formed by purified recombinant Sp1 (Figure 4(a)). An Sp3 specific antibody reduced the intensity of the second band and caused the appearance of a supershifted band. These results confirm that the upper band corresponded to Sp1 protein bound to the Sp-consensus oligonucleotide forming a protein/DNA complex and the second band to the presence of an Sp3 protein/DNA complex. An Sp2 antibody had no effect on the pattern of DNA/protein complexes, implying that Sp2 is not present in the endothelia. A similar pattern of binding was seen with nuclear proteins from all three endothelial cell types, with four DNA binding proteins including Sp1 and Sp3, and two unidentified family members in the two faster migrating complexes.

Functional activity of YY1 in endothelia

By scanning the DNA sequence of the HTR promoter fragments we found several candidate binding sites for YY1. In order to analyse the specific activity of YY1, EMSAs were carried out in the presence of a YY1-consensus dsDNA probe and nuclear extracts from the endothelial cells. For these and subsequent experiments, we also used a bone marrow endothelial cell line (BMEC) as an additional control since these cells had been immortalised with SV40 large T antigen as was the hCMEC/D3 line. Figure 5(a) shows the autoradiograms of the EMSA with the YY1 DNA/protein complexes formed. One distinct protein-DNA-complex was visible after incubation of the BMEC nuclear extracts with the YY1 probe. A similar pattern of binding was seen with LMVEC, although LMVEC contained a lower level of YY1 DNA binding activity. Only a faint trace of binding was seen with hCMEC/D3 and DMVEC extracts.

To confirm that the DNA binding protein was YY1, an anti-YY1 antibody was added to the binding reaction, which should prevent the formation of the complex or cause a supershift. As seen in Figure 5(b), formation of the main DNA/protein complex was inhibited and the appearance of a supershifted band, was formed by the anti-YY1 antibody confirming the production of a specific YY1/DNA complex by BMEC (lane 9) and LMVEC nuclear extracts (lane 14). Gel shift competition experiments were carried out in order to further confirm the specificity of the YY1 DNA-protein interactions. An excess of unlabelled competitive oligonucleotide, with a consensus binding site for YY1, inhibited DNA/protein complex formation with BMEC and LMVEC nuclear extracts, (lanes 7 and 12), but mobility was not inhibited by an excess of an oligonucleotide with an unrelated NF- κ B site (lanes 10 and 15), indicating that the binding was specific. The faster migrating DNA/protein complex was non-specific as it was not inhibited by unlabelled YY1. A mutant YY1 radiolabeled oligonucleotide probe mutated in the YY1 consensus sequence failed to interact with any of the proteins in the BMEC or LMVEC nuclear extracts (Figure 5(b)), and no protein/DNA complexes were formed.

Functional activity of TFIID in endothelia

Analysis of the endothelial cell nuclear extracts with a TFIID consensus probe in the EMSA showed that brain endothelium contains four specific proteins that bind to the TFIID probe (Figure 6(a)). Bone marrow endothelium contains one of these proteins, but the three faster-migrating proteins, were either greatly reduced or absent. None of the TFIID-consensus-binding proteins could be detected in LMVEC or DMVEC. Unlabelled, cold competitive TFIID consensus-oligonucleotide inhibited the formation of all four bands in the brain endothelium, whereas unlabelled NF- κ B had no effect on the TFIID DNA binding, confirming the specificity of the DNA/TFIID interaction (Figure 6(b)). Since TFIID is a component of the basal transcription machinery, the results suggest that protein(s) in the non-brain endothelial nuclear extract, are modulating the functional activity of TFIID.

Subcellular localization of transcription factors by immunofluorescence

The absence of a YY1/DNA complex in EMSA with the hCMEC/D3 nuclear extracts and the YY1 consensus probe indicated three possibilities:

- a) The cells do not express YY1.
- b) YY1 present in these cells is not capable of binding to the DNA.
- c) YY1 is present in the cells, but is not localised to the nucleus.

In order to determine the amount and localisation of the transcription factors, we carried out immunofluorescence on whole cells and western blotting on cell extracts.

Figure 7 shows the expression of YY1, Sp1 and Sp3 on the three endothelial cell types. All three transcription factors are detectable on all three endothelia and show a predominantly nuclear localisation. However some cytoplasmic staining was also present (see for example, YY1 staining on DMVECs). There appeared to be no variation of these three transcription factors with the cell cycle, since recently-divided (paired) cells show similar staining levels to isolated cells. However, the levels and profiles of the transcription factors are dependent on the endothelial cell type.

We also carried out immunofluorescence for transcription factors in primary human brain endothelium (passage 1), to establish whether the results seen with the endothelial cell line were representative. The results in Figure 8 show that the primary brain endothelium also has a high

Sp3:Sp1 ratio. YY1 is present in the nucleus of these cells, and the level appears to be higher than in the line cells. The predominantly nuclear localisation of all three transcription factors is comparable in primary endothelium and the hCMEC3/D3 line. Although one would ideally carry out all assays with primary human brain endothelium, insufficient donor brain tissue was available for EMSAs and Western blotting.

Analysis of YY1 isoforms

Since YY1 protein was shown to be present in nuclei of brain endothelium, we used Western blotting to determine whether the YY1 was present in different isoforms in cytosolic and nuclear extracts of the endothelia. Figure 9(a) shows comparable levels of YY1 protein expression in cytoplasmic extracts from all 4 cell types, as indicated by the presence of a protein of Mr=70kDa that was recognized by the YY1 antibody. The hCMEC/D3 cells also expressed a protein of Mr=43kDa that was recognized by the anti-YY1 antibody (Figure 9(a)). Since the shorter isoform, seen in brain endothelium was detected by antibody to the whole YY-1 (Figure 9(a)), but not by antibody to the C-terminus (Figure 9(b)), this implies that the short isoform lacks a C-terminal segment. YY1 proteins of these sizes have been shown previously by in vitro translation and Western blots,²² to be YY1 and a truncated form of YY1 at the C-terminus, respectively.²³ This suggests that in hCMEC/D3 cells YY1 is present, but truncation of the YY1 protein prevents its interaction with DNA - note that the C-terminus of YY1 contains its four DNA-binding zinc-fingers. Taken together, the immunofluorescence data and Western blots indicate that YY1 protein is present in hCMEC/D3 cells as well as non-brain endothelium, but that the levels and isoforms depend on the cell type.

Functional activity of YY1 in brain endothelium

Several models have been proposed to account for the alternative actions of YY1 in activating or repressing genes, each of which involve complex interactions with other transcription factors in association with the specific target gene. Initially we aimed to establish whether YY1 played any functional role in the regulation of the HTR gene. We therefore used RNA interference with siRNA to knockdown YY1-mRNA and reduce YY1 expression. Transfection with YY1 siRNA into hCMEC/D3

and dermal endothelium led to downregulation of endogenous YY1 protein levels to about 50% of that in control cultures as measured by Western blotting (Figure 10). This knockdown of the YY1 gene resulted in a decrease in HTR expression in brain endothelium, but only a small decrease was observed in dermal endothelium (Figure 10). Clathrin (used as a house-keeping gene control) was not reduced in the brain endothelium by YY1 knockdown (Figure 10). These results show that YY1 plays a role in regulation of HTR expression in brain endothelium

Analysis of Sp-family transcription factors and their interaction with YY1

We also examined the expression and size of Sp1 and Sp3 proteins by Western blotting in nuclear and cytoplasmic extracts. Proteins of 90-95 kDa and 100-110 kDa were detected for Sp1 and Sp3 respectively in all four cell types. Moreover there appeared to be additional Sp3 bands in the hCMEC/D3 cells, suggesting the Sp3 may have undergone some post-translational modification in brain endothelium (Figure 11).

Because of the proximity of the YY1 and Sp-family target sites in the transferrin-receptor promotor (Figure 3), we examined the possibility that Sp3 was interacting with YY1 in brain endothelium. The results of immuno-coprecipitation experiments are shown in figure 12. In these experiments, nuclear lysates from different endothelia were precipitated with antibody to one transcription factor and the precipitate was then analysed by western blotting for the presence of that transcription factor (positive control) or the other transcription factor, as evidence of interaction. (For these experiments, we could only use antibodies from rabbit for the precipitation and the blot, consequently the blots all have one non-specific band at 55kDa corresponding to rabbit Ig heavy chain). The results show that in hCMEC/D3 cells, Sp3 precipitation causes the coprecipitation primarily of the smaller YY1 variants: We detected some coprecipitation of YY1 by anti-Sp3 in dermal endothelium, but not lung endothelium. The result was confirmed when the coprecipitation was carried out the other way around – YY1 precipitation causes coprecipitation, primarily of the larger variant of Sp3 in brain endothelium, but not from lung endothelium. Coprecipitation of Sp3 with anti-YY1 from dermal lysates was barely detectable. The results show that there is a strong association of YY1 and Sp3 in brain endothelium

but not in non-brain endothelium. There is some evidence that the interaction preferentially involves the shorter variants of YY1 present in brain endothelium.

Finally, we attempted to knockdown Sp1 and Sp3 using siRNA (Santa Cruz), however we were unable to identify a time/dose that reduced the expression of the transcription factors without killing the cells, including conditions that work on other cell types.

Discussion

The broad aim of this investigation was to identify transcription factors that control expression of a set of proteins that are present in fully-differentiated brain endothelium. This study focused on HTR, which was strongly expressed in hCMEC/D3 and primary brain endothelium, weakly expressed in DMVEC and absent from LMVEC.

The HTR gene promoter has binding sites for various transcription factors, including YY1, TFIID and Sp1. hCMEC/D3, DMVEC and LMVEC nuclear extracts contained at least four DNA binding factors that bind to an Sp1 target, including Sp1 itself and Sp3. Sp1 is the prototype of a family of transcription factors that binds GC rich boxes and regulates the expression of many different genes.²¹ Sp-family members play an important role in proliferation and differentiation and participate in the regulation of genes that are ubiquitously expressed, as well as those expressed in a tissue specific manner.²⁴ For example, Sp1 binds to a GC element within the *pgp-1* promoter and is required for its basal expression in a number of cell lines.^{25,26} The complex formed between the 330bp segment and hCMEC/D3 nuclear extract indicated a large DNA-binding protein associated with the promoter. Thus it is possible that an Sp-family member is a component of this complex. The positioning of Sp1 relative to that of upstream regulatory elements can be critical for its transcriptional activity. For example, Sp1 cooperation with Ets and GATA is required for core promoter transcription of several endothelial genes such as ICAM-1.²⁷ Sp1 generally acts as a transactivator and has been reported to act cooperatively at the level of both DNA binding and promoter transactivation.²⁸

Regulation of Sp3 transcriptional activity is more complex and it has been described as an activator or an inhibitor.²¹ hCMEC/D3 and primary brain endothelial cells expressed more Sp3 protein than

DMVEC, LMVEC or BMEC, with a high Sp3:Sp1 ratio in the nuclear extracts and a distinct form of Sp3. Moreover Sp3 competes effectively with Sp1 for DNA-binding sites,²⁹ so one would expect Sp3 to bind preferentially to the TFR promoter in brain endothelium. Hence, Sp3 is a likely candidate as one element in the transcription factor complex required for endothelial HTR gene expression.

TFIID is a multisubunit complex that initiates transcription. The critical subunit, TATA binding protein (TBP), is directly involved in promoter recognition and also has coactivator subunits termed TBP-associated factors. *In vitro* TBP-associated factors act as specific coactivators by direct interaction with transcriptional activator proteins such as Sp1.³⁰ Hence, cell-type specific transcription is directed by differential activity of TFIID. The TFIID sites in the HTR promoter are in very close proximity to the YY1 sites (Figure 3). Moreover, YY1 and TFIID have similar DNA-binding requirements. Consequently TFIID and YY1 could compete with each other for HTR promoter binding. Alternatively bound YY1 could prevent other transcriptional activators such as Sp1 or Sp3 from interacting with TFIID.

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,^{31,32} and YY1 is known to regulate the cell cycle and differentiation both in differentiated cells,^{32,33} and during embryonic development.³⁴ YY1 is subject to complex regulatory mechanisms in different cell types which affect its functional activity.³⁵ The promoter sequences surrounding YY1 binding sites,¹⁵ its relative concentration,³⁶ or post translational modifications,³⁷ 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,³¹ including various cellular factors and adaptor proteins,³⁵ which are themselves, coactivators, corepressors or transcription factors.

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.²⁰ This mechanism has been demonstrated in the HPV-16 long control region, where YY1 competes with Sp1.³⁸ The proximity of the YY1 and TFIID sites in the HTR promoter suggests that direct

competition for DNA-binding could occur, and that active YY1 in LMVEC could displace TFIID and thereby repress the transferrin receptor gene in these cells. However, YY1 is also present in the nuclei of brain endothelium (Figures 7,8,9) but in this case appears to be functionally inactive (Figure 5). This raises the question of how YY1 in brain endothelium, differs from that in non-brain endothelium.

In addition to direct competition for sites on the DNA, it is known that YY1 interacts with a number of key transcription factors such as Sp1, ¹⁹ TFIID, TBP, ³⁹ myc ^{32,40} and ATF/CREB factors independently of DNA-binding.⁴¹ In addition, this study indicates that YY1 can also interact directly with Sp3 (Figure 12). In many experimental systems YY1 can also activate transcription and it is unclear how this is accomplished. Increasingly, evidence shows that YY1 interaction with other Sp family members may account for its ability to regulate tissue-restricted gene expression.

The presence of two YY1 isoforms, in hCMEC/D3 cells, is consistent with reports of several YY1 variants in different cells and tissues.⁴² This however is the first time that YY1 isoforms have been correlated with distinctive cell phenotypes. The truncated form of YY1 in hCMEC/D3 cells lacks the C-terminal DNA binding domain, but retains segments that interact with other transcription factors. The variant isoforms could explain the different activity of YY1 in brain and non-brain endothelium. Alternatively, the ability of YY1 to bind DNA may be modified by phosphorylation or acetylation.^{31,37} The data presented here show that the normal form of YY1, is associated with repression of HTR expression in non-brain endothelium, whereas expression of the 45 kDa truncated form in brain endothelium is associated with HTR expression. We propose therefore that HTR expression in endothelium depends on a balance of activating and repressing forms of YY1. In brain endothelium, the YY1 variants preferentially interact with Sp3 and transcription is permitted, whereas in lung endothelium the YY1 can bind DNA, and transcription is prevented. Knockdown of YY1 by siRNA in brain endothelium disrupts this control system so that repression is favoured and HTR expression reduced.

In summary, the distinctive characteristics of brain endothelial transcription factors, that potentially bind the HTR promoter are:

- a. Low functional expression of YY1, but high functional expression of TFIID;

- b. An isoform of YY1 that lacks the C-terminal zinc-fingers;
- c. Evidence that YY1 levels modulate the expression of HTR in brain endothelium;
- d. A high Sp3;Sp1 ratio, and the presence of a post-translational variant of Sp3;
- e. Evidence that Sp3 interacts with YY1 in brain, but not non-brain endothelium.

The data lead us to propose a model with the following characteristics:

1. Transcriptional activation of HTR depends on TFIID, in association with Sp3, which is strongly expressed and active in brain endothelium.
2. TFIID and YY1 compete for binding sites in the HTR promoter.
3. YY1 is expressed in distinct isoforms in different endothelia but only binds its target DNA in the HTR promoter in non-brain endothelium.
4. The balance between activating and repressive variants of YY1 determines whether the HTR gene is transcribed in different endothelia. YY1 can directly interact with Sp-family transcription factors, but could additionally engage with chromatin-modifying enzymes to establish long-term regulation of the endothelial phenotypes.

The promoter regions of occludin, claudin-5 and pgp-1 also have target sites for Sp-family transcription factors, TFIID and YY1. We therefore suggest that control by YY1 could be a common element in the switch mechanism for several genes which are selectively expressed in brain endothelium.

Materials and Methods

Endothelial cell cultures

We have used an immortalised brain microvascular endothelial cell line, derived from human adult brain tissue (hCMEC3/D3). This cell line shows a conserved endothelial cell phenotype and expression of BBB markers (transferrin receptor and tight junction proteins, ZO-1, occludin and claudin-5). The line also expresses p-glycoprotein (pgp-1) and breast cancer resistance protein.¹⁷ The cells were grown on collagen coated plates and maintained in EGM-2–MV medium (Biowhittaker, Wokingham, Berks, UK) supplemented with 5% FCS; 0.1% FGF2 and gentamycin; 0.025% VEGF,

IGF, EGF and ascorbic acid and 0.01% hydrocortisone. The cells were grown to confluence and rested for two days, prior to experiments.

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 line¹⁷.

Dermal and lung endothelium were purchased from Clonetics/Biowhittaker (Wokingham, Berks, UK) and grown in EGM-2 MV medium according to the manufacturers recommendations. These primary cells are from individual donors and were used at passage 5-8. The transformed human BMEC line was kindly donated by Babette Weksler.¹⁸ The cells were cultured in Dulbecco's modified Eagle medium (Gibco) with low glucose (1 mg/ml) and 2 mM glutamine, supplemented with 10% FCS. A detailed description of the culture conditions and characteristics of these cells is given in Hillyer *et al.*³

Amplification of HTR promoter by specific nested PCR.

One gene segment from the 5' promoter region of the HTR gene (sequence X05339, position -307 to -854; see figure 3) was prepared by nested PCR using normal human genomic DNA as a template (25 µg/ml, Promega). For the first PCR, HTR promoter region specific primer pairs, 5'-CCA AGG CCC AGA AAC GGA T-3', and 5'-GGC CTG AAG GTC AGT TTA TGT GC-3' from Invitrogen were used. PCR was carried out in a reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 µM of forward and reverse primers, 1U Taq DNA polymerase and 1 µl genomic DNA in a total volume of 20 µl. Fragments were amplified with the following PCR cycles: 94°C for 5 minutes: 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds, and finally 72°C for 7 minutes. Products of the first round of amplification were diluted 1 in 20 and amplified in a second nested PCR with primers 5'-CTA ACC GGC GGT TTA TAG CCT G- 3' and 5'-CTG ACC TTG ACC AAC CTC CAG TC-3', in the same conditions as the first round of PCR, for

30 cycles, with a final extension of 72°C for 10 minutes. The PCR products were separated and analysed on a 1% agarose gel, stained with ethidium bromide, and visualised under a UV light.

The 550 bp band was excised from the gel, extracted, purified with a Novagen kit and ligated into the pCR 2.1-TOPO cloning vector (Invitrogen). After transformation into E coli TOP10F' bacterial cells (Invitrogen), plasmids were screened by digestion with restriction endonucleases and the insert was sequenced to confirm its identity. Fragments were released from the vector by flanking restriction enzymes. Large scale plasmid preps were purified using a commercially available kit (Qiagen). The 550 bp fragment was digested with Acc1, to generate 2 fragments of 220 and 330 bp suitable for use in electrophoretic mobility shift assays (EMSA).

Nuclear protein purification

Nuclear protein extracts were isolated from cells according to the method of Staal *et al.*⁴³ Cells were grown to confluence and rested for 48 hours, then washed twice in ice cold PBS and scraped into 0.4 ml of cell lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF, 0.2 mM NaF, 0.2 mM Na orthovanadate and protease inhibitors). The cells were allowed to swell on ice for 15 minutes before 25 µl of 10% Nonidet P-40 was added, and the cells were vortexed vigorously for 15 seconds and centrifuged for 30 seconds in a microcentrifuge. The nuclear pellet was resuspended in 50 µl of nuclear extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, 0.2 mM NaF and 0.2 mM Na orthovanadate) by pipetting and rotating on a platform (4°C) for 20 minutes. The samples were centrifuged for 5 minutes, the supernatants removed and stored at -80°C. The protein concentrations were determined using the BioRad protein assay.⁴⁴

Electrophoretic Mobility Shift Assays (EMSAs)

Double stranded oligonucleotides containing various transcription consensus sites, obtained commercially (Promega or Santa Cruz), or cloned HTR restriction fragments of 220 and 330 base

pairs were end-labelled with [γ - ^{32}P] ATP (Amersham) using T4 polynucleotide kinase, at 37°C for 30 minutes. The oligonucleotide sequences were as follows:

Sp1, 5'-ATTCGATCCGGGCGGGGCGAGC-3';

YY1, 5'-CGCTCCCGGCCATCTTGGCGGCTGGT-3';

YY1(mutant), 5'-CGCTCC**GCGATT**ATCTTGGCGGCTGGT-3', with the mutated bases in bold;

TFIID, 5'-GCAGAGCATATAAAAATGAGGTAGGA-3';

NF- κ B, 5'-AGTTGAGGGGACTTCCCAGGC-3', with the factor binding sites underlined.

The labelled double stranded oligonucleotides were purified through a Probe Quant G50 microcolumn (Amersham, Bucks) into a final volume of 30 μl . Five micrograms of nuclear extracts were incubated in 20 μl DNA binding buffer, containing 20 mM HEPES (pH 7.5), 4% Ficoll, 1 μg of poly (dI-dC), 0.1 mM MgCl_2 , 0.1 mM dithiothreitol (DTT) and 1 μl ^{32}P -end-labelled double stranded oligonucleotide probe (about 10, 000 dpm or 10fmol) for 35 minutes at room temperature. After mixing with 1 μl of loading buffer (250 mM Tris-HCL, pH 7.8, 0.2% bromophenol blue, 40% glycerol), the resulting DNA/protein complexes were separated from free oligonucleotide, by subjecting them to electrophoresis in a precooled and pre-run 4 %, non-denaturing polyacrylamide gel (29:1, acrylamide:bisacrylamide) and electrophoresed in 0.25 X TBE buffer, for 10 minutes at 240 V and a further 5 hours at 120 V. Following electrophoresis, the gel was dried under vacuum for 45 minutes at 80°C, and visualised by autoradiography by exposure to Kodak X-Omat film at -70°C with intensifying screens.

To determine the specificity of the gel shift complexes, competition studies were performed, incubating 200-fold excess of an unlabeled oligonucleotide, in the binding buffer prior to separation of the protein/DNA complexes in the polyacrylamide gel. In parallel EMSAs, specific supershift antibodies (2 μg) were added to the binding reaction, and incubated for 30 minutes, at room temperature prior to the addition of the ^{32}P labelled probes. The Sp1 (PEP2), Sp2, Sp3 and YY-1 (C20) rabbit polyclonal antibodies were from Santa Cruz Biotechnology (X versions). After this incubation, the probe was added and incubated for a further 30 minutes, prior to loading on the gel.

UV Cross-linking followed by SDS-PAGE

Nuclear proteins were incubated with the radiolabeled transferrin receptor promoter fragments in the same binding conditions as in EMSAs, followed by cross linking the protein to its regulatory sequence using UV light (40000 $\mu\text{W}/\text{cm}^2$ for 50 minutes) and resolving complexes on an 8% SDS polyacrylamide gel. After electrophoresis the gel was dried and visualisation was performed by autoradiography.

Western Blotting

Identification of HTR and other endothelial markers was done by Western blotting. Cells were scraped into 40 μl SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% sodium dodecylsulfate, 5% glycerol, 50 mM dithiothreitol, 1 mM orthovanadate, 0.05 $\mu\text{g}/\text{ml}$ bromophenol blue) at 4°C. Protein concentration was measured by the Biorad protein assay and samples heated at 95 °C for 5-10 minutes. Equal amounts (20 μg) of cell lysates were resolved by an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in non-reducing conditions. Proteins were electroblotted onto BA23 nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) at 0.15 mA overnight at 4°C. Membranes were blocked in 5% non-fat dried milk at room temperature in PBS containing 0.05% Tween 20, for 2 hours. Membranes were washed and subsequently incubated with 1 in 1000 dilution of primary antibody in blocking buffer for 2 hours at room temperature. Antibodies to HTR, occludin and claudin-5 were from Zymed. Anti-pgp-1 (C219) was from Merck Biosciences. Polyclonal rabbit anti-YY1 antibodies were purchased from Santa Cruz Biotechnology; C20 was raised against the C-terminal 20 residues while H414 was raised against full length YY1 protein. Polyclonal rabbit anti-Sp1 and Sp3 antibodies were purchased from Santa Cruz. After exhaustive washing in PBS containing 0.05% Tween-20, membranes were incubated in 1:15,000 secondary antibody in blocking buffer for 1 hour at room temperature, - HRP-conjugated goat anti-rabbit or anti-mouse IgG (Pierce, Chester, UK), as appropriate. Membranes were washed with 6 changes of PBS containing 0.05% Tween-20 and once with PBS alone. Immunoblots were visualised by enhanced chemiluminescence (ECL, Amersham, Bucks,UK).

Immuno-coprecipitation assays

Confluent hCMEC3/D3, dermal or lung endothelial cell monolayers were scraped into 1ml of ice-cold RIPA lysis buffer (Sigma, Dorset, UK) and held at 4°C for 15 min. The lysates were then centrifuged at 14000 rpm for 15 min to remove cell debris. Lysates were immunoprecipitated overnight at 4°C with protein G beads (Pierce, Chester, UK) previously incubated for 1 h at 4°C with specific antibody. After three washes in RIPA lysis buffer, beads were resuspended in SDS sample buffer and the immune complexes separated on 10% SDS-PAGE gels and proteins detected by western blotting.

Immunofluorescence confocal microscopy and image analysis

Cells grown on collagen coated coverslips, were rinsed three times with cold PBS and fixed at room temperature with 4% paraformaldehyde in PBS for 10 minutes. After three washes in PBS, cells were permeabilised with 0.2% Triton X-100 in PBS for 10 minutes and then incubated with 0.2µg of either Sp1, Sp3 or YY1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. Cells were washed extensively and incubated with the appropriate FITC-conjugated secondary antibody (Vector Labs, Burlingame, CA). After three PBS washes, the coverslips were mounted in Dako fluorescent mounting medium (Dako Corp, Carpinteria, CA) and fluorescent images obtained with a confocal microscope Leica TCS (Leica Microsystems, Mannheim, Germany). After correction for background intensity, boxes were drawn around the nuclei of each cell and the mean pixel intensity was determined on a scale 0-255. This measurement was repeated for ~40 cells each from three experiments done on different days.

Cell culture with small interfering RNA (siRNA)

All siRNA reagents were from Santa Cruz Biotechnology and were used according to the manufacturer's recommended protocols and concentrations. The human YY1 siRNA was a 20-25 target specific nucleotide. Twenty four hours before transfection hCMEC/D3 cells or dermal

endothelium were plated on collagen coated 6 well dishes at 50-60% confluency, in growth medium without antibiotics. 48 hours after incubation at 37°C with the YY1 siRNA complex, protein lysates were extracted from the cells. mRNA silencing was confirmed by Western blots with a YY1 specific antibody. Preliminary analysis had shown that YY1 reduction was optimal at 48 hours after treatment.

Acknowledgments

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Legends

Figure 1.

Western blot analysis of endothelial cell lysates from HCMEC/D3 brain endothelium (B), DMVECs (D) and LMVECs (L) resolved by 8% SDS PAGE, under non-reducing conditions. 20µg of protein was loaded on each lane. 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 as loading controls, shown below each blot.

Figure 2.

Analysis of DNA-binding proteins in nuclear extracts of HCMEC/D3 (B), DMVEC (D) LMVEC (L) and bone marrow (bm) endothelium. EMSA was performed with dsDNA probes of 330 bp (a) and 220 bp (b) spanning the human transferrin promoter. A distinctive DNA/protein complex (arrowed) is present in brain endothelium using both probes. The position of free probe (FP) is indicated. Lane 1 on each gel is a negative control without protein (-). Protein/DNA complexes formed by UV-cross-linking the 330 bp probe to the nuclear extracts were resolved by 8% SDS PAGE and autoradiography (c). A brain specific (*) and a lung specific (**) band are arrowed.

Figure 3.

Human transferrin receptor promoter region, indicating the position of the 330 bp and 220 bp gene segments and location of known transcription factor binding sites.

Figure 4.

(a) Binding of nuclear extracts from brain (B) dermal (D) and lung (L) endothelium to an Sp-consensus oligonucleotide analysed by EMSA compared with the shift produced by recombinant Sp1 protein (rSp1). Four Sp-family members were identified (arrows).

(b) EMSA of an Sp-consensus oligonucleotide alone (-) or in the presence of recombinant Sp-1 (rSP1), or brain nuclear extract (B) and in the presence of an unlabelled competitive oligonucleotide Sp1, or

an unlabelled non-competitive oligonucleotide NF κ B. The Sp1 oligonucleotide competes with all four Sp-family bands in the brain nuclear extract, but binding was unaffected by NF κ B oligonucleotide.

(c) Identification of individual members of the Sp family of transcription factors by EMSA and antibody-induced supershift. Nuclear extracts from brain dermal and lung endothelium were incubated with an Sp-consensus oligonucleotide and antibodies to Sp1, Sp2, Sp3 or no antibody (-). The position of bands supershifted by the Sp1 antibody and the Sp3 antibody are arrowed. Antibody to Sp2 did not shift any of the bands generated by the endothelial nuclear proteins.

Figure 5.

(a) Binding of nuclear extracts from brain (B) dermal (D), lung (L) and bone marrow (bm) endothelium to a YY1-consensus probe, analysed by EMSA. FP indicates free probe. The bold arrow indicates a specific YY-/DNA complex.

(b) Binding of nuclear extracts from brain endothelium (lanes 1-5), bone marrow endothelium (lanes 6-10) and lung endothelium (lanes 11-15) to a YY-1-consensus probe either alone (lanes 1, 6, 11) or in the presence of excess cold YY1-consensus inhibitor (lanes 2,7,12) or a control NF- κ B-binding oligonucleotide (lanes 5,10,15). A mutated YY-1 probe (lanes 3,8,13) did not form complexes with the nuclear proteins. Inclusion of antibody to YY-1 generated a super-shifted band (*), when preincubated with the protein/YY1-probe complexes (lanes 4,9,14). Arrow indicates the YY1-specific complexes and NS indicates a non-specific band.

Figure 6

(a) Binding of nuclear extracts of brain (B) dermal (D), lung (L) and bone marrow (bm) endothelium to a TFIID-consensus probe, analysed by EMSA. FP indicates free probe. Four bands containing different isoforms present in brain endothelium are indicated.

(b) Inhibition of electrophoretic mobility shift by cold TFIID oligonucleotide (lane 3) compared with non-competitive cold NF-kB oligonucleotide (lane 2) and no inhibitor (lane 1).

Figure 7

Expression of transcription factors in brain (B) lung (L) and dermal (D) endothelium using 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. Data shown is representative of 3 independent experiments. The histogram shows average pixel densities plotted in arbitrary units for the different transcription factors in the three cell lines.

Figure 8

Expression of transcription factors Sp1, Sp3 and YY1 in primary human brain endothelium stained in parallel. Data shown is representative of 2 independent experiments.

Figure 9

Western blots of cytoplasmic (Cyt) and nuclear (Nuc) extracts of brain (B), dermal (D), lung (L) and bone marrow (bm) endothelium (20µg of protein per lane) stained with antibody H414 (a) against full length human YY1 and antibody C-20 (b) against a carboxy-terminal peptide of human YY1. Arrow indicates the full-length YY1 (70 kDa). * indicates a C-terminal truncated form of YY1 (~45 kDa) found only in brain endothelium. ** indicates a C-terminal truncated form of YY1 (~60 kDa) found only in lung endothelium.

Figure 10

Western blot analysis of proteins (20µg per lane) in dermal (D) and brain (B) endothelium (hCMEC/D3) using cells treated for 48hours with siRNA to knockdown YY1 expression, in comparison with control cells treated identically, but without the siRNA. Expression of YY1, transferrin receptor (HTR) and clathrin was measured on the same preparation by sequentially

staining, stripping and reprobing the blots. YY1 knockdown substantially eliminates HTR expression in brain endothelium but reduction of expression in dermal endothelium is limited.

Figure 11

Western blots of cytoplasmic (Cyt) and nuclear (Nuc) extracts of brain (B), dermal (D), lung (L) and bone marrow (bm) endothelium stained with antibody to Sp3. 20µg of protein was loaded on each lane. Arrow indicates expected position of bands.

Figure 12

Cell lysates of hCMEC/D3 cells (D3), lung and dermal endothelium were immunoprecipitated with protein-G beads coated with normal rabbit IgG (RbIgG, control) or with antibody to transcription factors (Sp3 or YY1). 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 immunoprecipitation was with anti-Sp3, to detect coprecipitation of YY1; the lower 6 blocks show experiments in which immunoprecipitation was carried out with anti-YY1 to detect coprecipitation of Sp3. Arrows indicate evidence of coprecipitation, seen in the centre lane of each block.

Fig. 1

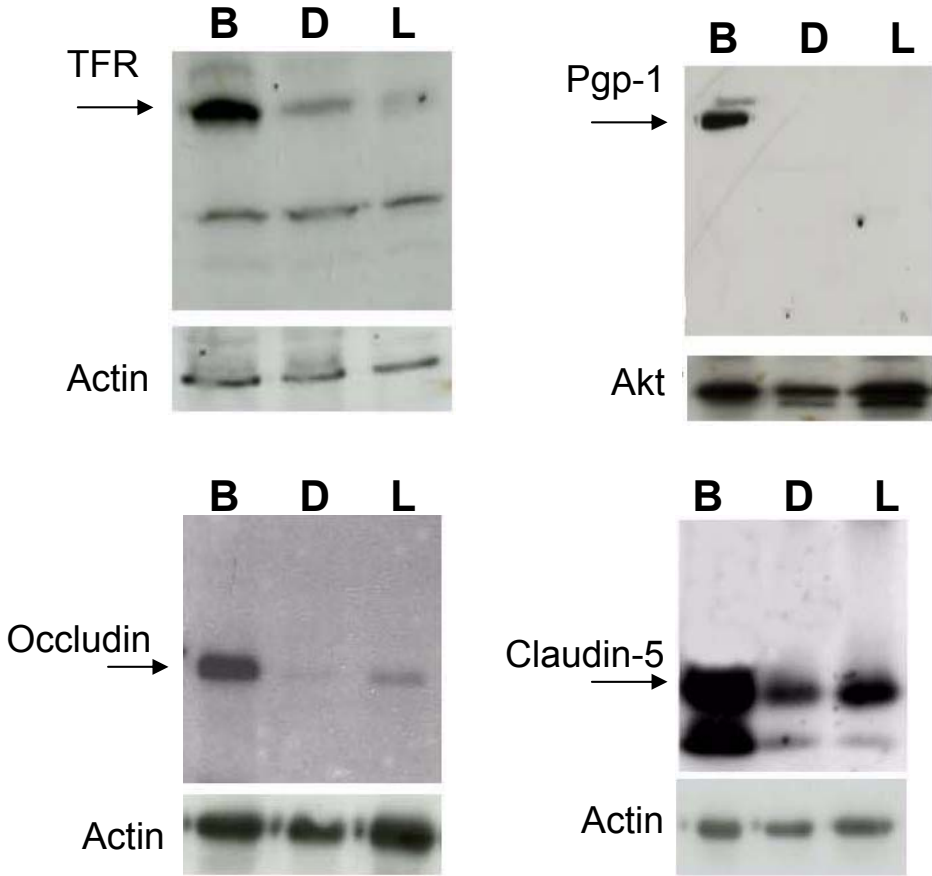


Fig. 2

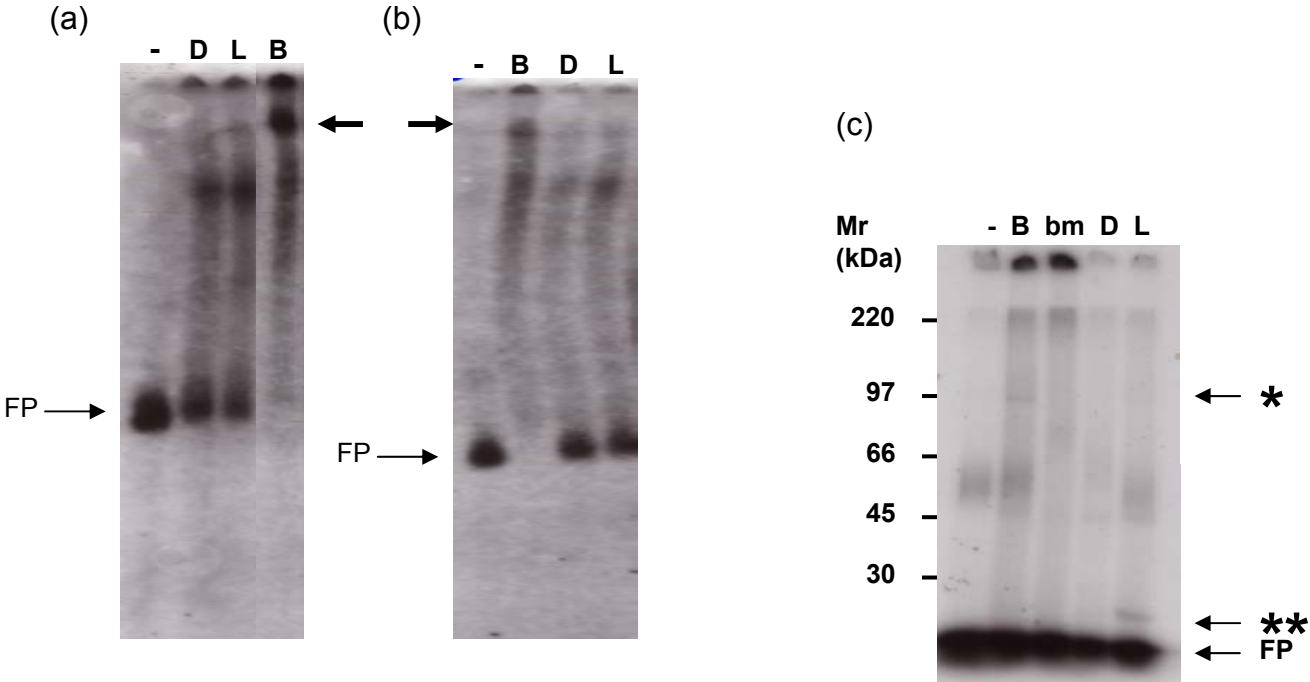


Fig. 3

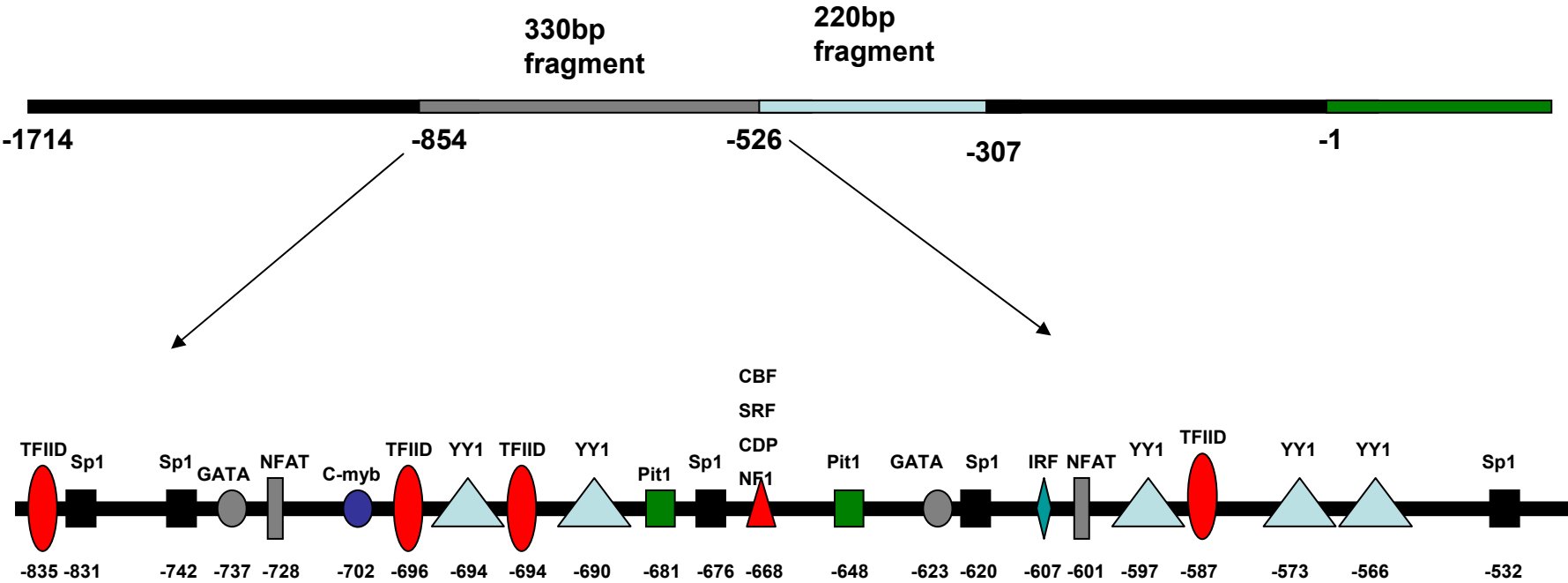


Fig. 4

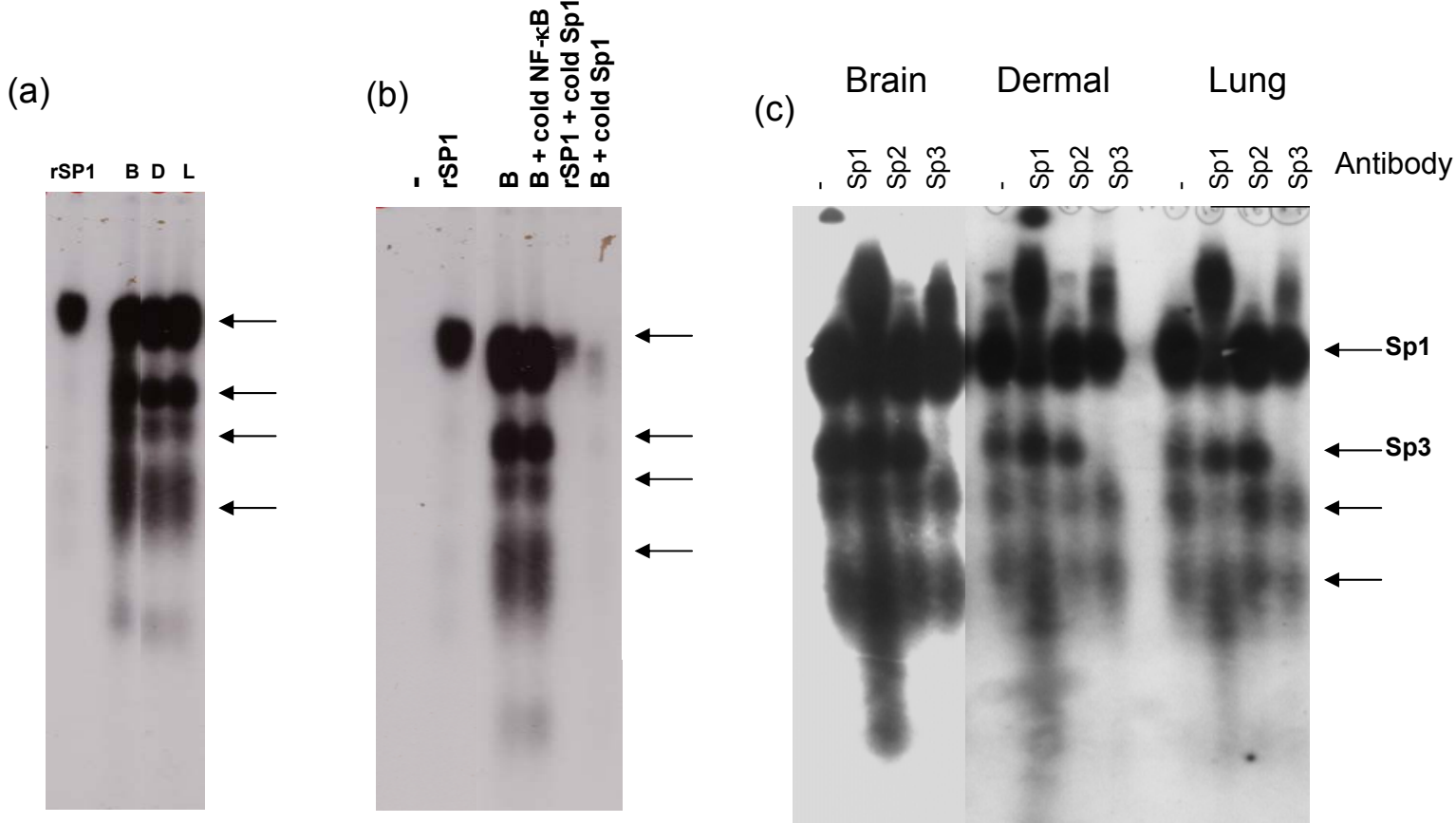


Fig. 5

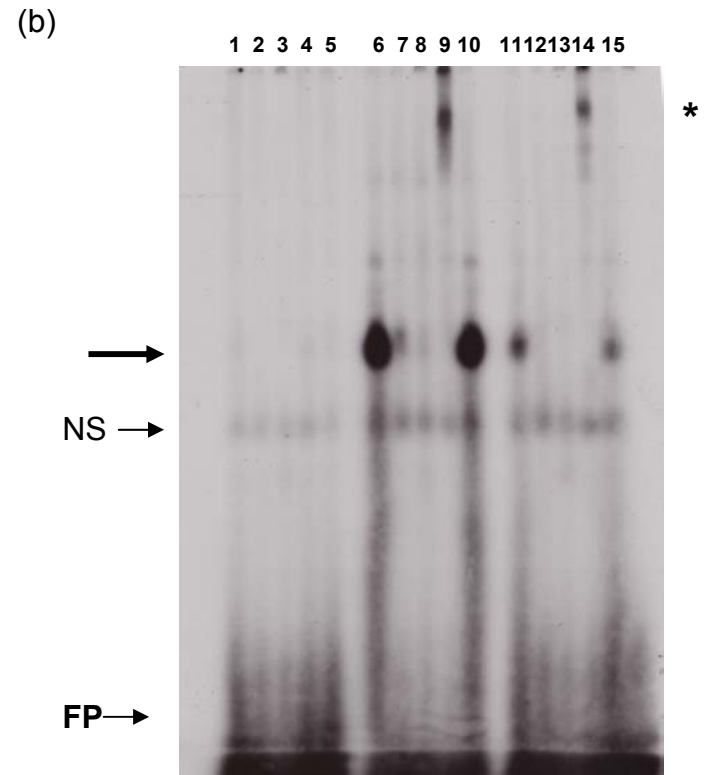
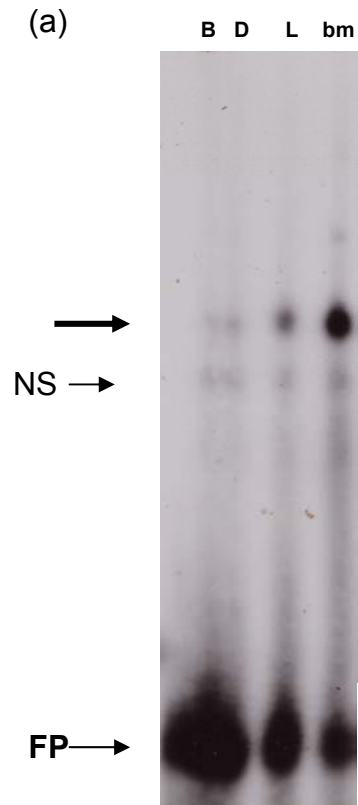


Fig. 6

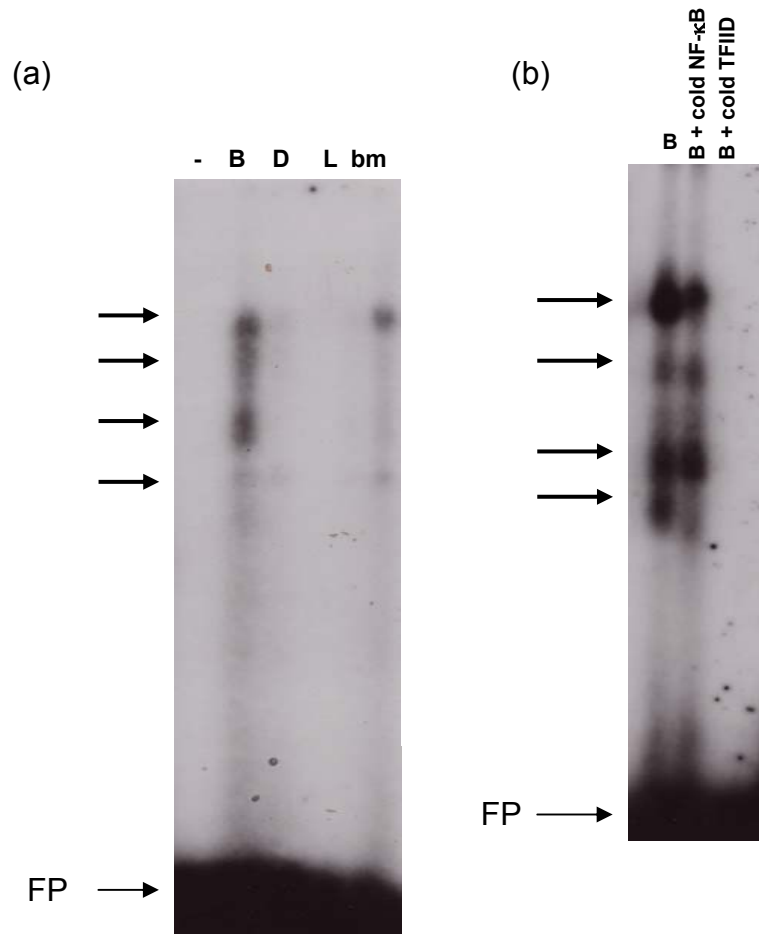


Fig. 7

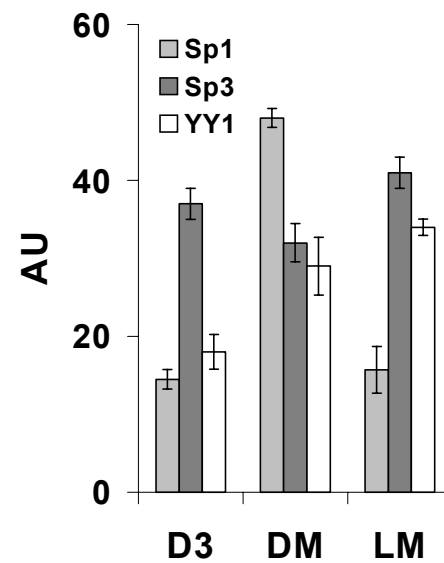
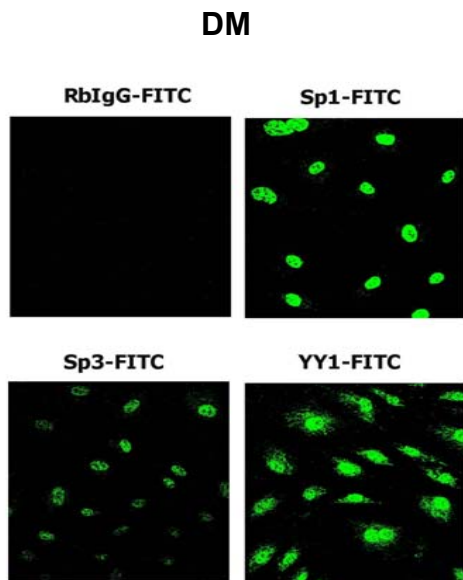
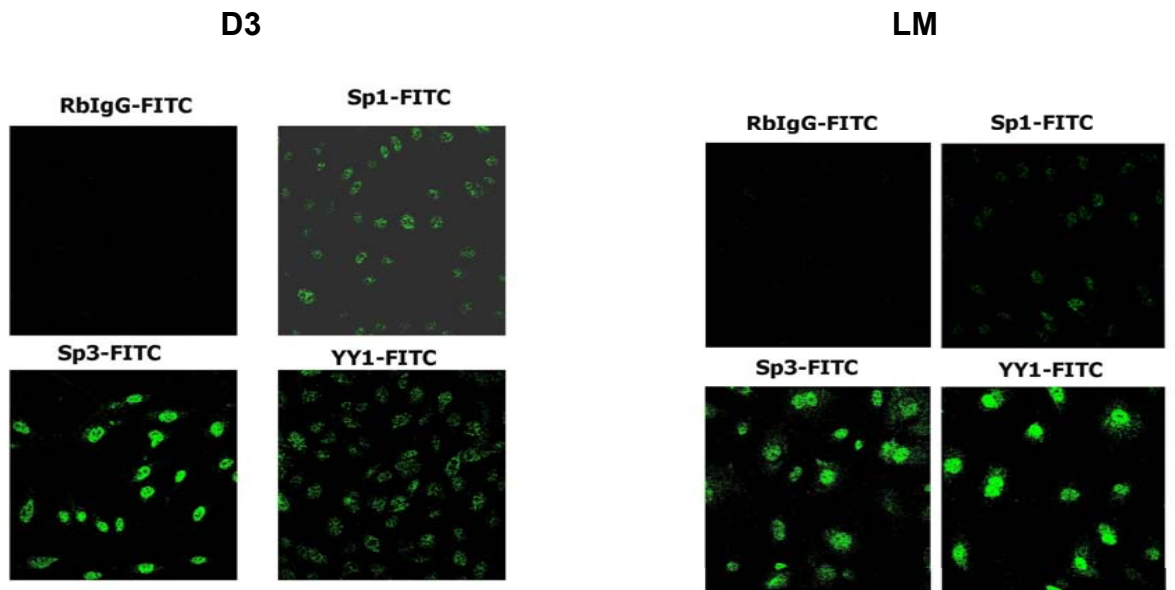


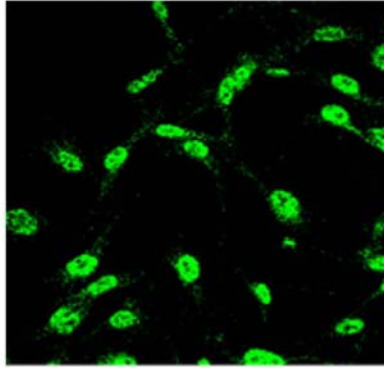
Fig. 8

Primary brain endothelium

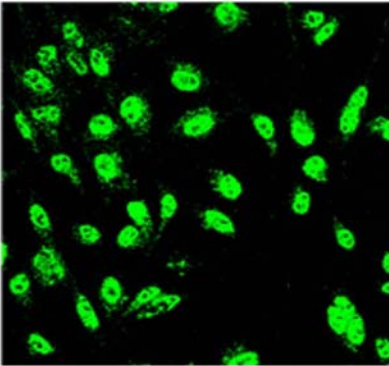
RbIgG-FITC



Sp1-FITC



Sp3-FITC



YY1-FITC

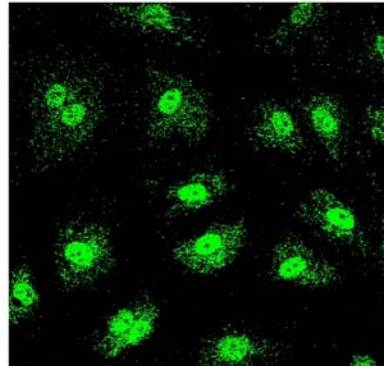


Fig. 9

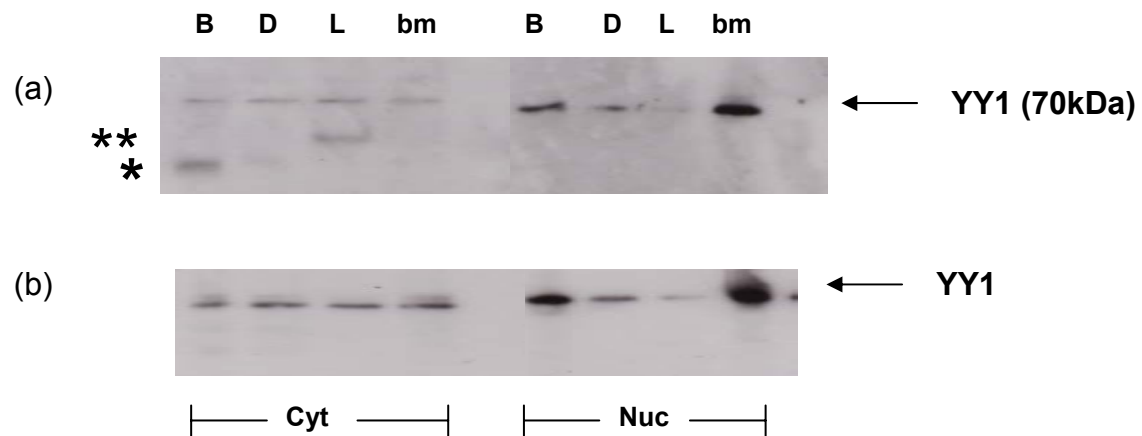


Fig. 10

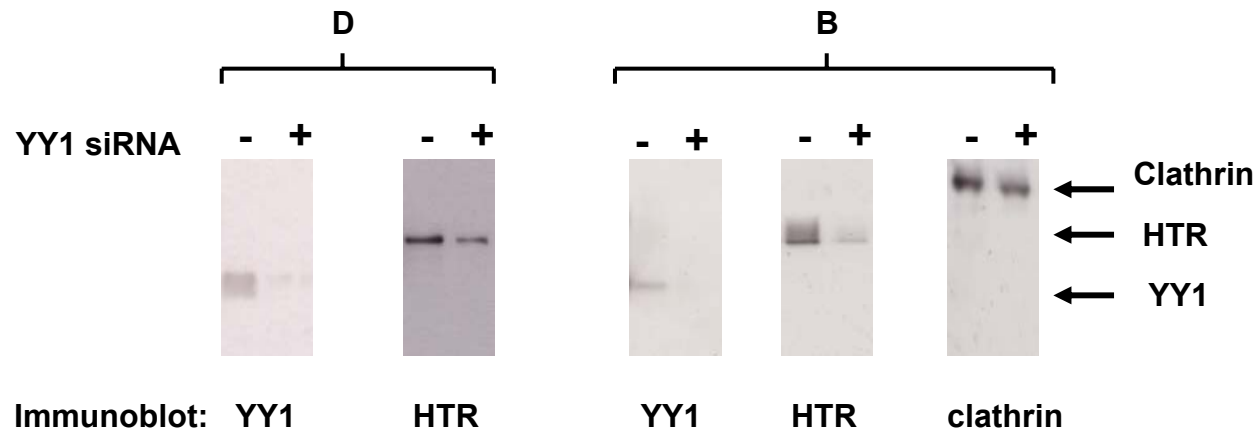


Fig. 11

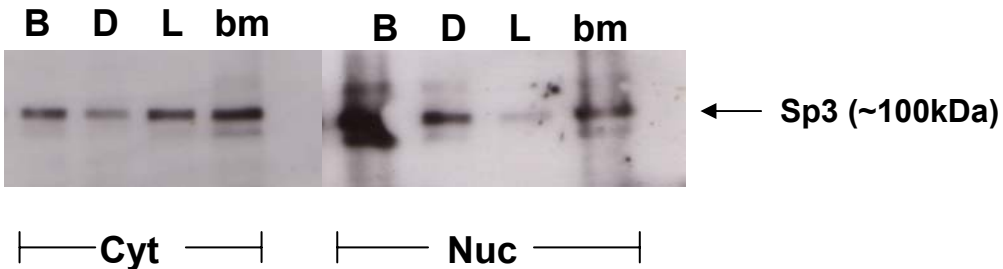


Fig. 12

