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THROMBOMODULIN EXPRESSION IN ADULT AND DEVELOPING MICE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Virginia Adrianne Ford

December, 1992

DEDICATION

To my father, Albert Binkley Dickas,

whose faith in me never wavered,

and to Thomas Edward Elliott,

who helped make the journey fun.

ACKNOWLEDGMENTS

I would like to thank the members of my committee, Dr. Eugene Rinchik, Dr. Larry Waters, Dr. Julian Preston and most importantly Dr. Steve Kennel for their excellent guidance and support. For their technical help, friendship, and understanding, I thank Linda Foote, and Trish Lankford. For expertly sectioned tissue I thank Jim Wesley, and for help in preparing the manuscript I thank Charlotte Rains, Sylvia Allen, and Nette Crowe.

This research was sponsored jointly by the National Institutes of Health Cancer Training Grant NIHCA 09104, the University of Tennessee, and the Office of Health and Environmental Research, United States Department of Energy, under Contract DE-AC05-840R21400 with Martin Marietta Energy Systems, Inc.

ABSTRACT

Two rat monoclonal antibodies were previously developed that bind separate epitopes on a murine glycoprotein, P112, which is expressed primarily in the lung capillary endothelium. Partial amino acid and cDNA sequence analyses demonstrated a strong similarity between P112 and thrombomodulin (TM), an endothelial cell glycoprotein involved in clot regulation. A subtractive monoclonal antibody assay, followed by Western blot analysis using anti-TM antibodies coupled with a biochemical assay for TM, showed that P112 was indistinguishable from TM. Quantitative analysis of both protein and mRNA levels in different murine tissues showed that the lung contained seven times more TM/cm^2 of vessel wall surface than did the kidney, whereas the liver displayed negligible amounts of TM. Analysis of eight different tissues suggests that TM levels are highest on continuous type endothelia, and much less prevalent on fenestrated and sinusoidal endothelia.

Sequence analysis of the promoter region of the TM gene demonstrated a significant difference in two strains of inbred mice. One strain, BALB/cBD, contains an intracisternal-A particle (IAP) proviral DNA sequence upstream of the initiation codon while another strain (that used for the MUSTHROMBO sequence listed in Genbank) lacked this insertion. Examination of ten different strains of laboratory mice showed that five contained the insert while the rest did not. Northern blot analysis for TM mRNA levels in these strains, showed

that there were different levels of TM expression in six-week-old mice, but these differences were not correlated with the presence or absence of the IAP proviral DNA.

In adult mice, 70% of the total body levels of TM are found in the lung microvasculature. The other 30% is distributed in the vessels of other organs, except the brain and liver, which have no detectable TM. Immunohistochemical studies of developing BALB/cBD (containing the IAP proviral insertion) and A/J (lacking the provirus) embryos exhibited an indistinguishable pattern of TM expression. As early as Day 6.5 post coitum to mid-gestation, TM is located in high amounts in the parietal endoderm of the placenta. High levels of TM are also found in the lung bud epithelium, and the meninges of the brain and spinal cord, suggesting that TM may play a role in development that is different from the role of clot regulation in the adult vasculature.

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PROLOGUE

Work in our laboratory has involved the study of cell surface molecules in relation to metastasis. Specificly, mouse lung tumor cells and normal lung cells have been studied. In the course of this work two monoclonal antibodies (MoAb), 273-34A and 411-201B (Rorvik *et al.*, 1988; Kennel *et al.,* 1988), were found that bind preferentially to the lung following *i.v.* injection. Further work indicated that the two MoAbs defined separate epitopes on the same glycoprotein, P112, and that this protein was expressed predominantly in the lung microvasculature (Kennel *et al.,* 1988). A MoAb two-site assay for determining the relative amounts of P112 in a protein lysate was created and used to show that the lung contained most of the P112 in an adult mouse (Kennel *et al.*, 1988). Further analysis using the MoAb for immunogold electron microscopy localized the binding site of P112 to the luminal surface of the lung microvessels (Kennel *et al.*, 1988).

The goal of my dissertation research was to identify the function of P112. Three approaches were used. One was to clone and sequence a cDNA for P112 for comparison to the sequences of proteins with known functions. Second, cloning and sequencing of the 5' promoter region for P112 would be performed, to locate sequences which may govern the endothelial cell specific expression of this protein. Finally, the pattern of P112 expression during murine development would be studied, to look for a specific role of P112.

After obtaining partial sequence for a P112 cDNA clone, it became evident that P112 was identical to an endothelial cell surface protein, thrombomodulin (TM). The major known function of TM is the control of clot formation in the vasculature.

Coagulation is a complex, multifaceted process involving a series of coordinated reactions requiring serum clotting factors, platelets, endothelial cells, and the vessel musculature. The cascade of reactions leading to clot formation involves a series of zymogen to protease transitions, which culminate in the conversion of soluble fibrinogen to insoluble fibrin. Under normal conditions, all of the factors necessary for clot formation are present, circulating in the vascular system as inactive zymogens. Clotting is triggered by exposure of subendothelial matrix proteins (intrinsic pathway, Figure 0.1) or by release of thromboplastin from damaged endothelial cells (extrinsic pathway) (for review see Mann *et al.*, 1988; Davie and Ratnoff, 1964; Macfarlane, 1964; Zwaal and Hemker, 1986).

When the vasculature is injured, the subendothelial cell matrix and blood come into contact. Platelets within the blood bind to the subendothelial matrix and are activated to secrete a variety of proteins (Tracey *et al.*, 1980). These secreted proteins lead to aggregation of more platelets at the wound site, vasoconstriction of the damaged vessel, and activation of factor XII to factor XIIa. Factor XIIa then catalyses the cleavage of Factor XI to factor XIa. Factor XIa cleaves factor IX to its active form, which in turn activates factor

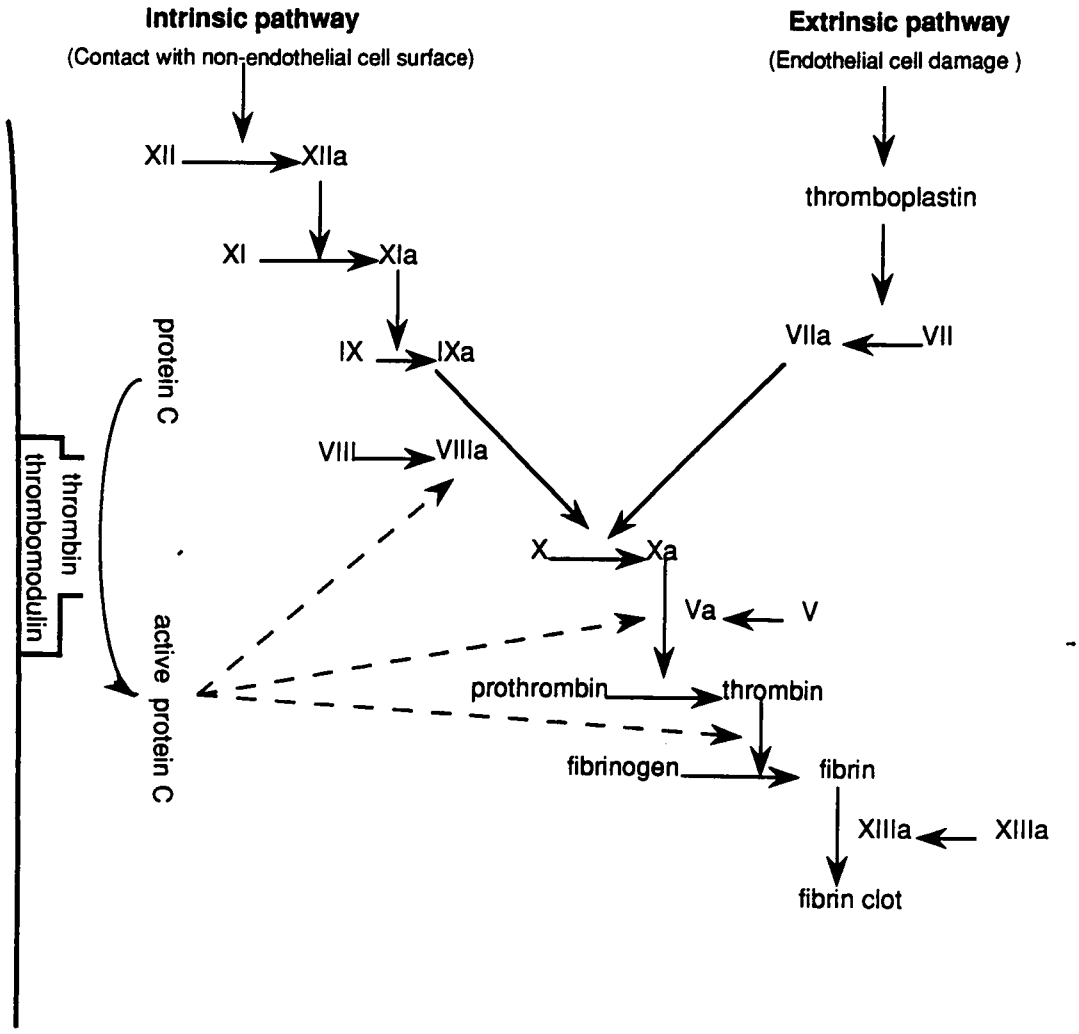


Figure 0.1. The Coagulation Cascade

--- dotted lines depict inhibitory reactions

VIII in the same manner. Factor VIIIa converts factor X to factor Xa, which enzymatically cleaves prothrombin to thrombin. Thrombin then cleaves fibrinogen, to form fibrin, which is the main component of the fibrin clot (see Figure 0.1). Like many of the other coagulation factors, thrombin also serves in a feedback loop to activate some cascade proteases. Thus thrombin can also activate platelets, factors XIII, V (Foster *et al.*, 1983; Nesheim *et al.*, 1979), and VIII (Steitz *et al.*, 1969; Fujikawa *et al.*, 1974; Robertus *et al.*, 1972) which, in turn, lead to the production of even more thrombin. Like the other coagulation factors, prothrombin circulates in the blood at levels higher than are necessary for clot formation (Waltz *et al.*, 1986; Mann *et al.*, 1988). Therefore, there is the potential for excess clotting that could lead to blood vessel occlusion. This does not happen under normal circumstances because of several regulatory mechanisms that act in opposition to the coagulation cascade. The tissues outside of the vasculature lack any means of clot inhibition, and therefore clotting is not hindered when blood flows into these tissues following vessel injury.

Endothelial cells secrete several proteins which inhibit the formation of clots. These proteins include prostacyclin, which inhibits platelet activation (Moncada *et al.*, 1976); endothelial cell-derived relaxing factor (Moncada *et al.*, 1988), which inhibits vasoconstriction (Bauer and Rosenberg, 1987; Hultin and Nemerson, 1978; Hultin, 1982); and plasminogen activator, which leads to plasmin formation and lysis of the fibrin clot (Comp *et al.*, 1981). Proteoglycans

containing heparin on the endothelial cell surface can accelerate antithrombin III activation which neutralizes thrombin by irreversible binding (Bauer and Rosenberg, 1987).

In addition to the mechanisms mentioned above, the endothelial cell surface contains TM, which can inhibit clotting at several points in the clotting cascade. First, TM forms a high affinity, 1:1 stoichiometric complex with thrombin with a Kd of 0.5 nmole/L (Dittman and Majerus, 1990; Esmon *et al.*, 1982b). This binding directly inhibits the ability of thrombin to convert fibrinogen to fibrin, activate platelets, and activate Factor V (Esmon *et al.*, 1982a; Esmon *et al.*, 1983a; Maruyama *et al.*, 1985; Jakubowski *et al.*, 1986; Polgar *et al.*, 1986). Analysis of this mechanism has shown that binding of thrombin to TM changes the electron spin resonance structure of the thrombin active site, indicating a conformational change (Musci *et al.*, 1988). TM has also been demonstrated to inhibit cleavage of prothrombin (Thompson and Salem, 1986, see Figure 1). Once thrombin is bound to TM, the complexed proteins in the presence of Ca^{++} can enhance the protein C anti-coagulation pathway.

The protein C anti-coagulation pathway is the most effective of the clot inhibitory mechanisms because it interferes with a number of the clotting factors. Once activated, protein C catalyzes the proteolytic inactivation of the regulatory factors Va and VIIIa. Factors Va and VIIIa are essential for the production of factors IXa and Xa; therefore, their inactivation is a strong inhibitor of coagulation (Suzuki *et al.*, 1983). Active protein C also stimulates

the release of plasminogen activator, which leads to fibrinolysis (Comp and Esmon 1981; Comp *et al.*, 1982), and inhibits the action of plasminogen activator inhibitor (Sakata *et al.*, 1985). Protein C can be activated directly by thrombin (Kisiel *et al.*, 1976; Kisiel, 1979; Amphlett *et al.*, 1981); however, it is activated 1000 times more effectively by the TM/thrombin complex (Esmon and Owen, 1981). In this manner, TM is able to convert thrombin from a procoagulant protease to a potent anticoagulant, by removing thrombin from the circulation and activating the anti-coagulant, protein C.

There are several other pathways by which clotting may be inhibited by TM. The TM/thrombin complex can be endocytosed leading to lysosomal degradation of thrombin and recirculation of TM, so that thrombin is no longer available for the coagulation cascade of reactions (Maruyama and Majerus, 1985). Rabbit TM has also been shown to exhibit heparin cofactor activity. Heparin is a proteoglycan that acts as a cofactor for antithrombin III, which inhibits the conversion of prothrombin to thrombin (Bourin *et al.*, 1986). This heparin activity has not been demonstrated in human, mouse or bovine TM (Jakabowski *et al.*, 1986). This may be due to the fact that neither human nor mouse TM contain the serine-glycine-X-serine signal sequence necessary for heparin like glycosaminoglycan attachment (Bourdon *et al.*, 1987).

A complete cDNA for human (Wen *et al.*, 1987), and incomplete cDNA for mouse (Dittman *et al.*, 1988) TM have been cloned and sequenced. A partial cDNA for bovine TM has also been cloned and sequenced (Jackman *et al.*,

1986). The genomic sequence for human TM has been determined (Shirai *et al.*, 1988), and the gene for TM has been localized to chromosome number 20 (Wen *et al.*, 1987). In both humans and mice this gene has been found to lack introns (Dittman *et al.*, 1988; Jackman *et al.*, 1987). Other known intronless genes include the hamster β-adrenergic receptor gene (Dixon *et al.*, 1986), mammalian α- and β-interferon genes (Nagala *et al.*, 1980), and some mammalian heat shock proteins (Mues *et al.*, 1986). It has been speculated that the purpose of intronless genes is to allow for rapid product synthesis due to a shortened mRNA processing time (Dittman and Majerus, 1990).

Purified human placental TM, when analyzed on SDS-PAGE, migrates as a protein with an apparent molecular weight of 75 KDa. Under reducing conditions this changes to 105 KDa, which is interpreted to indicate a secondary structure containing disulfide bonds (Salem *et al.*, 1984). Similar results have been obtained with isolated bovine TM (Esmon *et al.*, 1982b). Comparison of TM to the deduced amino acid sequence of other cloned genes has shown that the structural organization of TM is very similar to that of the low density lipoprotein (LDL) receptor (Jackman *et al.*, 1986, Shirai *et al.*, 1988), although no specific lipid binding function for TM has been demonstrated.

The amino-terminal methionine of the TM protein is followed by a leader sequence that consists of eighteen hydrophobic amino acids that are absent from the mature protein (see Figure 0.2). This leader sequence is thought to be a signal sequence for proper transport or processing of the molecule. The

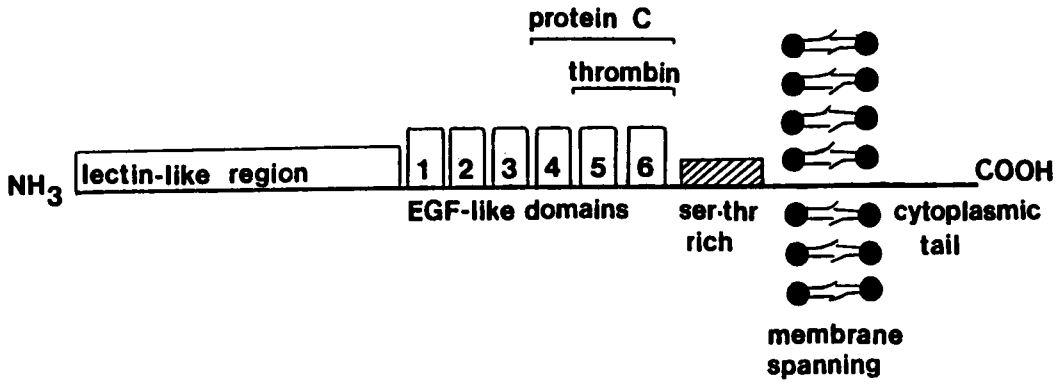


Figure 0.2. Schematic of the Thrombomodulin protein

amino-terminal 223 amino acids of the mature protein shares 20% homology with the hepatic asialoglycoprotein receptor and 31% homology with the rat cartilage proteoglycan (Peterson, 1988). Within this region, there are large areas of conservation between the human and mouse TM amino acid sequence, suggesting a conserved functional domain; however, this putative function remains obscure.

The next region of TM contains six epidermal growth factor (EGF)-like repeat domains (Doolittle *et al.*, 1984). Proteolytic digestion and isolation of fragments containing different groups of EGF-like repeat domains have demonstrated that EGF-like repeats 4, 5 and 6 are necessary for protein C activation (Stearns *et al.* 1989). Specific deletion of EGF domains from TM DNA and expression of deleted mutants in tissue culture cell lines have confirmed these results (Zushi *et al.*, 1989; Hayashi *et al.*, 1990). These methods also show that the thrombin binding site of TM requires EGF-repeats 5 and 6 (Kurosawa *et al.*, 1988; Suzuki *et al.*, 1989). The EGF-like regions of TM may function as a post-translational modification signal. It has been demonstrated that beta-hydroxylation of aspartic acid and asparagine occurs on some EGF-like repeat-containing coagulation factors (Fernlund and Stenflo, 1983) and on the LDL receptor (Stenflo *et al.*, 1988). EGF-like repeats 3 and 6 of rabbit TM contain the consensus sequence for beta-hydroxylation (Ohlin *et al.*, 1988). A beta-hydroxy aspartic acid residue has also been found in bovine TM (Stenflo *et al.*, 1988). The region following the EGF-like repeats

contains the lowest conservation of amino acid sequence among TM's found in different species. This region is a short serine-threonine rich region containing putative sites for O-linked glycosylation. Following this region is the putative transmembrane region which is composed of 23 hydrophobic amino acids.

Finally the cytoplasmic tail (38 amino acids) has been shown to have several potential phosphorylation sites. Phosphorylation has been associated with an increase in endocytosis and degradation of TM (Dittman *et al.*, 1988). Asparagine-linked sugar residues sites have been found in the cytoplasmic tail of TM (Stearns *et al.*, 1989).

There is conservation of asparagine-linked glycosylation sites in the mouse and human TM sequence at human amino acid residue number 115, and in human, mouse, and bovine TM at human residue 420. The rest of the putative glycosylation sites are not conserved (Dittman and Majerus, 1989). In both human and murine TM, there are two potential N-linked glycoslyation sites in the EGF-like domains (Jackman *et al.*, 1987).

A soluble form of TM has been isolated from human blood and urine (Ishii and Majerus, 1985). The apparent molecular weight of soluble TM is 85 KDa when determined by SDS-PAGE on reduced samples, indicating loss of part of the protein (Ishii and Majerus, 1985). The soluble form is believed to represent a truncated form of TM that is lacking the cytoplasmic tail and the transmembrane region, however, this has not been proven. Soluble TM may be formed by proteolytic cleavage of bound TM. *In vitro* studies indicate that

the soluble form can function in the same manner as the transmembrane form, except that soluble TM requires 3-5 times the amounts of thrombin for similar activation of protein C (Parkinson *et al.*, 1990). As the serum concentration of soluble TM is very low, it is unlikely that soluble TM exerts much effect on circulating thrombin levels.

Northern blot analysis for TM mRNA has found a single mRNA species of about 3,700 bases in mice and humans. In humans, the mRNA contains a 146-base 5' untranslated region, a 1,725-base coding region, a 3' untranslated region of 1,779 bases, and a 40-base poly A tail (Wen *et al.*, 1987). The 3' untranslated region is highly conserved (87% over 167 nucleotides) between human and mouse and is hypothesized to be involved in governing the rate of translation or message stability (Dittman and Majerus, 1990). In all species examined the 3' region contains the conserved sequence TTATTTAT. This sequence is thought to be associated with short mRNA half-lives (Shaw and Kamen, 1986). However, the half-life of mouse TM mRNA has been determined to be about 9 hours (Dittman *et al.*, 1988), which is rather long; therefore, this sequence does not appear have much effect on the half-life of mouse TM. Perhaps other binding functions in the long 3' untranslated region may be the reason for maintenance of this site.

The 5' genomic promoter region of the TM gene has been cloned and sequenced in mice (Dittman *et al.*, 1991, Genbank accession # M74231) and in humans (Jackman *et al.*, 1987). In mice, the TATA box is located at position -

33 and a CAAT box is found at position -116. The murine promoter region also contains five putative retinoic acid response elements of 4 nucleotides, which are located at positions -2225, -2147, -2022, -1781, and -545 (Dittman *et al.*, 1991, Accession # M74231). RNAse protection and primer extension assays have mapped the transcriptional start point to a position approximately 170-bp upstream of the initiation codon (Bird *et al.*, 1992) which, correlates well with cDNA data. In humans, a probable cap site is located at position -164, the TATA box is located at position -198 and the CAAT box is found at position -283. In addition to these transcriptional regulators in humans, three GGGCGG hexanucleotide sequences and one complimentary sequence (CCCGCC) are located at positions -302, -314, -338, -380, and -443 (Jackman *et al.*. 1987). These sequences are potential regions for SP1 interaction (McKnight and Tjian, 1986), which may regulate transcription (Dynan and Tjian, 1985).

Thrombomodulin has been isolated and studied in tissues from humans (Maruyama *et al.*, 1985; Salem *et al.*, 1984), rabbits (Esmon and Owen, 1981; DeBault *et al.*, 1986a), mice (Dittman *et al.*, 1988), and cows (Suzuki *et al.*, 1986), as well as in a variety of tissue culture cell lines (DeBault *et al.*, 1986b). Initially, TM was defined as an endothelial cell surface protein. Immunological analysis of rabbit tissues demonstrated the presence of TM on the blood vessel endothelium of all tissues studied (DeBault *et al.*, 1986a). According to this study, the tissue containing the highest amounts of TM was the lung, followed

by the heart and intestinal vessels. Thirteen different endothelial cell lines derived from the rabbit vasculature tested positive for TM expression (DeBault *et al.*, 1986b). TM has been found in the brain of rabbits (DeBault *et al.*, 1986), calves, and humans (Boffa *et al.*, 1991b). However it has not been detected in the central nervous system of mice (Maruyama *et al.*, 1985). Previous studies using human tissues showed that TM was absent from the human brain (Ishii *et al.*, 1986). These contradictory results are probably due to different methods of tissue fixation (Boffa *et al.*, 1991b). TM has been observed in the blood vessels and lymphatics in the syncytotrophoblast of human placenta (Maruyama *et al.*, 1985). For human umbilical vein endothelial cells (HUVECS), the number of TM molecules has been determined to be 30,000 to 55,000 per cell (Maruyama and Majerus, 1985). TM has been found on the surface of human platelets at a concentration of 60 molecules per platelet (Suzuki *et al.*, 1988). The small (85 KDa) form of TM has been found in human plasma at a concentration of 20 ng/mL (Ishii *et al.*, 1988). TM has recently been identified using a monoclonal antibody (MoAb) to human TM (IgG-TM) on human blood monocytes and macrophages in the synovial tissue lining (McCachren *et al.*, 1991).

The locations mentioned thus far are all vascular regions where one would expect to find an anticoagulant molecule. However, TM has also been localized to the squamous epithelium of epidermis using immunohistochemical methods (Yonesawa *et al.* 1987). TM has been found on a variety of cultured cells

including Chinese hamster ovary cells, NIH 3T3 cells (Dittman *et al.*, 1988), and A549 small cell lung cancer cells (Maruyama and Majerus, 1985).

Two MoAb, R1-242 and R1-1622, to murine TM were used to study TM distribution in Day 7.5 embryos (Imada *et al.*, 1987). This work demonstrated the presence of TM on the parietal endoderm of the placenta (Imada *et al.*, 1987). A polyclonal antibody was used to localize TM expression to the lung bud and neural epithelium of Day 10.5 embryos (Imada *et al.*, 1990a).

TM is subject to several different regulatory mechanisms. It has been demonstrated that internalization is one method by which the concentration of TM on the cell surface is controlled. The binding of thrombin to TM on A549 lung cancer cells induces endocytosis of the protein complex, degradation of thrombin in the lysosomes, and return of TM to the cell surface (Suzuki *et al.*, 1988). This process may be inhibited by the presence of inactive protein C, but not by active protein C, indicating that endocytosis cannot occur until protein C is activated and released into the blood (Maruyama *et al.*, 1984, Maruyama and Majerus, 1987). In hemangioma cells, addition of phorbol myristic acetate (PMA) induced TM endocytosis in the absence of thrombin and led to the degradation of TM (Dittman *et al.*, 1988). HUVECS treated with PMA demonstrated an initial loss of surface TM as indicated by a loss of protein C activity, followed by an increase in TM levels. This observation is consistent with the endocytosis of TM, followed by increased mRNA levels and protein synthesis of the molecule (Hirokawa and Aoki, 1990). Some endothelial cell

lines that have been tested did not exhibit the internalization of TM. This may reflect differences in the functions of the endothelial cells or an artifact of the different methods of measuring TM levels.

One of the cytokines, tumor necrosis factor (TNF), also exhibited a regulatory effect on TM through several pathways. TNF induced the production of the procoagulant, tissue factor, thus reducing the effectiveness of TM and protein C (Naworth and Stern, 1986). This was followed by an increase in the endocytosis and lysosomal degradation of TM (Moore *et al.*, 1989). Additionally, TNF has been shown to inhibit the transcription of TM mRNA (Conway and Rosenberg, 1988). Two other cytokines, interleukin-1 and endotoxin, decreased the activity of TM (Naworth *et al.*, 1986; Moore *et al.*, 1987); however, their mode of action has not been elucidated. Cyclic-AMP treatment of HUVECS (Maruyama *et al.*, 1991; Hirokawa and Aoki, 1991) and Chinese hamster ovary cells (Imada and Imada, 1982; Imada *et al.*, 1980) led to an increase in TM protein levels as determined by two-dimensional gel electrophoresis. HUVECS treated with retinoic acid also exhibit increased TM protein levels as determined by the enzyme-linked-immunosorbant-antibody method (Horie *et al.*, 1992).

Studies of mouse hemangioma cells have shown that TM transcription and synthesis were increased in the presence of exogenous thrombin. Treatment of these same cells with cycloheximide also led to an increase in transcription of TM (Dittman *et al.*, 1989). This evidence has been interpreted to suggest the

presence of a labile protein repressor for TM (Dittman *et al.*, 1989). Such a repressor might explain how TM expression in specific tissues is controlled, but one has not been isolated. Thrombin has also been shown to increase TM mRNA and protein levels in a hemangioma cell line (Dittman *et al.*, 1989).

While there are no known disorders that result from deficiencies or abnormalities of TM, TM has been shown to have some useful clinical applications. Both purified full-length TM and solubilized recombinant TM (lacking the transmembrane region and cytoplasmic tail) have been used to reduce clotting induced by injected thrombin in the mouse (Kumada *et al.*, 1988; Gomi *et al.*, 1990). Similar results have been obtained in rabbits (Ehrlich *et al.*, 1990) and rats (Solis *et al.*, 1991). The half-life of injected TM was found to be 3-7 hours, which is much greater than the 30 minute half-life of protein C (Ehrlich *et al.*, 1990), which has been used to inhibit clotting *in vivo* (Dittman, 1991; Taylor *et al.*, 1987). Therefore, TM may be a better choice for clot inhibition in humans.

TM may be useful as a target for drug delivery to the lung. Intravenous injection of MoAb to TM leads to rapid accumulation in the lung vasculature (Hughes *et al.*, 1989). The antibodies remain in the lung for an extended period (up to one week) and demonstrate a 24 hour half-life (Hughes *et al.*, 1989). MoAb coupled to liposomes directs efficient localization to the lung vasculature (Hughes *et al.*, 1989) where they are endocytosed into the vessel endothelial cells (Holmberg *et al.*, 1990). Experiments are under way to test if

this MoAb/liposome delivery system may be used to deliver anti-cancer drugs to the lung vasculature.

Thrombomodulin has also been demonstrated to have some useful diagnostic properties. An increase in plasma TM levels is indicative of endothelial injury (Dittman 1991; Ishii *et al.*, 1991; Boffa *et al.*, 1991a; Iwashima *et al.*, 1990). Increases in plasma TM levels have also been found to be a good indicator of renal dysfunction (Yoshida *et al.*, 1990), disseminated intravascular occlusion, adult respiratory distress syndrome, disseminated intravascular coagulation, and acute hepatic failure (Takano *et al.*, 1990). Three MoAbs to human TM, KA-2, KA-3, KA-4, have been isolated to facilitate these studies (Takano *et al.*, 1990). Immunohistochemical staining of various tumors indicates that TM may also be a good marker for endothelial cell derived vascular tumors (Yonezawa *et al.*, 1986).

Thus TM is a well studied molecule with potential for both therapeutic and diagnostic application. This dissertation work was aimed at furthering our knowledge about TM expression and function.

CHAPTER 1

Thrombomodulin is Preferentially Expressed in Lung Microvessels

Summary

Two rat monoclonal antibodies were previously developed that bind different epitopes on a murine glycoprotein, P112, which is expressed primarily in lung capillary endothelium. In this chapter we show that P112 is identical to the endothelial anticoagulant protein, thrombomodulin (TM). Several lines of evidence support this conclusion. First, amino acid analysis of P112 shows a high degree of homology to TM and both molecules exhibit the same mobility in gel electrophoresis. Second, P112 and TM share reactivity for two different monoclonal antibodies. Third, purified P112, like TM, acts as a cofactor for protein C activation.

Quantitative analysis of TM (P112) expression using a two-site monoclonal antibody assay demonstrates that significantly higher levels of TM are found in lung in comparison to other highly vascularized organs, i.e. the kidney and liver. Quantitative Northern blot data correlate with the two-site assay data and demonstrate that the high level of TM expression in lung is not due to preferential binding of the monoclonal antibodies to lung TM but rather to increased production of TM mRNA in the lung relative to other highly vascularized organs. It is suggested that expression of TM is highest in cells from continuous endothelium.

Introduction

Thrombomodulin (TM) is an endothelial cell, transmembrane glycoprotein, which was discovered on the surface of the aorta by Esmon and Owen (1981). The main function of TM is to bind to the procoagulant protease, thrombin, thereby changing it to an anti-coagulant (Dittman and Majerus, 1990; Esmon, 1987). Thrombomodulin acts as a cofactor for the activation of protein C (a serine protease that inactivates factor Va and VIIIa) (Esmon and Owen, 1981; Fulcher *et al.*, 1984; Kisiel *et al.*, 1977). Thrombomodulin removes thrombin from the circulation and hinders its ability to activate platelets and cleave fibrinogen (Esmon *et al.*, 1982a; Esmon *et al.*, 1983a). Thrombomodulin also inhibits factor Xa cleavage of prothrombin (Thompson and Salem, 1986).

P112 is defined by immunoprecipitation with two rat monoclonal antibodies (MoAbs), 411-201B and 273-34A (Kennel *et al.* 1987; Rorvik *et al.*,1988). Previous work with these MoAbs has demonstrated that P112 is found on the endothelial cell surface of the lung microvessels (Rorvik *et al.* 1988). These MoAbs accumulate preferentially in the lung when injected into the tail veins of mice (Kennel *et al.*, 1990), and can be used to target liposomes to the lung (Hughes *et al.*, 1989; Maruyama *et al.*, 1990). Data in this chapter show that P112 is identical to TM. Previous to this study, the best data available concerning the relative levels of TM throughout the vasculature have been collected using immunofluorescence which is difficult to quantify (DeBault *et al.*, 1986a). MoAb 273-34A and 411-201B bind to different epitopes on P112(TM)

and form the basis of a two-site assay (Kennel *et al.*, 1988). The two-site assay is a highly specific, quantitative assay that enables accurate measurement of the levels of TM in organs with different endothelial cell types. Data in this chapter show that the tight-junction endothelium of lung expresses more TM than does endothelium in kidney or liver, indicating that anti-thrombolytic action may be accomplished by different means in different types of capillary beds.

Materials and Methods

Antibodies

MoAbs 273-34A and 411-201B are rat subclass IgG_{2a}, the binding specificities of which have been previously described (Kennel *et al.*, 1987; Rorvik *et al.*, 1988). The origin and specificity of rat MoAb 135-13C has also been previously described (Kennel *et al.*, 1981; Kennel *et al.*, 1989). Rat MoAb 135-14, used as a negative control, was isolated from the same fusion as MoAb 135-13C, but has no known binding activity.

Polyclonal antibody to P112 was prepared in New Zealand white rabbits by subcutaneous injection of 10 μg purified P112 in complete Freund's adjuvant. Three booster injections in incomplete Freund's adjuvant were given at two week intervals before serum collection. The antiserum specifically immunoprecipitated a single major protein band corresponding to P112 from a lysate of ^{125}I-lactoperoxidase surface-labeled, normal mouse lung cells.

Cell Lines

The normal mouse lung cell line (NMLC) was generated from a mixed population of dissociated BALB/cBD normal mouse lung cells. The line has not been cloned and has been continuously passaged for approximately 150 bi-weekly subcultures (Kennel *et al.*, 1988).

Amino Acid Analysis

P112 was purified from a detergent lysate derived from a BALB/cBD lung microsome fraction by immunoaffinity chromatography as described previously (Kennel *et al.*, 1988). P112 was subjected to NH_2-terminal amino acid sequence analysis on an Applied Biosystems 470A gas phase sequencer as previously described (Kennel *et al.*, 1989). Additionally, cyanogen bromide fragments of P112 were generated by overnight incubation at a protein:cyanogen bromide ratio of 2:1 (Laemmli, 1970). Peptides were separated on a 12% polyacrylamide gel in sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970) and transferred to Immobilon polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) membranes for sequence analysis (Masudaira, 1987).

P112 Purification and Analysis

MoAbs were coupled to Sepharose 4B (Pharmacia, Uppsala) to final concentrations of 10 mg/ml (MoAb 411-201B and MoAb 135-13C) and 25 mg/ml (MoAb 273-34A) as previously described (Kennel *et al.*, 1986). A protein extract was made from phosphate buffered saline (PBS) perfused BALB/cBD lungs. The lung tissue was taken up in a lysis buffer (10 ml/g wet

weight tissue)(PBS) containing 0.5% NP-40, 0.01% phenylmethylsulfonylfluoride (PMSF), 20 μg/ml leupeptin, and 0.01 M sodium azide and homogenized in a Polytron (setting #6) (Brinkman Instruments). The insoluble material was removed by serial centrifugations for 10 min at 200g, 10 min at 1200g, and 1 hr at 150,000g. The lysate containing the soluble material was divided into three five-ml portions and incubated batchwise with 100 μl of the MoAb coupled Sepharose at 4C overnight. After five washes with 2 mls PBS the Sepharose was treated with 1 ml of 3M KSCN containing 0.05% NP-40 to elute the antigen. Eluates were dialyzed against H_2O for two hrs, dried in a Speed Vac Concentrator (Savant) and redissolved in 1 ml H_2O. All three lysate samples were exposed to the MoAb-containing Sepharose twice, and the amount of P112 left in the lysate following each extraction was determined by a MoAb two-site assay.

The treated lysates and eluates were analyzed on SDS-PAGE followed by Western blotting (Kennel *et al.*, 1988). Briefly, proteins in gels were transferred to a PVDF membrane in a Hoefer Scientific TE mini transfer electrophoresis unit for 1.5 hrs at 200 mA. The protein blot was blocked for 1 hr at room temperature with blotto (20% fetal calf serum, 10% non-fat dry milk in PBS containing 0.01% Tween 20). The blot was then incubated for 1 hr at room temperature with a 1/200 dilution of rabbit anti-rat TM antiserum (a gift from Dr. PW Majerus, St. Louis, MO) (Lollar and Owen, 1980) in blotto. The secondary antibody, horse radish peroxidase conjugated goat anti-rabbit IgG

(Biorad, Richmond, CA) was also incubated for 1 hr in blotto. Detection of immunologically reactive protein bands was accomplished with diaminobenzamine and H_2O_2. SDS-PAGE high molecular weight markers (Biorad, Richmond, CA) were stained with 0.2% Amido Black.

Immunoprecipitation

Immunoprecipitation was accomplished as previously described (Kennel *et al.*, 1987). Briefly, confluent dishes of NMLCs were surface radiolabeled with ^{125}I using lactoperoxidase. Labeled cells were solubilized in 0.5% NP40, 0.01% PMSF, 20 μg/ml leupeptin, and 0.01 M sodium azide. The lysates were then treated, individually, with MoAb 411-201B to TM, rabbit anti-P112/TM polyclonal serum, and rabbit anti-rat TM serum. Normal rabbit serum and normal rat serum served as negative controls. Protein immunoconjugates were precipitated from solution using secondary polyclonal antisera. Immunoprecipitates were analyzed on 8% SDS-PAGE.

Protein C Activation Assay

Human Protein C (1.32 μg) (American Diagnostica Inc., Greenwich, CT) was incubated with 1.43 μg of bovine thrombin (Sigma, St. Louis, MO) and approximately 1μg of immunopurified P112 or bovine thrombin purified TM (Salem *et al.*, 1984) (a gift from Dr. Xiang Gao, Knoxville, TN). A negative control for protein C activation was carried out without the addition of any of the other components. A control for thrombin activated protein C was also included. Activation was carried out in 0.01M Tris-HCl pH 7.4 buffered saline

(0.15M) containing 0.25 mM Ca^{++} at 37C for 1 hr in a total volume of 100 μL (Kurosawa and Aoki, 1985). The activated protein C was assayed for amidase activity on the substrate Boc-Leu-Ser-Thr-Arg-MCA (Sigma, St. Louis, MO). Incubation with the substrate (50 nM) was carried out at 37C for 10 min and AMC (7-amino-4-methyl coumarin) liberation was fluorometrically determined (excitation at 380 nm and emission at 460 nm). The amount of AMC liberated was determined using a standard curve constructed with AMC (Sigma, St. Louis, MO).

Two-site Assay for P112/TM

The two-site assay was performed essentially as previously described (Kennel *et al.*, 1988). Briefly, two μg of HPLC purified MoAb 273-34A diluted in 0.05 M sodium bicarbonate were bound at 4C overnight to each well of Immulon II Removawell strips (Dynatech, Broomall, PA) Homogenates of normal tissues were prepared as described in P112 purification and analysis. Fifty microliters of six two-fold dilutions of each tissue homogenate were incubated in duplicate in antibody coated wells for 2 hrs at 4C. Wells were washed with PBS and the bound antigen (P112) was detected by addition of 50 ng of ^{125}I MoAb 411-201B in 50 μl of 5 mg/ml BSA with 0.05% NP-40 for 2 hrs at 4C. Wells were washed, separated, and analyzed for ^{125}I. Plots of amount (ng) of ^{125}I MoAb bound versus wet wt (g) of tissue were constructed. For tabulation of data, the amount of ^{125}MoAb 411-201B bound/g of tissue

homogenate analyzed was determined at the point on the curve at which 1 ng of ^{125}I MoAb was bound.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Kennel *et al.*, 1988). Briefly, tissues were fixed for 24 hrs in buffered formalin, embedded in paraffin and sectioned at approximately 5 μm thickness. Sections were deparaffinized in xylenes, rehydrated through graded alcohol, blocked with normal serum and incubated with 0.1 μg/mL of ^{125}I labeled antibody. Slides were washed in PBS and H_2O, dried, dipped in Kodak NTB-2 emulsion, and developed after two weeks in the dark.

cDNA Analysis

Polyadenylated RNA was isolated from BALB/cBD lung tissue (see RNA analysis) and the mRNA was sent to Clontech Laboratories for conversion into a λ gt11 "cDNA stretch" library (Clonetech Labs, Palo Alta, CA). The library was screened with the polyclonal rabbit anti-mouse P112 antiserum, according to the method of Young and Davis (1983). The isopropyl-thio-β-D galactopyranoside treated nitrocellulose filters were incubated on the plates for 5 hrs at 37C. Bound antibody was detected as for Western blot analysis. Positive plaques were picked and cloned.

The two cloned phage stocks (P112.1 and 15Ab.2) were amplified and λ DNA was prepared by the plate-lysate method (Sambrook *et al.*, 1989). Purified λ DNA was digested with *Eco*RI (Gibco BRL, Gaithersburg MD) and

analyzed by agarose gel electrophoresis. Insert cDNA was isolated from agarose and subcloned into the *Eco*RI site of pGEM-7Z- (Promega, Madison, WI). DNA prepared from clones containing the insert (Morelle, 1989) was sequenced using Sequenase (United States Biochemical Corp, Cleveland, OH) according to the supplier's directions. Sequence comparison was performed using an IBM PC Model 50 and DNASIS (V3.00) software (Hitachi).

RNA Analysis

Total RNA was isolated from frozen BALB/cBD tissues by extraction with guanidine isothiocyanate followed by pelleting through 5.7 M CsCl (Kingston *et al.*, 1989). Polyadenylated RNA was isolated from a column containing 100 μL oligo dT cellulose. For Northern blot analysis 5μg of mRNA was run on a 1% agarose gel containing 2.2M formaldehyde in 10 mM sodium phosphate buffer for 5 hrs at 5 V/cm (Lehrach *et al.*, 1977). The RNA was transferred to Genescreen (New England Nuclear, Boston, MA) by blotting overnight in 20X SSC. After a 1 hr bake at 80C and 2 min of UV irradiation (258 nm) at 800 $\mu W/cm^2$, the blot was washed in 2 M NaCl, 10 mM Tris-HCL pH 8.0, 0.1% SDS for 20 min at 45C. Prehybridization and hybridization were carried out in 10 ml of 7% SDS, 0.5M sodium phosphate buffer pH 7.2, 2 mM EDTA, and 1% non-fat dry milk at 45C for 18 hrs (Church and Gilbert,1984). The DNA probe, a 200 bp *Eco*RI/*Bgl* I fragment from clone P112.1, was purified from Sea Plaque (FMC Bio Products, Rockland, ME) low melt agarose and labeled by random primer DNA synthesis with ^{32}P dCTP (New Research Products, Boston,

MA) and an oligo labeling kit (Pharmacia, Uppsala). This fragment spans position 1236 to 1435 in the cDNA sequence (Dittman *et al.*,1988). Markers were the 0.25 -9.5 kB RNA ladder (Gibco BRL, Gaithersburg, PA).

Band density on autoradiograms was analyzed on an LKB 2202 Ultroscan Laser Densitometer using the LKB Apple Gelscan program.

Results

Amino Acid Analysis

The amino terminal sequence obtained from immunopurified P112 matches the mature murine TM sequence deduced from cDNA (Dittman *et al.*, 1988) from position 19 through 32 except for one ambiguous amino acid at position 28 and the Cys residue at position 30 (not detected by sequence analysis without carboxymethylation). Position 19 in TM is the amino terminal amino acid that follows the 18 amino acid signal peptide. Sequence of a 42,000 kDa cyanogen bromide fragment was determined after purification by SDS-PAGE and transfer to PVDF membranes. This sequence matched the TM sequence from position 81 through 97 with the exceptions of positions 85 and 93, where ambiguous results were obtained, and position 94, which is a Cys residue. Position 80 is a Met residue, consistent with the cleavage chemistry of cyanogen bromide. Thus amino acid sequence data are consistent with the hypothesis that TM and P112 are identical.

Cross-reactivity of MoAb's to P112 with Thrombomodulin

Two MoAbs that react with different epitopes on the P112 molecule (Kennel *et al.*, 1988) were tested for reactivity with TM using immunoabsorption followed by Western blot analysis. Crude homogenates of BALB/cBD lung, a rich source of P112 and TM (Kennel *et al.*,1988; DeBault *et al.*,1986), were extracted with MoAb bound to Sepharose. The two anti-P112 MoAbs and a control MoAb (135-13C, which does not react with P112) were used. Two extractions with each MoAb were performed and the level of P112 remaining in the lysate after each extraction was determined by MoAb two-site assay (Table 1.1). After two treatments with either MoAb 273-34A or 411-201B, greater than 98% of the detectable P112 had been removed from the lysate while the control MoAb 135-13C had removed none of the P112. The increase in detectable levels of P112 in the control are most likely due to removal of extraneous protein after repeated freezing, thawing, and centrifugation. Western blot analysis of the depleted lysates and column eluates shows that removal of P112 from the lysate results in a loss of rabbit anti-rat TM reactivity (Fig. 1.1)(compare lanes 1, 2, and 3), whereas the MoAb 411-201B and 273-34A eluates are recognized by the antiserum against TM (lanes 5 and 6). TM reactivity is not immunoabsorbed with the negative control MoAb 135-13C (lane 4 and 7). In addition to this work, Western blot analysis shows that MoAb 273-34A reacts with authentic TM purified on immobilized thrombin (Salem *et al.*, 1984; Kurosawa and Aoki, 1985), and that both TM and P112

Table 1.1. P112 Extraction Experiment

Sample	P112 (ng MoAb bound/g wet wt)[a,b]	% P112 left in lysate
lysate control	35000	100
After 1st extraction with		
MoAb 273-34A	1800	5
MoAb 411-201B	11000	30
MoAb 135-13C	41000	120
After 2nd extraction with		
MoAb 273-34A	00	0
MoAb 411-201B	600	2
MoAb 135-13C	49000	150

P112 concentration/mL of BALB/cBD lung protein lysate as determined by MoAb two-site assay after extraction with Sepharose.

[a] Values for ng ^{125}I MoAb 411-201B bound/g tissue analyzed.

[b] Values listed fall within 10% confidence limits.

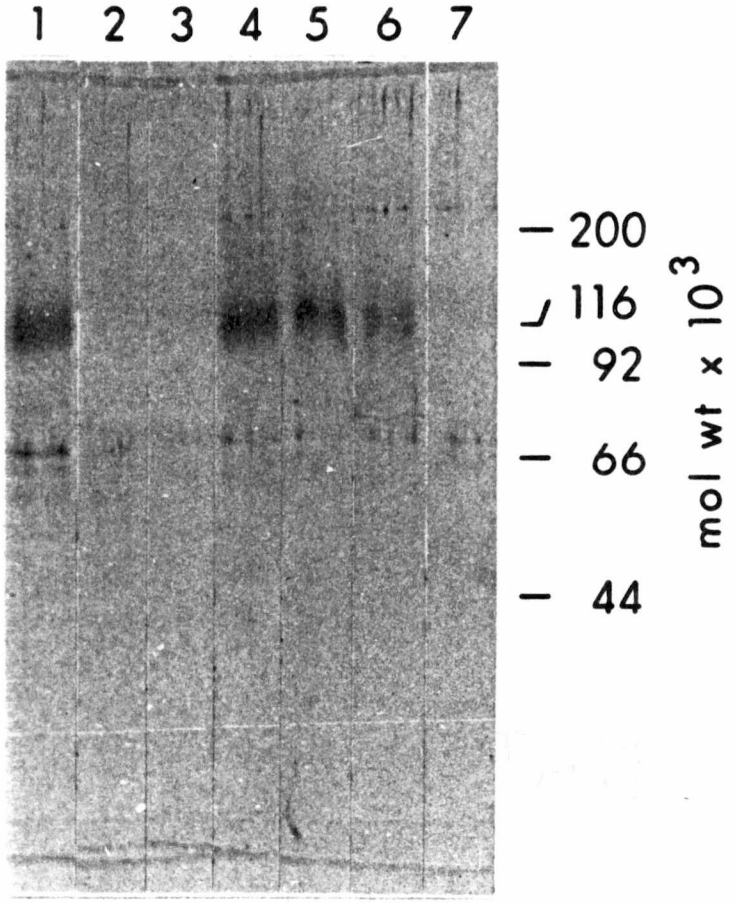


Figure 1.1. Western blot analysis of MoAb-coupled-Sepharose extracted BALB/cBD lung lysate. Blot stained with rabbit anti-rat TM antiserum. Lane 1, untreated lysate; lane 2, MoAb 273-34A treated lysate; lane 3, MoAb 411-201B treated lysate; lane 4, MoAb 135-13C treated lysate (negative control). Eluates from each Sepharose column are in lanes 5-7. Lane 5, MoAb 273-34A eluate; lane 6, MoAb 411-201B; and lane 7, MoAb 135-13C (negative control).

exhibit identical mobility on SDS-PAGE under reducing (112 kDa) (Fig. 1.2) or non-reducing (75 kDa) conditions. (data not shown).

Protein C Activation

An enzyme assay for TM has been developed (Kurosawa and Aoki, 1985), which measures a fluorescent compound generated by activated protein C. Immunopurified P112 was tested in this system for cofactor activity in the thrombin catalyzed activation of protein C. Results (Table 1.2) demonstrate that P112 is indistinguishable from TM as a cofactor for protein C activation. Thrombin alone, which slowly activates protein C in the absence of cofactor, was used to control for background levels of activation.

cDNA Cloning and Sequencing

BALB/cBD lung mRNA was used to prepare a λ gt11 cDNA expression library. After screening over 10^6 plaques with a rabbit anti-mouse P112 antiserum, two positive plaques were identified. These clones, P112.1 and 15Ab.2, were 950 bp and 1600 bp in length, respectively. Partial sequence analysis of these clones showed 100% identity to the published sequence for mouse TM (Dittman *et al.*, 1988). Clone P112.1 is identical to the TM sequence from bases 1241 to 1356, which translates into the fourth epidermal-growth-factor-like region of the mature protein. Clone 15Ab.2 matches the TM sequence from bases 719 to 774 and 2062 to 2157 (data not shown). The first region of homology falls within the sequence coding for the amino terminal lectin-like region of the molecule, whereas the second region is located within

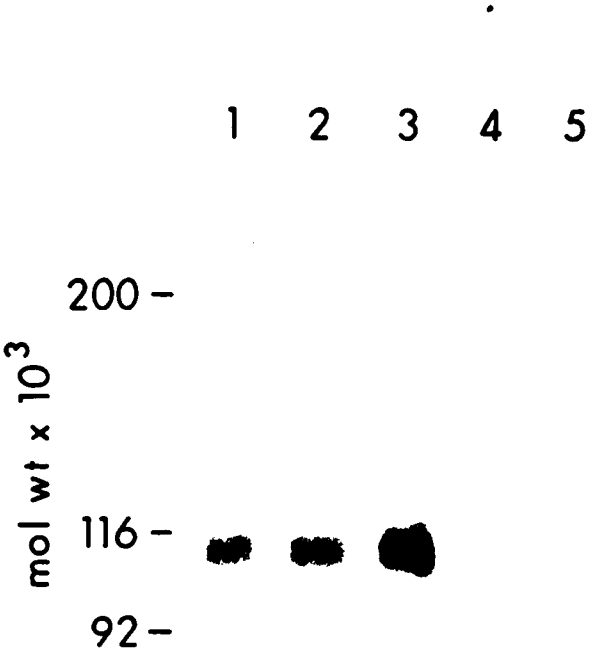


Figure 1.2. SDS-PAGE of immunoprecipitates of ^{125}I-labeled NMLC surface proteins solubilized with detergent. Lane 1, rabbit anti-rat TM treated lysate; lane 2, MoAb 411-201B treated lysate; lane 3, rabbit anti-P112/TM treated lysate; lane 4, normal rabbit sera control; lane 5, normal rat sera control.

Table 1.2. Activation of Protein C.

Incubation mixture	nM AMC released[a]
Protein C	0
Thrombin, Protein C	3.1[b]
Thrombin, Protein C Thrombomodulin	13.3
Thrombin, Protein C P112	13.2

[a] The amount of AMC released after a 10 min. incubation correlates to the levels of activated protein C.

[b] Thrombin can activate protein C independently of thrombomodulin.

the 3' untranslated region of the full length mRNA. Since this work demonstrated 100% identity to three different regions of the murine TM cDNA sequence, further sequence analyses were not done.

Two-site Assay for P112/TM.

The MoAb two-site assay has been used to quantify TM levels in nine organs from 8-12 week old female BALB/cBD mice (Table 1.3). Results show that the lung contains the highest levels of TM (110,000 ng of Ab bound/g wet weight), with the kidney (2900 ng of Ab/g wet weight), and heart (2100 ng of Ab/g, wet weight) having considerably lower values. Some data are available concerning the capillary surface area of some of the organs tested. Assuming that the relative capillary surface areas are similar in dogs, cats, and mice, this information can be used to calculate the levels of TM/capillary surface area (Table 1.3). This tabulation clearly shows the highest levels of TM (38 ng Ab/cm^2) in the lung. Kidney (3-8 ng Ab/cm^2), another highly vascularized organ, contains only one-fifth to one-twelfth the levels of TM in comparison to the lung. Other well-vascularized organs, such as the heart (4 ng Ab/cm^2) and brain (2 ng Ab/cm^2), contain only one-ninth and one-nineteenth the levels of TM, respectively.

Immunohistochemistry

Immunohistochemical staining of mouse lung sections using radiolabeled MoAb 273-34A shows that most of the TM is located within the alveolar capillaries and venules, with small amounts present in the larger arteries and

Table 1.3. Quantitation of P112/TM by Two-site assay and Northern Blot

Tissue	P112/TM[a] (ng MoAb/g wet wt)	mRNA Intensity	Surface Area (cm^2/g wet wt)	P112/TM (ng MoAb/cm^2)	Ref.
lung	110000	5200	3000[b]	38	(Wiebel, 1973)
intestine	2900	n.d.[c]	n.d.a.[d]	-	--
kidney	2800	100	350-820[b]	3-8	(Crone, 1984 Renkin and Gilmore, 1973)
spleen	6500	170	n.d.a.	-	--
uterus/ ovary	12000	330	n.d.a.	-	--
liver	500	n.d.	n.d.a.	-	--
heart	2100	90	500[b]	4	(Bassingthwaighte et al., 1974)
thymus	1900	n.d.	n.d.a.	-	--
brain	300	n.d.	150[e]	2	(Pawlik et al., 1981)

[a] Values for ng ^{125}I MoAb bound/g wet wt tissue analyzed at the point on the curve where 1 ng MoAb is bound.
[b] Surface area values calculated from dog organs
[c] Not determined
[d] No data available
[e] Surface area values calculated from cat organs

veins (Fig. 1.3, panel A). Staining was demonstrated to be specific since there is no staining in the negative control (panel B). Immunohistochemical staining of the kidney, another highly vascularized organ is also shown in Fig. 1.3. Most of the staining is found in the large renal arteries, with a much lesser degree of staining in the vessels of the medulla and cortex (panel C). Virtually no staining is present within the kidney glomeruli. The control antibody shows no staining in the kidney (panel D). Experiments to determine the microdistribution of different doses of i.v.-injected ^{125}I MoAb 273-34A show a similar distribution of TM expression (data not shown).

mRNA Analysis

The two-site assay for TM concentration shows that the lung contains considerably higher levels in comparison to other organs. One possible explanation for this result is that there may be different isoforms for TM in each tissue that are not recognized by the MoAbs, even though TM is encoded by a single copy, intronless gene (Jackman *et al.*, 1987). To test this possibility a Northern blot of mRNA isolated from different mouse organs was hybridized to a 200-bp restriction fragment from the cDNA clone P112.1. These data show that lung contains at least ten times more TM mRNA than the other tissues tested (Fig. 1.4). The relative intensity of mRNA bands in each organ mimics the relative protein levels found with the two-site assay (Table 1.3) in that lung has from 16 (uterus) to 5100 (liver) times the mRNA found in the other organs. Reprobing this blot with the human gamma actin message

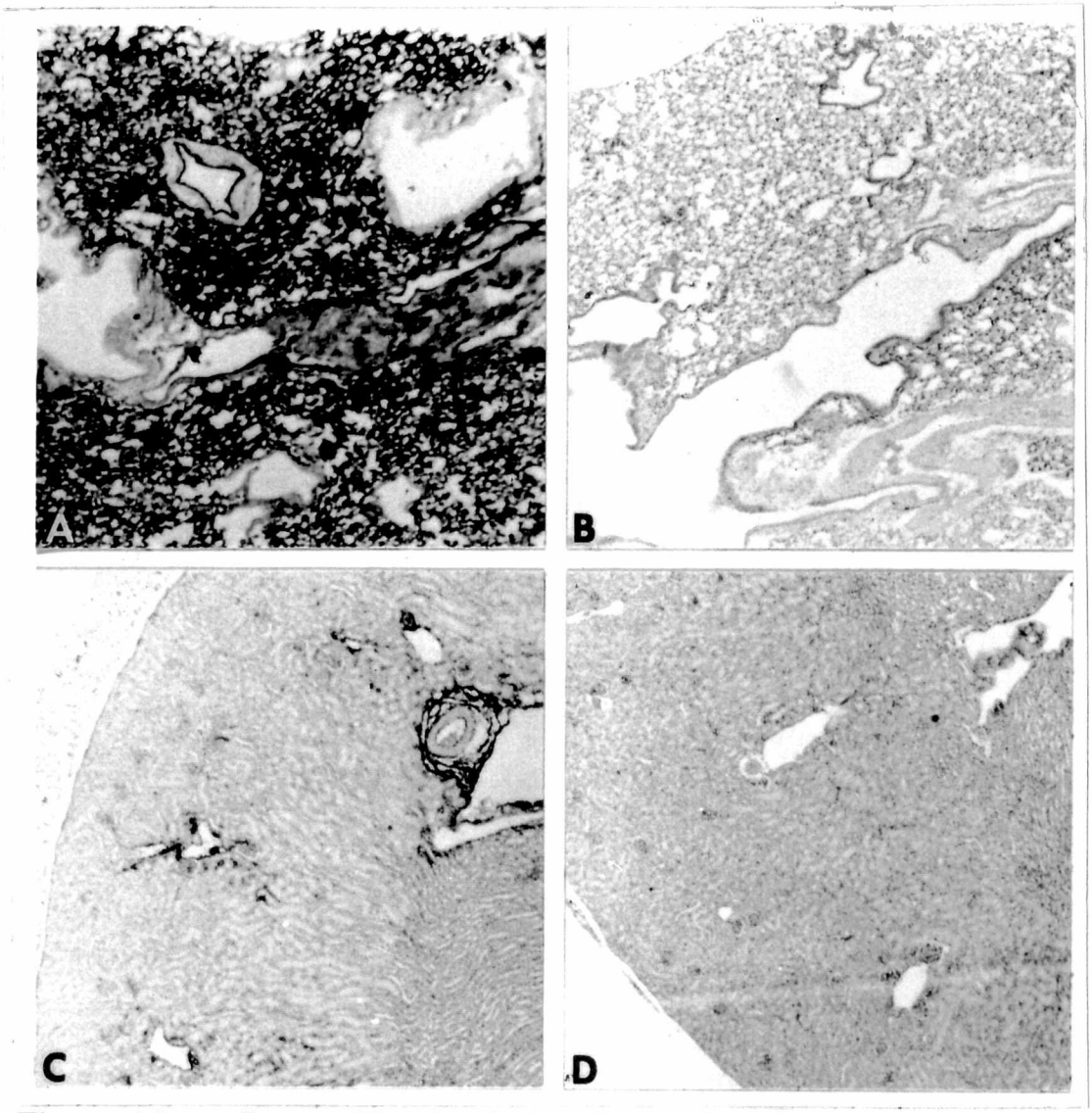


Figure 1.3. Immunohistochemical staining of buffered formalin fixed BALB/cBD lung and kidney. Panel A, lung section (25X magnification) stained with ^{125}I MoAb 273-34A, Panel B lung negative control stained with ^{125}I MoAb 135-14, panel C, Kidney section (25X magnification) stained with ^{125}I MoAb 273-34A, Panel D, kidney negative control stained with ^{125}I MoAb 135-14.

Note: ^{125}I MoAb 411-201B showed the same staining pattern as MoAb 273-34A.

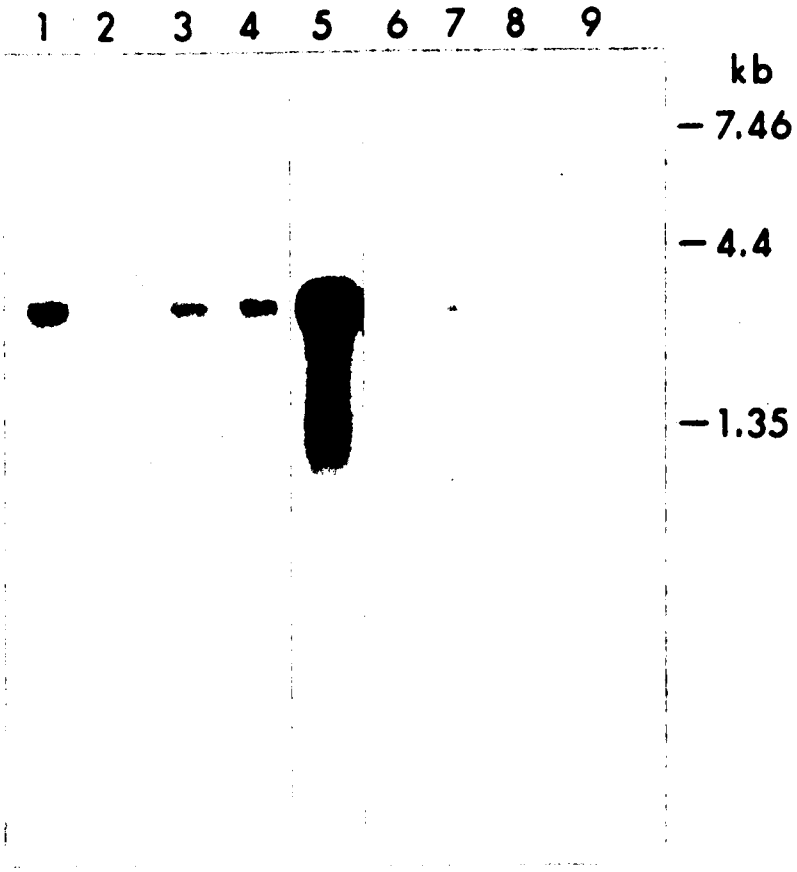


Figure 1.4. Northern blot analysis of RNA samples from murine tissues. Poly A RNA from uterus, 8 μg (lane 1), testes, 8 μg (lane 2), stomach, 8 μg (lane 3), spleen, 8 μg (lane 4), lung, 8 μg (lane 5), liver, 8 μg (lane 6), kidney, 7 μg (lane 7), intestine, 8 μg (lane 8), heart, 8 μg (lane 9). The blot was probed with a 200 bp, ^{32}P random hexamer primed labeled cDNA *Eco* RI/*Bgl* I digested fragment from clone P112.1.

(Gunning *et al.*, 1983) showed that each lane contained roughly equal amounts of mRNA.

Discussion

Thrombomodulin is an endothelial cell protein involved in maintaining blood homeostasis within the vasculature by regulating part of the anticoagulant pathway (Esmon and Owen, 1981; Dittman and Majerus, 1990; Esmon, 1987). We have demonstrated through several different methods, that the endothelial cell protein, P112, is identical to TM. First, it was shown that rabbit anti-rat TM reactivity is lost when P112 is specifically removed from a lung lysate, and that immunopurified P112 is recognized by the same anti-TM serum. These results strongly suggest that both MoAbs to P112 also recognize TM. Second, we have shown that MoAb 273-34A recognizes mouse TM purified with bovine thrombin and that P112 and TM show the same mobility on SDS-PAGE (data not shown). Finally, we demonstrated that P112 is indistinguishable from TM in an enzyme assay for protein C activation. Since it is known that the gene for TM is intronless and present in only one copy in both the human and mouse (Dittman and Majerus, 1990; Jackman *et al.*, 1987), it is unlikely that we have isolated a closely related yet different cDNA. We conclude that P112 and TM are the same molecule. Early work by Lollar and Owen (1980) demonstrated that the majority of injected radiolabeled thrombin could be found in the lung after 10 minutes circulation time. Our data on the distribution of radiolabeled

MoAb to TM (Kennel *et al.*, 1990; Hughes *et al.*, 1989) shows that distribution varies with dose and time of circulation. It is difficult to calculate the exact concentration of TM in various tissues from distribution data due to these and other variables. In addition to this work, immunohistochemistry has shown that TM is found on the surface of all endothelial cells in the rabbit vasculature (DeBault *et al.*, 1986). In the human, TM has been found on arteries, veins, and lymphatic vessels of the placenta (Maruyama *et al.*, 1985). While these studies demonstrate nicely where TM is found, they offer only estimates of TM concentration. Using the two-site assay, we demonstrate that the quantities of TM expressed in various organs do not correlate exactly with the estimates of endothelial surface area from these organs (Table 1.3).

If all endothelial cells express the same amount of TM, one would expect the lung and kidney to have similar levels of TM since both organs are highly vascularized. Comparison of TM levels to relative surface area demonstrates that the lung contains 5-12 times more TM/cm^2 surface area than the kidney. It seems unlikely that this could be due only to differences in the number of endothelial cells per organ. One possible explanation for this result is that there are different isoforms of TM in each tissue. Since the MoAbs used in the two-site assay were generated against lung protein, they may recognize lung-specific epitopes on TM and thus react with other TM isoforms to a lesser degree. If this were the case, we would expect the two-site assay to show that there was an abundance of TM in the lung but little in other tissues. To

eliminate this possibility, Northern blot analysis of nine organs was used to determine the relative amounts of TM mRNA in each tissue. A scan of the intensity of the TM mRNA band in each organ was used to estimate the levels of TM message in each organ. Comparison of the mRNA intensity data and the two-site assay data demonstrates a similar pattern of expression, which suggests that there is only one form of TM mRNA in all tissues and that the lung expresses the highest amount of TM mRNA.

Since all of the organs tested express some TM, these results cannot be explained in terms of organ-specific expression of proteins. Rather, we hypothesize the explanation lies in the type of endothelial cell on which TM is expressed. It is known that there are three different types of endothelium: continuous, fenestrated, and sinusoidal (Palade *et al.,* 1979). The lung, which contains the highest levels of TM, is composed of a continuous endothelium. The kidney also contains a mixture of continuous and fenestrated endothelium (Pino, 1989). Since most of the immunohistochemical staining for TM is found within continuous vessels (lung microvessels and large arteries of the kidney), we suggest that TM is expressed in greater quantities on the continuous endothelium as opposed to fenestrated or sinusoidal endothelium. An extension of this reasoning would indicate that if anti-thrombolytic activity is required in fenestrated and sinusoidal endothelium, it is likely to be mediated by a mechanism that does not involve TM.

CHAPTER 2

An Intracisternal A-particle DNA Sequence Interrupts the 5' Region of the Thrombomodulin Gene in some Strains of Laboratory Mice.

Summary

Sequence analysis of the 5' end of the thrombomodulin (TM) gene has identified a difference in two strains of laboratory mice, BALB/cBD (maintained at the Oak Ridge National Laboratory) and the strain used for the sequence listed in the Genbank database. An intracisternal A-particle (IAP) provirus was present in the BALB/cBD sequence but absent from the Genbank sequence. Thus, there are two different 5' regions for the TM gene in mice. Southern blot and polymerase chain reaction analyses on other mouse strains demonstrated the presence of this proviral sequence in five out of ten strains tested. Northern blot analyses demonstrated that there were differences (up to 3-fold) in the steady-state levels of TM mRNA among the strains. However, TM mRNA levels did not correlate directly with the presence of the intracisternal A-particle DNA upstream from the TM gene. This is in contrast to other loci (the interleukin-3 gene, c-*mos*, and the immunoglobulin C_k gene), in which the IAP element is known to affect levels of RNA transcription. While the IAP provirus does not appear to have an effect on TM transcription under normal conditions, it is possible that transciption levels altered by factors like thrombin and tumor necrosis factor are affected by the IAP provirus.

Introduction

Thrombomodulin (TM) is an endothelial cell, transmembrane protein that functions in the regulation of coagulation (Dittman and Majerus, 1990; Esmon, 1987). In the adult mouse, TM is found predominantly on the surface of the lung microvessels (Ford *et al.*,1992). Small amounts of TM have also been reported on platelets (Suzuki *et al.*, 1988), in serum and urine (Ishii and Majerus, 1985), and on serveral nonvascular surfaces of body cavities (Boffa *et al.*, 1987). During development, TM has been identified in the atrium, lung bud, nueroepithelium, and head mesenchyme of day 10.5 embryos (Imada *et al.*, 1990) and is expressed in the prietal endoderm of F9 embryonal carcinoma cells following treatment with retinoic acid and dibutryl cyclic AMP (Imada *et al.*, 1987).

Previous work in our laboratory involved the cloning of the BALB/cBD TM cDNA (Ford *et al.*, 1992). The fact that TM is an intronless gene makes it a good candidate for the study of 5' promoter and enahancer sequences. During the course of the sequencing of this region, a Genbank FastA search (Bilofsky and Burk, 1988; Pearson and Lipman, 1988) identified a sequence for the 5' promoter region for the TM gene that extends to position -2633 upstream from the initiation codon (MUSTHROMBO, Accession #M74231). The two sequences shared greater than 98% identity except at the extreme 5' region, where at position -2429, the BALB/cBD sequence and MUSTHROMBO completely diverge. The region spanning position -2767 to

-2429 of the BALB/cBD sequence is shown to be 98.5% identical to the 3' long terminal repeat (LTR) of an intracisternal A-particle (IAP) DNA sequence (MUSIAPIL-3, Accession #X04120). Thus at least two different types of 5' promoter regions appear to be present in laboratory mice: one strain carries an inserted provirus upstream of the initiation site and another strain does not.

IAP elements are defective retroviruses (Kuff and Lueders, 1988) that are found budding from the endoplasmic reticulum in preimplantation mouse embryos (Biczysko *et al.*, 1973; Kelly and Condamine, 1982; Poznanski and Calarco, 1991), mouse myeloma tumors (Shen-ong and Cole, 1982), and rarely in normal tissue (Wivel and Smith, 1971). Proviral IAP DNA is reiterated in the mouse genome at roughly 1000 copies per haploid genome (Lueders and Kuff, 1977). Promoters and enhancers within the LTR of retroviruses can lead to changes in gene-transcription levels, regardless of the LTR orientation (Christy *et al.*, 1985; Christy and Huang, 1988). The presence of an IAP element in or near the transcription start site has led to an increase in transcription levels (Ymer *et al.*, 1985; Canaai *et al.*, 1983; Chang-Yeh *et al.*, 1991; Mold *et al.*, 1991), whereas in another case, a decrease in transcription of a gene was observed after IAP insertion (Hawley *et al.*, 1984). In this study we have used polymerase chain reaction (PCR) and Southern blot analysis to show that of ten different mouse strains tested, half contain the provirus integrated in the TM gene 5' promoter region and

half do not. Northern blot analysis of total lung RNA from ten different strains of laboratory mice was also used to investigate the role that the inserted IAP provirus plays in the normal transcriptional regulation of the TM gene. We demonstrate that there are indeed significant differences in lung TM mRNA and protein levels among mouse strains, but the levels do not appear to be correlated with the presence or absence of the IAP element.

Materials and Methods

Library Construction and Screening

A BALB/cBD liver DNA genomic library was constructed using a Promega LambdaGEM-11 Genomic Cloning Vector System (Promega, Madison, WI) according to the manufacturers suggested protocol (Promega Protocols). Briefly, liver DNA was partially digested with *Sau*3AI and the fragments were ligated into LambdaGEM-11 *Xho*I half-site arms. The resulting ligation products were packaged into phage particles using the Gigapack Gold Phage Packaging System (Stratagene, LaJolla CA). The recombinant phage were grown in the bacterium LE392 on NZCYM agar plates. Replicate plaque lifts onto nitrocellulose filters (Schleicher and Schuell, Keene, NH) were washed in 0.1% SSC, 1.0% SDS (sodium dodecyl sulfate) for 30 min at 65C, prehybridized in 5X SSC, 1X Denhardt's, 1% SDS, and 100 μg/ml salmon sperm DNA for 3 hrs at 42C, and then

hybridized with radiolabeled probe in 5X SSC, 1% SDS, 10% dextran sulphate, 50% formamide, and 100 μg/ml salmon sperm DNA (Rinchik *et al.*, 1989). Filters were washed at 65C with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS. The probe was a 552-bp *Sau*3AI/*Bam*HI restriction fragment from the TM gene (Fig 2.3, P-3)[position 701 to 1253 on MUSTHRM DNA sequence)(Dittman *et al.*, 1988] that was labeled with an Oligolabeling Kit (Pharmacia, Piscataway, NJ) according to the manufacturers protocol. Positive clones were digested with *Eco*RI and *Bam*HI and the resulting fragments subcloned into pGEM 7Z-(Promega, Madison, WI).

Sequence Analysis

Sequence analysis was carried out utilizing first the SP6 and T7 primers from pGEM 7Z- and, thereafter, using primers synthesized with a PCR-Mate 391 DNA Synthesizer (Applied Biosystems, Foster City, CA). The sequencing reaction was carried out using Sequenase (U.S. Biochemical Corp., Cleveland OH) according to the manufacturer's suggested protocol.

Southern Analysis

Genomic spleen DNA was isolated by grinding the tissue to a fine powder after freezing in liquid nitrogen and extraction of the DNA into 10 mM Tris-Cl (pH 8.0), 0.1 M EDTA, 20 μ/ml pancreatic RNase, 0.5% SDS, and 100 μg/ml proteinase K (Sambrook *et al.*, 1989). Three micrograms of high molecular weight DNA was digested with the appropriate restriction

endonuclease and separated on a 0.7% agarose gel for *Eco*RI digestions, and a 0.4% agarose gel for *Eco*RV digestions. The DNA was transferred to Genescreen (NEN Research Products, Boston, MA) by capillary transfer in 10X SSC (Sambrook *et al.*, 1989). Hybridization conditions were the same as described above.

PCR Analysis

PCR reactions were set up with 800 ng of genomic DNA and 800 ng of each primer using the Amplitaq Sequencing Kit (Perkin Elmer Cetus, Norwalk, CT) and amplification was done with a DNA Thermocycler 480 (Perkin Elmer Cetus). The thermocycler was set for one cycle of melting at 94C for 3 min, annealing at 66C for 1 min, and synthesis at 72C for 2 min, followed by 30 cycles of melting at 95C for 1 min, annealing at 66C for 1 min, and synthesis at 72C for 1 minute. Ten percent of the resulting reaction product was analyzed on a 5% polyacrylamide gel.

PCR amplification of the whole IAP element required longer synthesis times. The reaction was carried out using 800 ng of BALB/c DNA and 800 ng each of the primers 539 and 532 (see Table 2.1 for sequence and Fig 2.2 for position). Reaction conditions were: one cycle of melting at 94C for 3 min; annealing at 66C for 1 min; and synthesis at 72C for 5 min; followed by 25 cycles of melting at 95C for 2 min; annealing at 65C for 1 min; and synthesis at 72C for 2.5 min. The entire sample was then precipitated with ethanol, run on a 0.8% agarose gel, and transferred to Genscreen. Transfer

and hybridization conditions were the same as listed above.

Northern Blot Analysis

Total RNA was isolated from frozen tissues by extraction with guanidine isothiocyanate followed by pelleting through 5.7 M CsCl (Kingston, 1989). For Northern analysis, 3-5 μg of RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde in 10 mM sodium phosphate buffer for 5 hrs at 5V/cm (Lehrach *et al.*, 1977). The RNA was transferred to Genescreen by blotting overnight in 10X SSC. After exposure to 3 min of UV irradiation (258 nm) at 800 $\mu W/cm^2$, the blot was washed, prehybridized, and hybridized with the TM gene probe as indicated above. X-ray films from autoradiography of Northern blots and ethidium bromide intensities of stained gels were digitalized using a Hewlett Packard Scangel 2 with Hewlett Packard Scanning Gallery 5 Software and analyzed using Quantiscan (Microbial Systems Ltd., Biosoft).

Two-site Assay for TM

The two-site assay was performed as previously described (Kennel *et al.*, 1988; and Chapter 1). Briefly, 2 μg of purified mAb 273-34A was bound to each well of Immulon II Removawell strips (Dynatech, Broomall, PA). Homogenates of normal tissues were prepared by sonication (setting 6 Brinkman Instruments Cell Disruptor 185) in lysis buffer (0.5% Nonindet P-40, 0.01% phenylmethlsulfonyl flouride, 20 μg/ml leupeptin, and 0.01 M sodium azide) at a ratio of 1 g tissue to 10 ml lysis buffer. Undissolved

tissue was removed by centrifugation (see Chapter 1 for more detail). Fifty μL of six two-fold dilutions of each tissue homogenate were incubated in duplicate in antibody-coated wells for 2 hrs at 4C. Wells were washed with phosphate buffered saline (PBS), and the bound antigen (TM) was detected by the addition of 50 ng of ^{125} I-labeled monoclonal antibody (MoAb) 411-201B for 2 hrs at 4C. Wells were analyzed for ^{125}I. For tabulation of data, the amount of ^{125}I-411-201B bound/mL lysate was determined at the point on a curve at which 1 ng of ^{125} MoAb was bound.

Animals

BALB/cBD mice were maintained in a specific pathogen free environment at the Oak Ridge National Laboratory. *Mus spretus* and C3Hf mice were a gift from Dr. E. Rinchik and came from the ORNL Mammalian Genetics Animal Facility. BALB/cByJ, C58/J, 129/J DBA/2J C57L/J A/J, and *Mus musculus molossinus*/Ei were obtained from the Jackson Laboratory, Bar Harbor, ME. Six-week-old female mice were used exclusively for all experiments.

Results

Genomic Library and Sequence Analysis

Screening of a λ phage genomic library constructed from BALB/cBD liver DNA with a 552-base pair restriction fragment from the TM cDNA, identified two positive clones. *Eco*RI and *Bam*HI restriction digestion and

Southern analysis of these two clones revealed that one contained a 4.0-kb fragment that hybridized to the TM gene probe. Based on the restriction map of the TM gene (Dittman *et al.,* 1988), we concluded that this piece of DNA contained 2.7 kb of 5' flanking DNA and 1.2 kb of the TM gene. This *Eco*RI-*Bam*HI fragment was ligated into pGEM 7Z- for sequence analysis. The 5'-region genomic DNA was sequenced and was found to be virtually identical to the 5' sequence MUSTHROMBO (Dittman *et al.*, 1988), with the exception of the 5'-most 338 base pairs of the BALB/c sequence, which spanned position -2767 to -2429 of the TM gene (Figure 2.1). This region of divergence from MUSTHROMBO sequence was found to be 98.5% identical to the long terminal repeat region of the IAP element, MUSIAPIL3 (accession #X04120). MUSIAPIL3 refers to the complete sequence of the IAP element that had inserted into the promoter region of the interleukin-3 gene in WEHI-3B cells (Ymer *et al.*, 1985). Thus, some BALB/c substrains contain a IAP provirus LTR inserted 2.4 kb upstream of the TM gene (BALB/cBD), whereas other substrains do not. A schematic of both 5' regions is depicted in Figure 2.2.

Southern Blot Analysis

Southern blot analysis of ten strains of mice was performed to determine whether restriction fragment length polymorphisms (RFLPs) could be correlated with the presence or absence of an IAP proviral element insertion

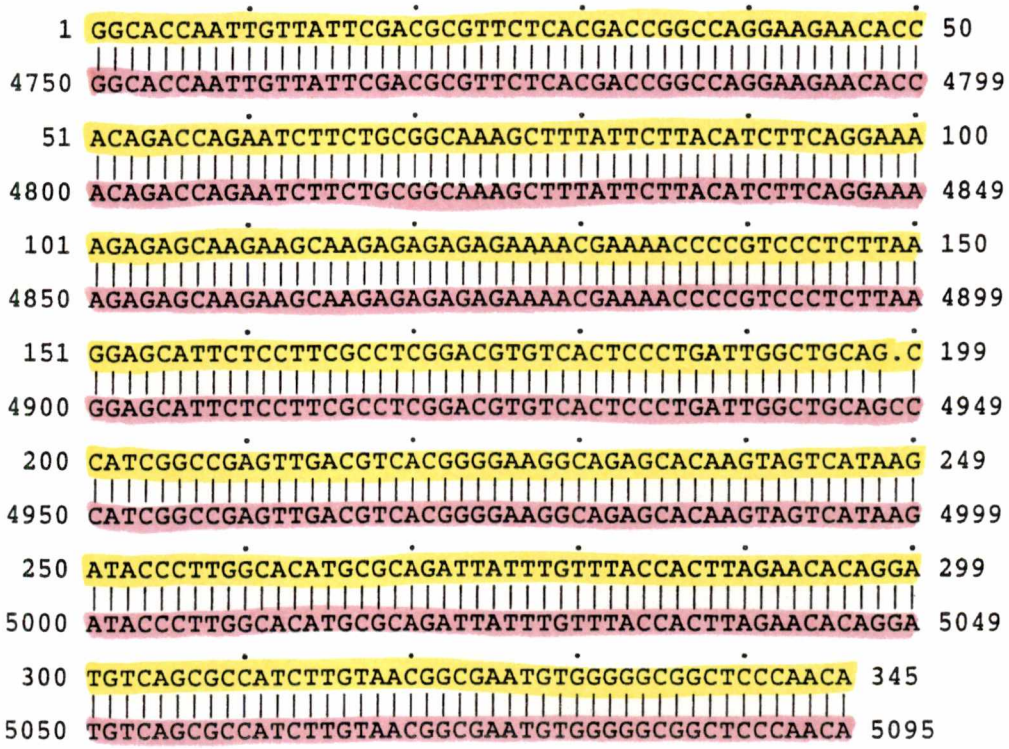


Figure 2.1 Sequence comparison of the 5' region of the TM gene.

a. Comparison of the BALB/cBD 5' promoter region sequence to MUSIAPIL3.

top = BALB/cBD sequence
bottom = MUSIAPIL3 sequence

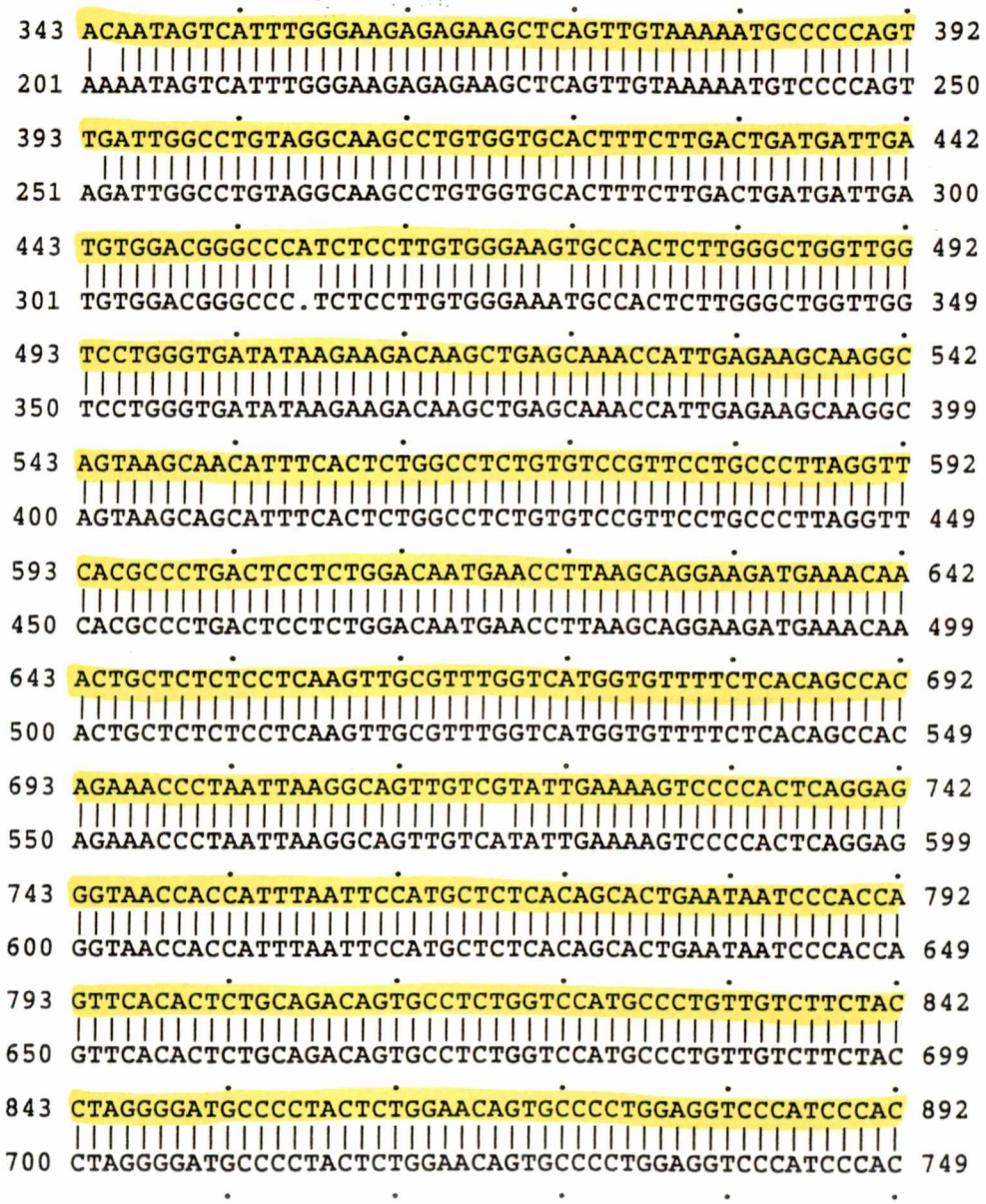


Figure 2.1 (continued).

b. Comparison of BALB/cBD 5' promoter region sequence to MUSTHROMBO (Accession # M74231).

top = BALB/cBD sequence
bottom = MUSHTROMBO sequence

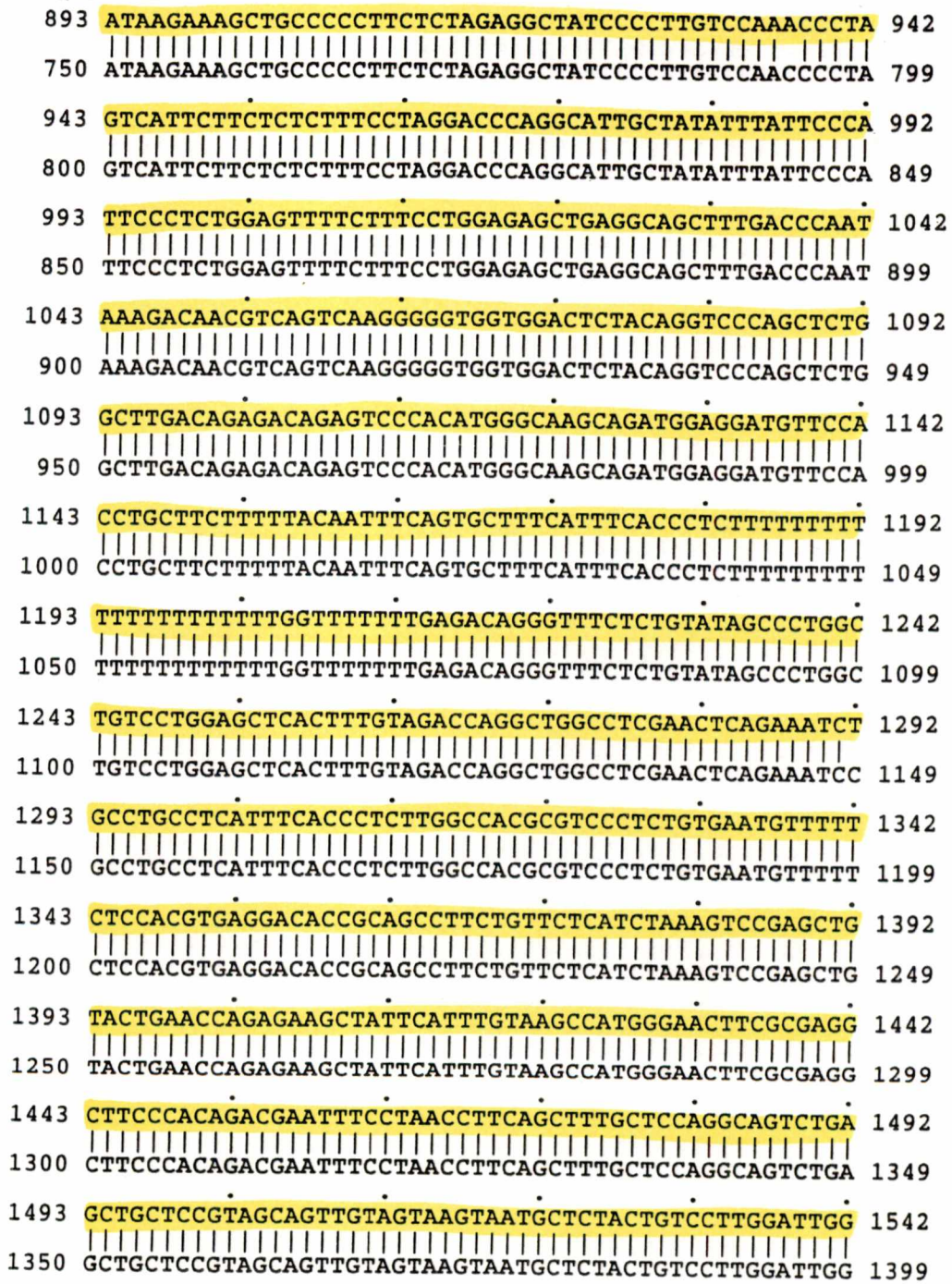


Figure 2.1 b (continued).

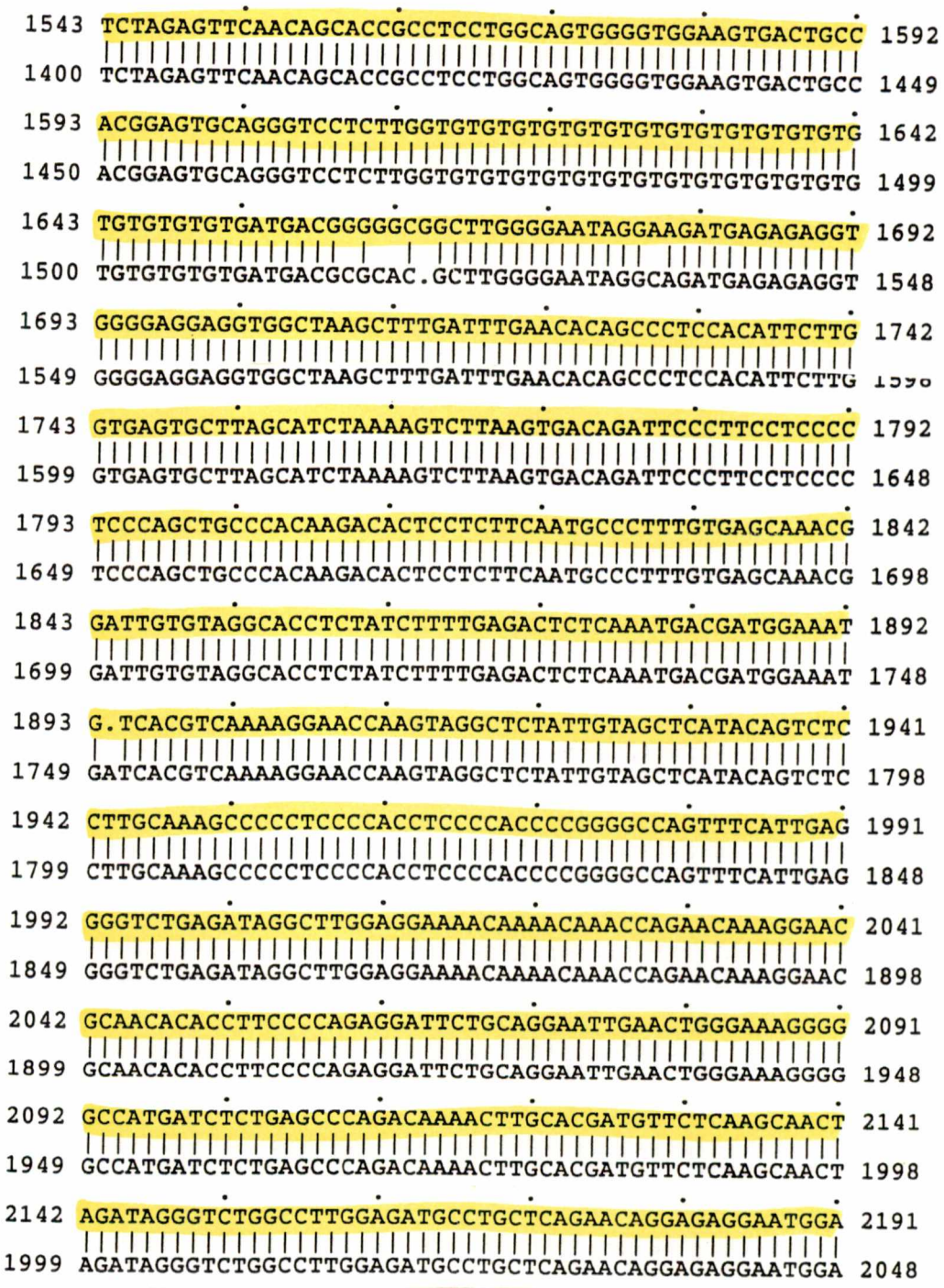


Figure 2.1 b (continued).

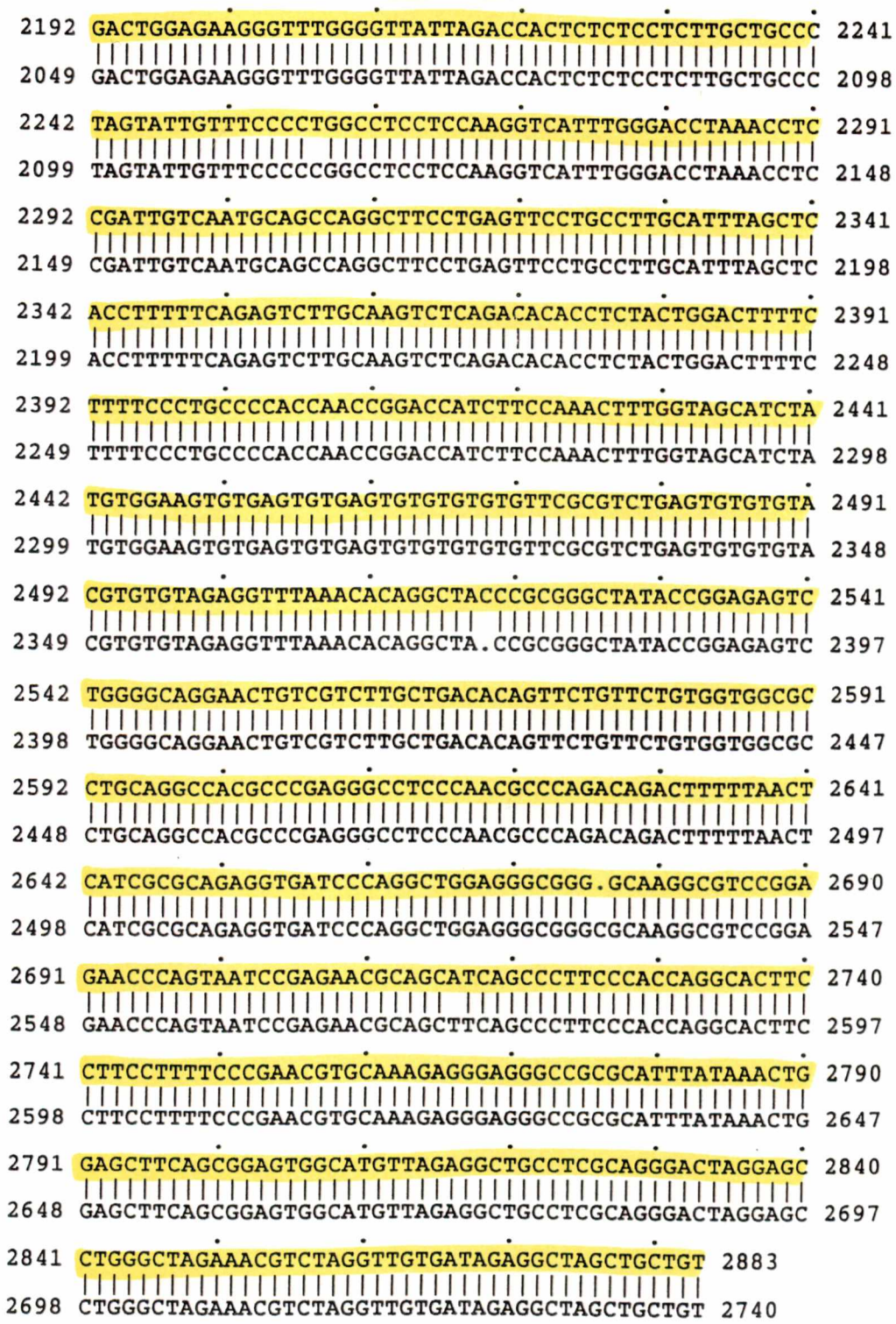


Figure 2.1 b (continued).

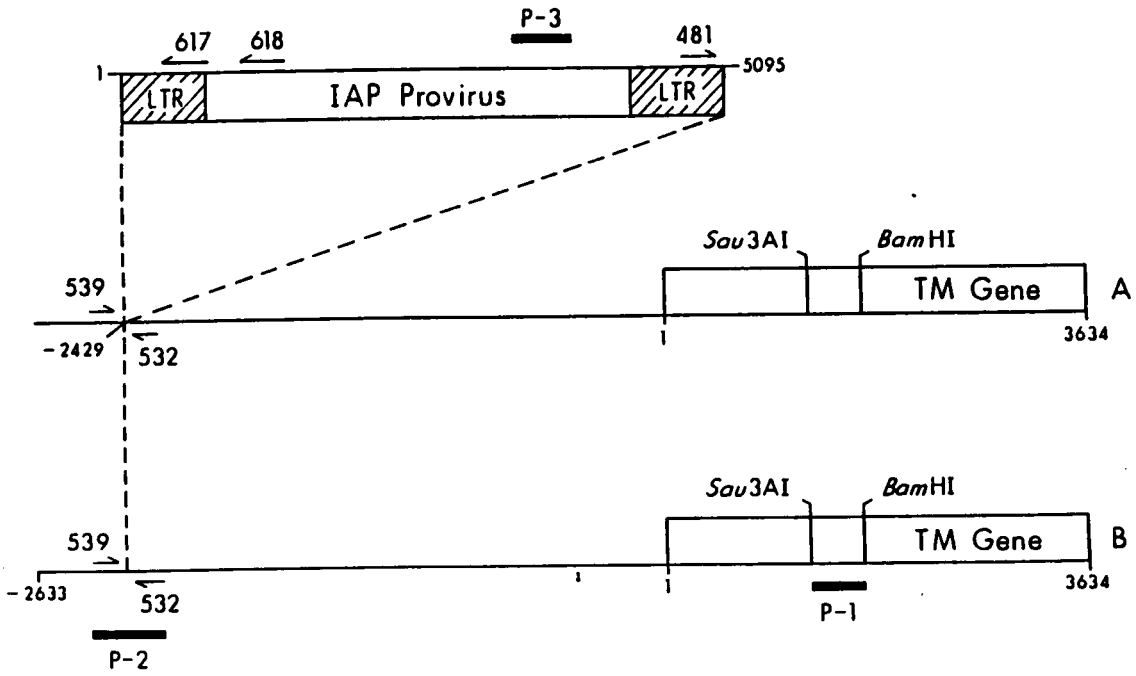


Figure 2.2. Diagram depicting the 5'region of the TM gene from BALB/cBD that was subcloned into pGEM 7Z-(A) and MUSTHROMBO (B). Position 1 marks the beginning of the TM gene sequence. The sequence for MUSIAP-IL3 is also marked from position 1 to 5095 and refers to the nucleotide sequence listed in Genbank. The promoter region called MUSTHROMBO is denoted by negative integers to the left of the TM transcription start site. Bold lines labeled P-1, P-2, and P-3 indicate regions of DNA used for hybridization probes (see text). Half arrows with numbers 532, 539, and 481 depict PCR primers and the direction of synthesis.

5' to the TM gene. The BALB/cBD IAP contains an *Eco*RI site within the LTR that is lacking in the strains which do not contain the IAP provirus. Blots of *Eco*RI-digested DNA were hybridized with a TM gene probe, P-1 (Figure 2.2 and methods). This analysis demonstrated that four of the mouse strains (BALB/cByJ, C3Hf/Rl, C57L/J, and 129/J) had a fragment of 12.5 kb. This was identical in size to the fragment found in BALB/cBD that had been shown to contain an IAP LTR. The other five strains examined (A/J, C58/J, DBA/2J, *Mus spretus*, and *Mus musculus molossinus*) had a band of 15.9 kb (Figure 2.3), indicating a rearrangement in the DNA relative to the BALB/cBD sequence, possibly due to the absence of an IAP provirus.

PCR Analysis for the Presence or Absence of the IAP LTR.

PCR analysis of the ten strains was conducted to determine whether the IAP proviral sequence identified in BALB/cBD DNA was present or absent. To test whether the 12.5 kb *Eco*RI fragment of BALB/cByJ, C3Hf, C57L/J, and 129/J genomic DNA also indicated the presence of IAP DNA, two primers were synthesized for PCR. One primer was made complementary to the LTR sequence (primer 576, Figure 2.2) while the other was made to DNA 3' to the provirus at position -1924 to -1949 of MUSTHROMBO (primer 532, Figure 2.2). All primer sequences are listed in Table 2.1. PCR using these primers should amplify an 818-bp fragment from BALB/cBD DNA and other DNAs which carry the IAP 5' to the TM gene. In this PCR

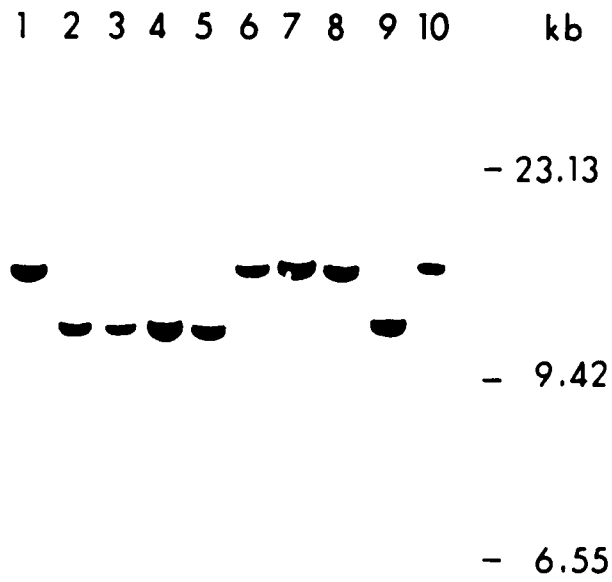


Figure 2.3. Southern Blot of *Eco*RI-digested mouse DNA hybridized with TM gene probe P-1. Strain DNAs are in the following order: A/J (lane 1), BALB/cBD (lane 2), BALB/cByJ (lane 3), C3Hf (lane 4), C57L/J (lane 5), C58/J (lane 6), DBA/2J (lane 7), *Mus spretus* (lane 8), 129/J (lane 9), and *Mus musculus molossinus* (lane 10).

Table 2.1: Primer Sequences

Primer #	DNA sequence	Position (5'-3')	Sequence	Accession number
481	MUSIAPIL3	4887-4911	CCGTCCCTCTTAAGGAGCATTCTCC	X04120
532	MUSTHROMBO	661-684	CATTGGACCAGAGGCACTGTCTGCA	M74231
539	MUSTHROMBO	29-54	GGGAGATTAGAAGCCAGAAGCCGTG	M74231
617	MUSIAPIL3	147-172	TGACACGTCCGAGGCGAAGGAGAAT	X04120
618	MUSIAPIL3	439-464	GCCTGTGAGCCTAAGAGCTAATCCT	X04120

reaction, template DNA from five strains (BALB/cBD, BALB/cByJ, C3Hf, C57L/J, and 129J) led to the formation of a fragment calculated to be 818 bp (Figure 2.4A). No PCR product was created with these two primers from DNAs from the other five strains, indicating that the DNA sequence corresponding to at least one primer binding site was absent. Since BALB/cBD, BALB/cByJ, C57L/J, C3Hf, and 129/J all contain the same size of PCR product, it is likely that the site of provirus insertion is identical in all strains and that insertion was a single event which occurred before the creation of the separate strains.

To test for the absence of the provirus, PCR analysis was carried out using a primer spanning positions -2554 to -2579 of MUSTHROMBO sequence (primer 539) and primer 532 (see Fig 2.2). In the absence of the IAP, these primers would generate a 652-bp PCR fragment. Figure 2.4b shows that template DNA from A/J, C58/J, DBA/2J. *Mus spretus*, and *Mus musculus molossinus* all lead to amplification of a 652-bp DNA fragment. DNA from the other strains that resulted in amplification of an IAP-specific junction fragment with primers flanking the insertion site, produced no detectable PCR product. The most likely possibility for this result is that a large piece of DNA (the IAP element) has inserted into the DNA making PCR less efficient.

Figure 2.4. PCR analysis for the presence or absence of an IAP LTR. Panels A and B depicts a 5% polyacrylamide gel of one tenth of PCR products. For panel A primers 481 and 532 were used. These primers should generate an 818-bp fragment (see arrow) in DNA that has an inserted LTR. Panel B depicts PCR products from the same strain DNAs using primers 539 and 532. These primers should generate a 652-bp fragment in strains that do not have an inserted LTR. Strain DNAs are in the following order: A/J (lane 1), BALB/cBD (lane 2), BALB/cByJ (lane 3), C3Hf (lane 4), C57L/J (lane 5), C58/J (lane 6), DBA/2J (lane 7), *Mus spretus* (lane 8), 129/J (lane 9), *Mus musculus molossinus* (lane 10). Lane 11 shows ΦX174 RF DNA/*Hae* III fragments as molecular weight markers.

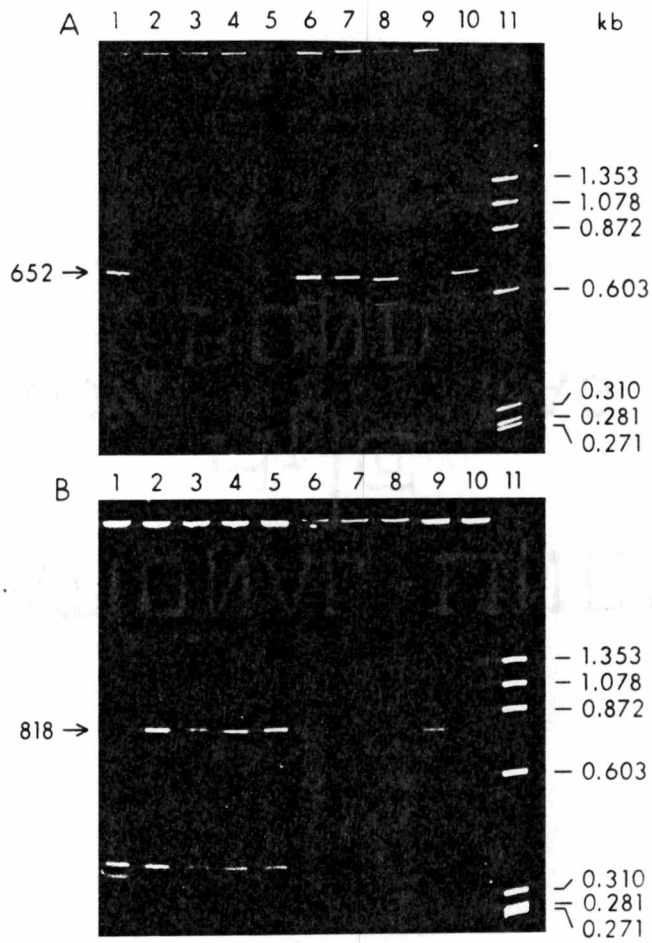

A
1 2 3 4 5 6 7 8 9 10 11
kb
652
1.353
1.078
0.872
0.603
0.310
0.281
0.271
B
1 2 3 4 5 6 7 8 9 10 11
818
1.353
1.078
0.872
0.603
0.310
0.281
0.271

Figure 2.4

Determination of Size and Type of IAP Element.

The accumulated data indicate the presence of an IAP proviral LTR in DNA of five of the mouse strains. However, these data did not indicate whether an entire IAP element was present. To determine the length and gain more information as to the type of IAP element present, we used Southern blot analysis, PCR, and sequencing information.

The sequence information accumulated from the LTR region showed 98.5% identity over a 346-bp overlap to the MUSIAPIL3 sequence (Ymer *et* al., 1985). In addition sequence analysis of the ends of the original BALB/cBD genomic λ clone, from which the construct in Figure 2.2 was derived, identified another region of 100% homology spanning the region 3780 to 3985 of MUSIAPIL3 (data not shown). To obviate the need for sequencing the entire element, we used PCR and Southern analysis to determine the actual size of this IAP element. For Southern analysis, genomic DNA from all ten strains of mice was digested with *Eco*RV [Sequence information from MUSIAP-IL3 (Ymer *et al.*, 1985), our lab, and MUSTHROMBO (Accession # M74231) indicated that there were no *Eco*RV sites in any of these sequences], blotted and hybridized with the P-1 DNA probe (Figure 2.2). A RFLP of about 5.5 kb was observed between the mouse strains that have the IAP provirus (19.6 kb) and strains which do not (14.1 kb)(Figure 2.5a). The prototype MUSIAP-IL3 is 5.095-kb in length, which is similar to the size difference determined by gel

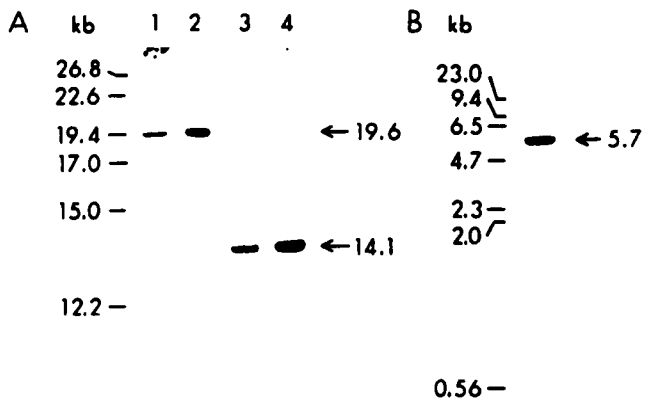


Figure 2.5. Southern (panel A) and PCR analysis (panel B) for determining the relative size of the inserted IAP element. Panel A shows a Southern blot of *Eco*RV-digested genomic DNA for strains A/J (lane 1), BALB/cBD (lane 2), C58/J (lane 3), and DBA/2J (lane 4), which was hybridized with the TM gene probe, P-1. Sizes of high molecular weight markers (Life technologies) are listed on the left side and sizes of hybridization fragments are listed on the right. Panel B shows a southern blot of PCR products using primers 532 and 539 on BALB/cBD DNA. The southern blot was hybridized with a probe to the IAP element DNA, P-3. Molecular weight markers (λ *Hind*III) are listed on the left.

electrophoresis (5.5 kb). Since these are such high molecular weight fragment DNAs, our size estimates could easily be off by 0.5 kb. In addition to the Southern analysis, PCR was preformed on BALB/cBD DNA using the primers 539 and 532, which flank the IAP element. A Southern blot was made from the PCR DNA and hybridized with probes to the flanking region of the IAP (see P-2 in Figure 2.2) and with an internal IAP probe (see P-3 in Figure 2.2). This analysis showed that the inserted DNA was 5.7 kb in length (Figure 2.5b). Since MUSIAP-IL3 is 5.095 kb and the primers used would generate flanking regions totaling .562 kb we would expect a PCR fragment of 5.657 kb. These results demonstrate that DNA with this IAP contains an insertion of about 5 kb, which is similar to the size of MUSIAP-IL3.

PCR analysis was also carried out for the presence of the 5'-most LTR of the IAP element DNA sequence. Two primers were synthesized: primer 617 was made to a region within the LTR whereas primer 618 was made to the region directly following the LTR (see Fig 2.2 and Table 2.1). Primers 539 and 617 should generate a 337-bp fragment in the DNA of mouse strains containing the IAP provirus (see Fig 2.6, lanes 1 through 5). Primers 539 and 618 should generate a 629 bp fragment (see Fig 2.6, lanes 6 through 10). These results demonstrate that the five strains that contain the provirus (BALB/cBD, BALB/cByJ, C3Hf, C57L/J, and 129/J) also contain the 5'-most LTR in its entirety. Therefore, we conclude that BALB/cBD, BALB/cByJ,

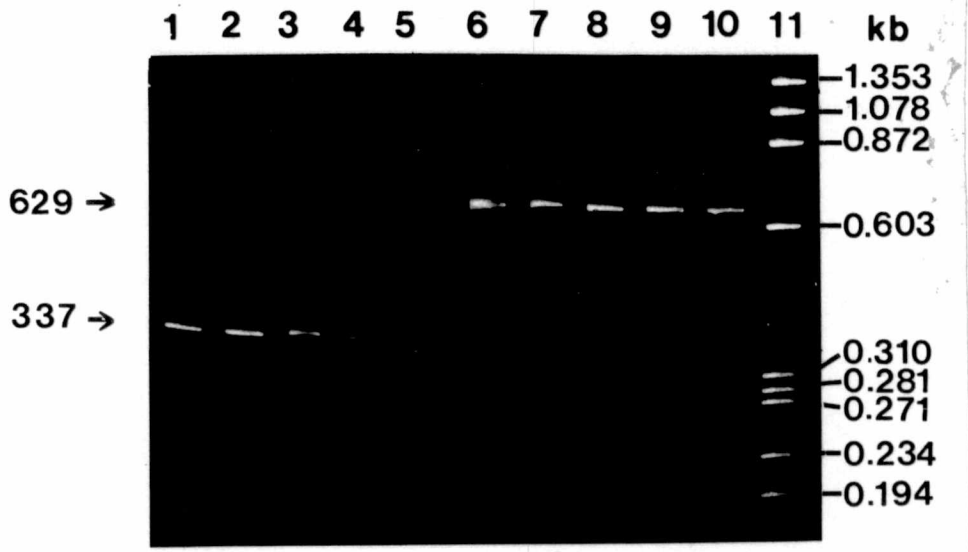


Figure 2.6. PCR analysis for the presence of the 5'-most LTR. Figure depicts a 5% polyacrylamide gel of one tenth of PCR reaction products. For lanes 1 through 5, primers 539 and 617 were used. These primers should generate an 337 bp fragment (see arrow) in all strain DNAs that have the IAP element. Strains are in the following order, BALB/cBD (lane 1), BALB/cByJ (lane 2), C3Hf (lane 3), C57L/J (lane 4), 129/J (lane 5). Lanes 6 through 10 depict the results of PCR reactions using primers 539 and 618. These primers should generate an 629 bp fragment if the entire 5' LTR is present. BALB/cBD (lane 6), BALB/cByJ (lane 7), C3Hf (lane 8), C57L/J (lane 9), 129/J (lane 10). Lane 11 shows ΦX174 RF DNA/*Hae*III fragments as molecular weight markers.

C3Hf, C57L/J, and 129/J have an IAP provirus inserted 5' to the TM gene that is quite similar, if not identical, to MUSIAP-IL3.

Analysis of Lung TM mRNA levels in mouse strains.

Because IAP elements have been shown to influence levels of transcription for a number of genes, it was of interest to determine if there were differences in the levels of TM mRNA among strains of mice, and whether these differences could be correlated to the presence or absence of the IAP. Total lung RNA from 6-week-old female mice was analyzed by Northern blot with a TM gene probe. Lung RNA was used because the lung is the only tissue that contains high enough levels of TM mRNA to be detected by Northern analysis of total RNA. The Northern blot was hybridized with the TM gene probe P-1 (Fig 2.7A) and later with a γ-actin probe [Fig 2.7B (Gunning *et al.*, 1983)] for normalization of RNA levels. In addition to the actin mRNA levels, we scanned a negative image of the RNA gel (Fig 2.7C) to determine the levels of ethidium bromide stained 18S RNA. A bar graph depicting the relative levels of each of these RNAs is presented in Figure 2.7D. Quantification of RNA on Northern blots is difficult, especially for small differences. Assuming 18S levels do not vary from strain to strain, a comparison of the relative levels of actin DNA with the levels of 18S RNA indicates a difference in the levels of actin mRNA from strain to strain. Variations in actin levels have been observed by others as well (Hollander and Fornace, 1990; Schneider *et al.*, 1988). Therefore, we have

Figure 2.7. Northern blot analysis of the relative levels of TM in mouse lungs. Panel A depicts the Northern blot for TM mRNA, Panel B the Northern blot for γ actin mRNA, and Panel C the ethidium-stained RNA gel. For all panels the samples are as follows; A/J (lane 1), BALB/cBD (lane 2), BALB/cByJ (lane 3), C3Hf (lane 4), C57L/J (lane 5), C58/J (lane 6), DBA/2J (lane 7), *Mus spretus* (lane 8), and 129/J (lane 9). Panel D depicts the levels of ethidium-stained 18S RNA, actin mRNA, and TM mRNA. Panel E depicts the relative levels of TM mRNA after standardization with both γ actin mRNA levels and 18S RNA levels. For panels D and E a ($^+$) following a sample number indicates the presence of the 5' IAP element and a ($^-$) indicates the absence of the element.

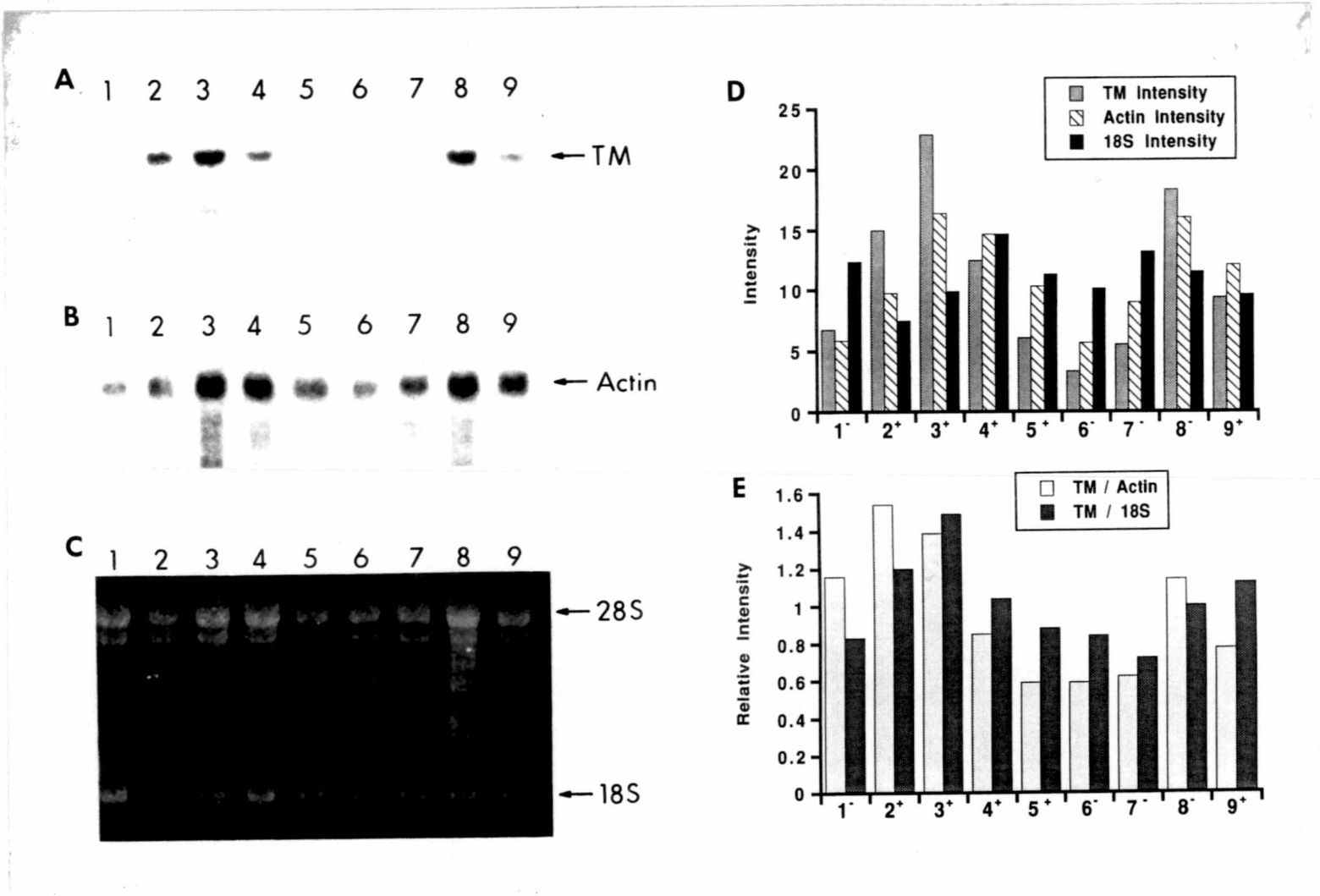

A
1 2 3 4 5 6 7 8 9
TM
B
1 2 3 4 5 6 7 8 9
Actin
C
1 2 3 4 5 6 7 8 9
28S
18S
D
TM Intensity
Actin Intensity
18S Intensity
Intensity
25
20
15
10
5
0
1- 2+ 3+ 4+ 5+ 6- 7- 8- 9+
E
TM / Actin
TM / 18S
Relative Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1- 2+ 3+ 4+ 5+ 6- 7- 8- 9+

Figure 2.7

standardized TM levels to two different measurements, actin mRNA levels and 18S RNA levels (Fig 2.7E). Regardless of which method was used to standardize TM mRNA levels, the data demonstrate that levels of lung TM RNA do vary slightly (up to 3-fold) from strain to strain, but the differences do not appear to correlate with the presence or absence of the 5' IAP provirus. This series of experiments has been repeated 4 times producing similar results each time. For simplicity, only one representative set of data are shown.

Two-site Assay for TM protein levels

The levels of TM protein in different organs from the strains were examined for comparison to mRNA levels in lung. TM protein levels in lungs as determined by MoAb two-site assay demonstrate similar strain differences (Table 2.2). Examination of the TM levels in two other organs, heart and kidney, are also shown in Table 2.2.

Discussion

Initially, sequence analysis of the 5' region of the TM gene was performed to better understand the mechanisms governing transcriptional regulation of the gene. The sequence generated from BALB/cBD DNA was found to be 98% identical to MUSTHROMBO (Accession # M74231), indicating there were other differences in the two sequences. However these differences were minor and likely to be due to ambiguous regions of

Table 2.2. Two-site assay for TM

Strain	Kidney (ng MoAb bound/ mL protein lysate)	Heart	Lung
A/J$^-$	222	51	557±44
BALB/cBD$^+$	159	59	816±23
BALB/cByJ$^+$	180	62	900±141
C3Hf$^+$	180	60	801±45
C57L/J$^+$	170	45	572±23
C58/J$^-$	180	44	785±22
DBA/2J$^-$	260	57	768±112
Spretus$^-$	33	20	429±83
129/J$^+$	125	71	775±392
MOLF/Ei$^-$	149	70	421±72

A ($^+$) following a strain name indicates the presence of IAP element DNA 5' to the TM gene whereas a ($^-$) indicates the IAP element is absent.

sequence analysis or strain variation. The major differences in the two sequences was the presence of a IAP provirus 3' LTR in BALB/cBD DNA, a difference which may have ramifications for the control of TM transcription. Since the presence of an IAP element has proven to affect transcription levels of nearby genes, our interest was in the effect that the IAP element might have on TM gene transcription. Southern blot analysis of ten strains of laboratory mice demonstrated a rearrangement in the DNA near the TM gene. PCR analysis showed that the DNA from five of the mouse strains examined contained the same LTR as found in BALB/cBD, whereas the other five lacked the IAP LTR.

Sequencing of the genomic λ clone revealed another 200 bases of sequence that matched MUSIAP-IL3 from position 3783 to 3986. Southern blot and PCR analysis was then used to show that the size of the insert was about 5 kb, very close to 5.095 Kb, which is the size of MUSIAP-IL3 (Ymer *et al.*, 1985). Therefore, it was concluded that the IAP element inserted 5' to the TM gene in five strains of laboratory mice was very similar, if not identical to MUSIAP-IL3. Because the PCR fragments spanning the insertion site of the IAP DNA were identical in size, indicating an identical insertion site in these strains, it was also concluded that insertion of the IAP element was a single event that occurred before the generation of the different inbred strains of mice. It is interesting to note that the two wild strains of mice *Mus spretus* and *Mus musculus molosinus* lack the IAP

element 5' to the TM gene, suggesting that this insertion did not occur before the generation of these strains. The one exception to this theory is the sequence reported in Genbank (MUSTHROMBO). The clone used for the MUSTHROMBO sequence was isolated from a genomic λ library purchased from Clontech (personal communication, Dr. W.A. Dittman) which was constructed using BALB/c (Andervont lineage) DNA. A sample of Clonetech genomic DNA (BALB/c, Andervont lineage) was purchased. PCR amplification of this DNA for both the 818-bp IAP specific fragment and the 652-bp fragment depicting absence of the IAP provirus experiment demonstrated that Clontech BALB/c (Andervont lineage) DNA actually contained the provirus (data not shown). This discrepancy suggests that either BALB/c DNA was not used to make the λ library used for the MUSTHROMBO sequence, or that the IAP provirus was lost during library construction. In either case, the theory that the IAP provirus insertion occured before the generation of inbred mouse strains, still holds.

The finding that there was a difference in the 5' region of the TM gene was of interest because the presence of IAP proviral DNA in the 5' region of mammalian genes has been demonstrated to effect the transcription levels of some genes. For example, insertion of an IAP provirus into the 5' region of the interleukin-3 gene leads to constitutive synthesis of the interleukin-3 gene in WEHI-3B leukemia cells (Ymer *et al.*, 1985), and IAP provirus insertion into the coding region of c-*mos* leads to activation of the oncogene in a

plasmacytoma cell line (Canaani *et al.*, 1983). Immunoglobulin C_K genes have been shown to be inactivated by IAP integration in a hybridoma cell line (Hawley *et al.*, 1984), whereas insertion of an IAP element 9 kb 5' to the angiotensinogen gene cap site does not appear to effect transcription levels (Clouston, 1990). Recently, an IAP LTR has been shown to promote the transcription of mouse IAP promoted placental gene (MIPP) in the placenta, parietal yolk sac and visceral yolk sac of day 13.5 mouse fetus (Chang-Yeh *et al.*, 1991; Mold *et al.*, 1991).

It has also been demonstrated that some IAP LTRs can effect transcription levels regardless of orientation of insertion (Christy *et al.*, 1985; Christy and Huang, 1988). Therefore, basal levels of TM transcription in the lungs were compared in mice that had the inserted IAP provirus versus mice that did not. Normalization of the levels of TM mRNA to both γ actin and 18S RNA levels demonstrate that there are definite differences in the basal levels of TM mRNA in the lungs of laboratory mouse strains, but these differences are not correlated with the presence or absence of the 5' IAP element. Because TM is a single-copy gene in mice (Dittman and Majerus, 1990), these results cannot be explained by the presence of another copy of the gene that is also transcriptionally active. Monoclonal antibody two-site assay analysis for TM levels in lung, kidney, and heart, also demonstrates strain differences that correlate with mRNA levels (data not shown), but these differences cannot be correlated with the presence of the IAP element DNA.

All ten strains of mice examined express TM, even though half contain a large insertion (5 kb) in the 5' promoter region of the gene. This fact suggests that all of the promoter and enhancer sequences necessary for TM transcription under normal conditions, are present within the region extending to position -2429. However, there are many different factors that have been demonstrated to induce an effect on the levels of TM transcription. For example, exposure of mouse hemangioma cells to cyclohexamide and thrombin leads to an increase in TM mRNA levels (Dittman *et al.*, 1989). Treatment with cyclic AMP and retinoic acid also lead to increases in TM protein levels in F9 embryonal carcinoma cells (Imada *et al.*, 1990a; Imada *et al.*, 1990b). In Chinese hamster ovary cells, treatment with cyclic AMP alone will lead to increased levels of TM (Imada and Imada, 1982). The presence of an IAP element 5' to the TM gene in some, but not all, strains of mice may be important when upstream transcriptional regulators are being studied. A DNA rearrangement of this magnitude may have moved enhancer elements further away from the TM gene while, at the same time, inserting a whole new set of transcriptional regulators. Therefore, it is possible that this proviral insertion has a biological effect on induced TM transcription. However, no specific effect of the IAP provirus on induced transcription of TM mRNA has been documented to date.

CHAPTER 3

Thrombomodulin Distribution During Murine Development

Summary

Thrombomodulin is a transmembrane glycoprotein involved in the regulation of clot formation. Previous work has shown that in the adult mouse, thrombomodulin is expressed primarily on the luminal surface of endothelial cells; whereas during development, thrombomodulin has been localized to the placental parietal endoderm, the neuroepithelium, head mesenchyme, lung bud, and atrium of Day 10.5 post coitum embryos. We examined the expression of thrombomodulin in the adult mouse and the developing embryo from Day 7.5 to Day 18.5 post coitum. The high expression of thrombomodulin in the lung bud epithelium, the developing gonads and meninges, and the parietal endoderm in comparison to the expression levels in the adult organs suggest that thrombomodulin may play a critical role in development that is separate from its role in clot regulation, namely, the inactivation of thrombin and the activation of protein C.

Introduction

Thrombomodulin (TM) is a transmembrane glycoprotein involved in the regulation of blood coagulation (Esmon and Owen, 1991; Dittman and Majerus, 1990). It has been shown that TM binds to the procoagulant thrombin in a 1:1 ratio (Esmon, 1987), and that formation of this heterodimer inactivates the coagulant properties of thrombin while activating the anti-coagulant molecule, protein C (Esmon *et al.*, 1983a; Jakubowski *et al.*, 1986; Esmon *et al.*, 1982a; Fulcher *et al.*, 1984; Kisiel *et al.*, 1977).

Structurally, TM consists of a small carboxy terminal cytoplasmic domain, a transmembrane spanning region, which is very similar to the transmembrane region of the low density lipoprotein receptor, and six epidermal growth factor-like (EGF-like) repeats. It is known that the thrombin binding site is contained in the region of EGF-like repeats 5 and 6, while the protein C activation activity requires EGF-like repeats 4, 5, and 6 (Suzuki *et al.*, 1989; Hayashi *et al.*, 1990; Stearns *et al.*, 1989; Zushi *et al.*, 1989; Bourin *et al.*, 1986; Kurosawa *et al.*, 1987). Near the amino-terminus a lectin-like region has been identified that has no known function (Dittman and Majerus, 1990; Petersen, 1988)

TM is found predominantly on the surface of endothelial cells (Esmon and Owen, 1981; DeBault *et al.*, 1986a; Maruyama *et al.*, 1985). However, the presence of TM has also been reported on platelets, (Suzuki *et al.*, 1988) in serum and urine (Ishii and Majerus, 1985), and on several nonvascular surfaces of body cavities, particularly the pleura, pericardium, and peritoneum (Boffa *et*

al., 1987). The distribution of TM in the vasculature of adult BALB/cBD mice has been studied extensively using both quantitative and qualitative methods (Ford *et al.*, 1992; Kennel *et al.*, 1988). Results of these studies show that the lung microvasculature contains the highest levels of TM, with other highly vascularized tissues expressing lesser amounts (Ford *et al.*, 1992).

TM has been found in the parietal endoderm of F9 embryonal carcinoma cells treated with retinoic acid and dibutryl cyclic AMP (Imada *et al.*, 1987). Studies of TM expression (fetomodulin) in development have been limited to Day 10.5 embryos where TM is found in the atrium, lung bud, neuroepithelium, and head mesenchyme (Imada *et al.*, 1990a). In this study we detail the distribution of TM in the developing BALB/cBD mouse fetus from Day 7.5 to Day 18.5 post coitum and compare it to the sites of expression in the adult mouse. The data show that in contrast to expression in the adult, TM is found in high concentrations in the meninges of the brain and spinal cord, and the developing reproductive system, suggesting that TM may have a function in embryogenesis different from the known function of clot inhibition. The TM gene of BALB/cBD mice contains an IAP provirus inserted 5' to the initiaion site. For purposes of comparison, a strain lacking this insertion, A/J, was also examined, and shown to have an identical pattern of TM expression during development, ruling out the possibility that TM expression during development is governed by transcription regulatory elements within the proviral DNA.

Materials and Methods

Embryos

BALB/cBD mice were maintained in a specific-pathogen-free-environment at the Oak Ridge National Laboratory, which is accredited by the American Association of Laboratory Animal Care (AALAC). A/J mice were obtained from the Jackson Laboratory and also maintained in a specific-pathogen-free-environment.

Embryos were obtained from mated female mice. The presence of a vaginal plug the following morning demonstrated successful mating and the time was designated Day 0.5. At one day intervals post-coitum from Day 6.5 to birth, pregnant females were sacrificed by cervical dislocation, the uterus was excised, and the embryos were fixed in neutral-buffered 10% formalin.

Antibodies

MoAbs 273-34A and 411-201B are rat subclass IgG_{2a}, the binding specificities of which have been previously described (Kennel *et al.,* 1987; Kennel *et al.,* 1988) Rat MoAb 135-14 (subclass IgG_{2a}), used as a negative control, has no known binding specificity.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Ford *et al.*, 1992). Briefly, tissues were fixed in neutral-buffered formalin for 24 hrs, embedded in paraffin, and sectioned at approximately 5 micrometer thickness. Sections were deparaffinized in xylenes, rehydrated through graded alcohols,

blocked with normal serum and incubated with 0.1 μg/mL of ^{125}I chloramine T labeled antibody (specific activity = 4 μCi/μg) for 2 hrs at room temperature. Slides were washed in phosphate buffered saline (PBS) and then H_2O, air dried and dipped in Kodak NTB-2 emulsion and developed after two (embryonic tissue) or four (adult tissue) weeks in the dark.

Results

Respiratory system

The lungs of adult BALB/cBD mice have been shown to express the highest amounts of TM in comparison to all other organs (Ford *et al.*, 1992). Data in Figures 3.1, 3.2, 3.3, and 3.4 show the distribution of TM in BALB/cBD and A/J tissue sections as detected by direct immunohistochemical staining with ^{125}I MoAb 273-34A and autoradiography. TM expression in the adult was localized to the vascular surface of lung microvessels and larger vessels (Figure 3.1a and 3.3a). The thoracic pleura also showed some expression of TM. TM was first detected in the developing respiratory system at Day 11.5 in the lung-bud epithelial submucosa (see Day 13.5 lung bud, LB, Figure 3.1d). As the respiratory system developed, strong staining for TM was seen in the bronchial mucosa, alveolar septa, all large vessels, and pleura from Day 13.5 on through to birth (Figures 3.1g, 3.4d, and 3.4g) Strong staining was also observed in both the tracheal submucosa and the nasal submucosa. In both of these regions, TM was first detected at Day 15.5 and was present thereafter throughout

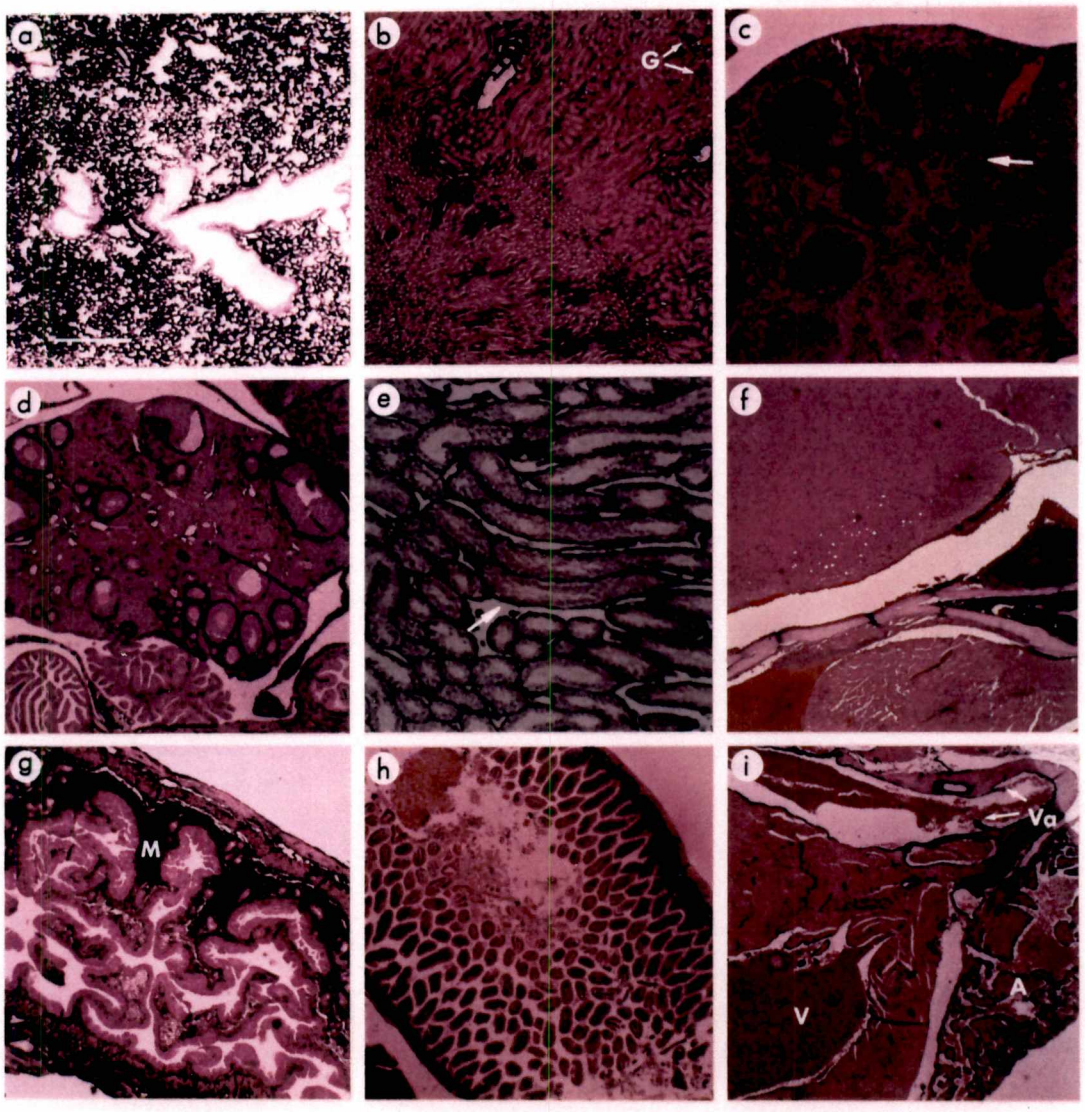


Fig. 3.1. Immunohistochemical staining of adult BALB/cBD tissue, autoradiographs exposed for four weeks. lung, (a); kidney, G=glomeruli (b); spleen, (c); ovary, (d); testes, (e); brain (pituitary), (f); uterus, M=myometrium (g); intestines, (h); heart, Va=valves, V=ventricle, A=atrium (i). Bar is equal to 400 microns.

Fig. 3.2. Immunohistochemical staining of developing BALB/cBD tissue, autoradiographs exposed for two weeks. Panels a-f, bar is equal to 100 microns, Day 15.5 kidney, (a); Day 17.5 ovary, (b); Day 15.5 spinal meninges (Me), (c); Day 13.5 lung bud (LB), (d); Day 18.5 heart valves (Va), (e); Day 9.5 embryo and extraembryonic tissue, PE= parietal endoderm, M= uterine myometrium (f). Panels g,h bar is equal to 400 microns. Day 15.5 lung and heart, (g); Day 17.5 intestines (I) and testes (T), (h); Panels i-l bar is equal to 1 millimeter. Day 9.5 embryo and extra-embryonic tissue, (i). Day 16.5 head, Me = meninges, Ep = ethmoid plate, Te = teeth, (j); Panel k is a ^{125}I MoAb 135-14C negative control.

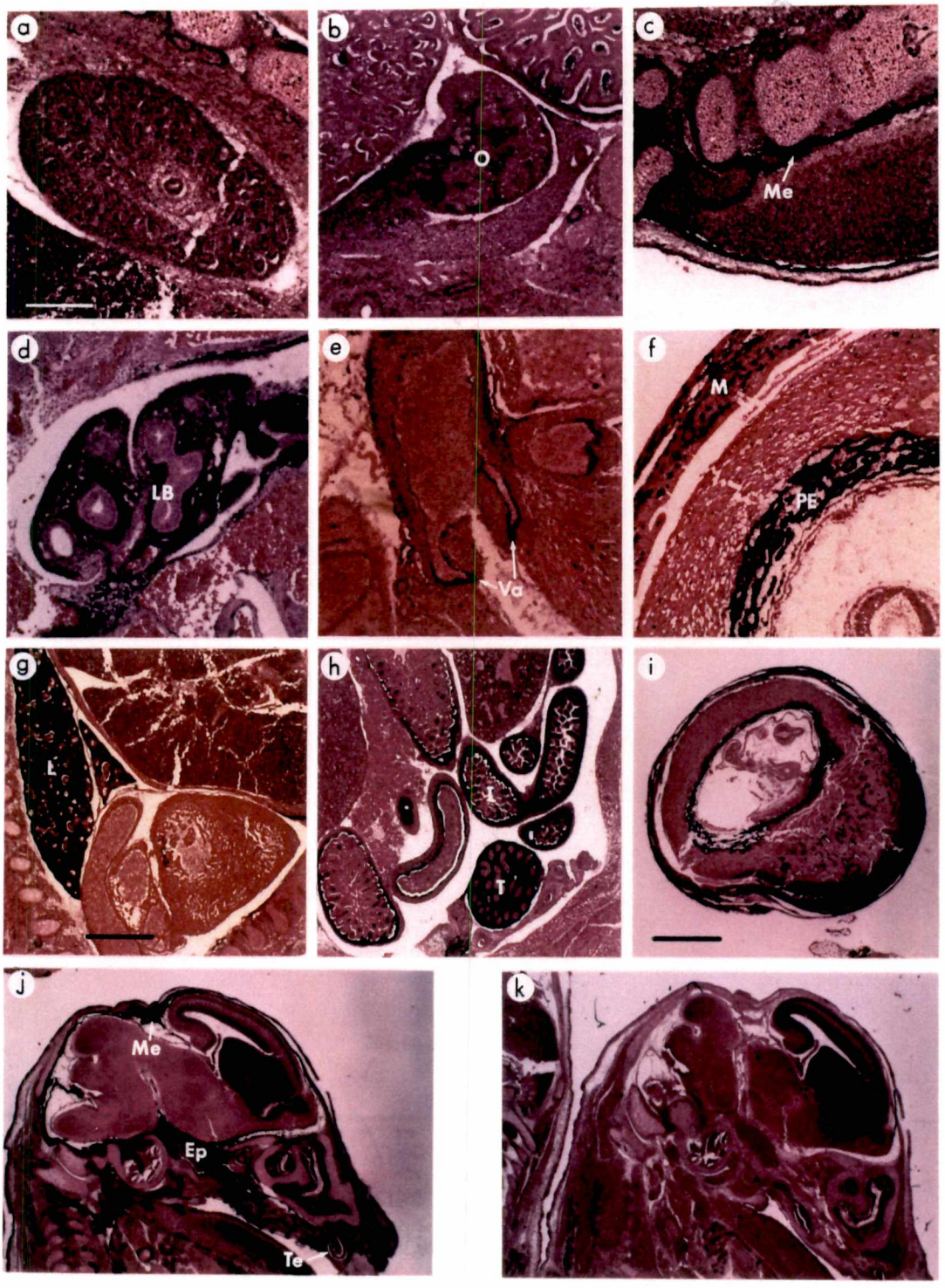


Figure 3.2

Figure 3.3. Immunohistochemical staining of adult A/J tissue, autoradiographs exposed for two weeks. lung, (a); kidney, (b); spleen, (c); ovary, (d); uterus, (e); intestine, (f); liver, (g), and heart, (h). Bar is equal to 400 microns.

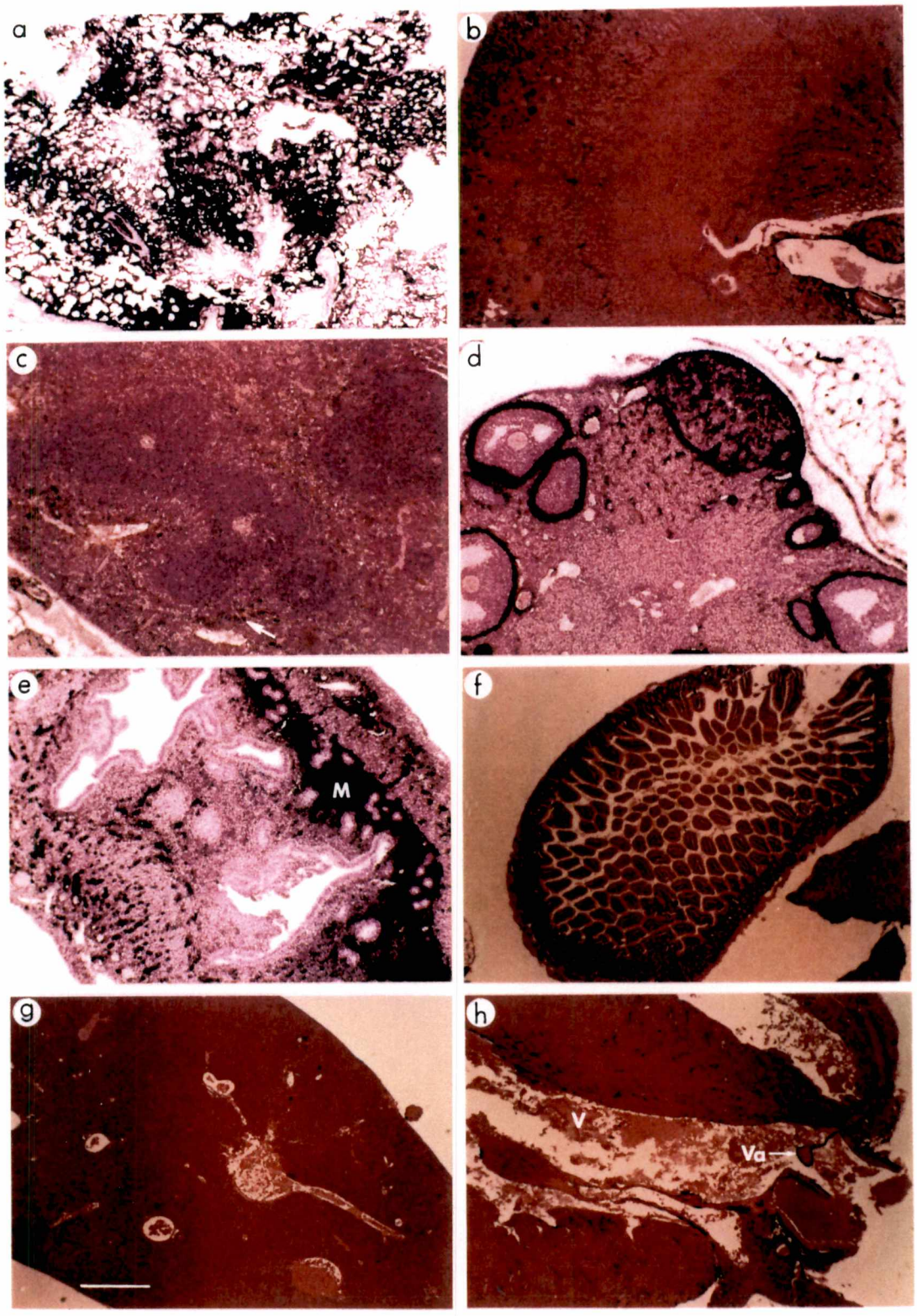


Figure 3.3

Figure 3.4. Immunohistochemical staining of developing A/J tissue, autoradiographs exposed for two weeks. Panels a-f, bar is equal to 100 microns, Day 15.5 kidney, (a); Day 14.5 ovary, (b); Day 15.5 spinal meninges (Me), (c); Day 14.5 lung (L), (d); Day 18.5 heart valves (Va), (e); Day 15.5 testes (T),(f). Panels g,h bar is equal to 400 microns. Day 17.5 lung (L),(g); Day 17.5 intestines (I),(h). Panels i-j bar is equal to 1 millimeter. Day 11.5 embryo and extraembryonic tissue, PE= parietal endoderm, (i); Day 15.5 head, Me = meninges, Na = nasal cavities, (j); Day 12.5 whole embryo, B = brain, (k).

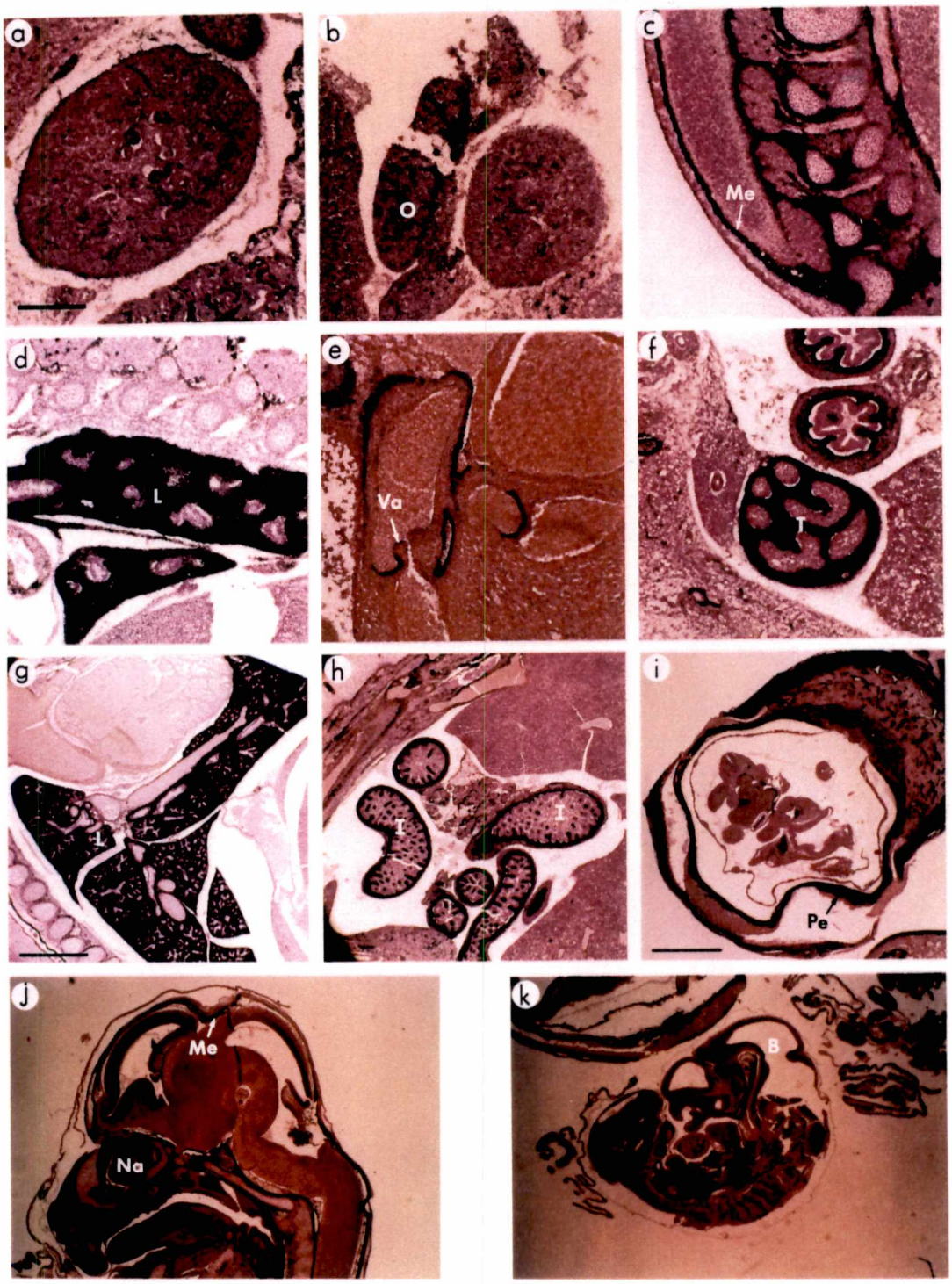


Figure 3.4

development. A summary of these data is presented in Table 3.1.

Circulatory system

In the adult mouse, the atrial endocardium and the heart valves show a higher degree of TM expression than was seen in the ventricular endocardium (Figures 3.1i and 3.3h). In the embryo, TM in the circulatory system was first seen in the developing aorta at Day 9.5. From this point in development onward, the endothelium of all large arteries and veins stained positively for TM. Within the chambers of the heart, the first visible TM staining was seen in the valves forming between the atria and ventricles at Day 13.5. Staining was always darker in the valves when compared to the endocardium. Figures 3.2e and 3.4e show Day 18.5 heart valves in BALB/cBD and A/J mice respectively. TM staining of the ventricular endocardium was evident at Day 13.5 and was more marked by Day 17.5. TM staining in the atrial endoderm followed the same pattern of expression as was seen in the ventricle.

Urinary system

In the adult kidney, TM was detected only in the arteries and veins of the medulla and cortex. Very little TM was found in the capillaries of the glomerular tufts or in the kidney capsule of the adult (Figures 3.1b and 3.3b), confirming earlier quantitative studies on adult mouse tissues (Ford *et al.*, 1992; Chapter 1). The tissues of the developing renal system did not show TM staining until very late in gestation. Figures 3.2a and 3.4a show Day 15.5 kidneys, which are negative for TM staining. The blood vessels of the kidney,

Table 3.1. Thrombomodulin in developing BALB/cBD and A/J mice.

	days post coitum											
TISSUE	9.5	10.5	11.5	12.5	13.5	14.5	15.5	16.5	17.5	18.5	19.5	Adult
Lung												
Vessels					+3	+3	+4	+4	+4	+3	+3	+4
Airway mucosa					+3	+4	+4	+4	+4	+4	+4	
Tracheal mucosa							+3	+4	+4		+3	+2
Nasal mucosa							+3		+3	+2	+4	
Lung bud			+2	+3								
Thoracic pleura												+1
Heart												
Atrial endocardium				-	+/-		+/-		+2	+1	+2	+4
Ven. endocardium				-	+/-		+/-	+/-	+1	+/-		+3
Valves				-	+2	+1	+3			+3	+3	
Aorta	+1		+1									
Intestines												
Submucosal vessels						+2	+4	+4	+4	+3	+3	+2
Vessels of the villi							+4	+4	+4	+3	+3	+2
Kidney												
Arteries & veins						-	-	+/-	+2		+2	+3
Glomeruli						-	-	-	-		-	+/-
Capsule						-	-	+/-	+1		+1	+1
Stomach												
Submucosal vessels					-	-	+2	+2	+1	+1	+2	+2
Peritoneum										+1	+1	+1
Liver												
Sinusoids					-	-	-	-	-	-	-	-

Table 3.1 (continued)

TISSUE	days post coitum 9.5	10.5	11.5	12.5	13.5	14.5	15.5	16.5	17.5	18.5	19.5	Adult
Reproductive Organs												
Uterus												
Mucosal vessels												+4
Myometrium												+3
Serosal membrane												+2
Ovary												
Secondary follicle theca												+3
Interstitium							+\-	+1		+1	+1	
Testes												
Interstitium							+2	+4	+4			+\-
Neural tissue												
Brain meninges	+/-		+1	+4	+3	+4	+4	+4	+4	+4	+4	+/-
Spinal meninges			+1		+4	+4	+4	+4	+4	+4	+3	
Vibrissae follicles							+3	+2	+2	+3		-
Teeth							+1	+2	+1	+1	+1	-
Developing cartilage							+4	+4	+3	+2		-
Spleen												
Red pulp												+
White pulp												-

Note. Thrombomodulin expression in developing BALB/cBD and A/J mice as measured by ^{125}I MoAb 273-34A immunohistochemistry. Intensities of autoradiograms were scored as (-), negative; (+), weakly positive to (+4), strongly positive. Blank spaces indicate that no section was available.

excluding the glomerular vessels, were positive for TM staining at Day 16.5. By Day 17.5 the distribution and levels of TM expression was similar to that seen in the adult. The kidney capsule began expressing TM at the same time as the larger vessels (Day 16.5). Vessels of the glomerular tufts were not positive for TM at any stage of development.

The Digestive System

In the adult intestine, TM staining was localized to the mucosal microvessels (Figs 3.1h and 3.3f) A very similar pattern was seen in the developing fetus although staining was more intense. In the developing intestines, TM staining was first visible at Day 14.5 and was present from this time onward. Figures 3.2h and 3.4h depict a Day 17.5 gut and (I) marks the intestines. The stomach showed the same pattern of expression, with TM first appearing in the mucosa at Day 15.5. Staining of the serosal surface was present at Day 18.5.

The neural system

In the adult mouse, the evidence of TM staining in the blood vessels of the brain was equivocal (Fig 3.1f) However, there was a slight staining of the meninges surrounding the brain. The developing animal exhibited a very different staining pattern for TM in neural tissue. Staining in the meninges of the developing brain was first seen at Day 9.5. By Day 12.5, this staining had intensified, and from this time on, TM expression was very high in the meninges of the brain (Fig 3.2j and 3.4j, Me) The meninges surrounding the spinal cord also showed high levels of TM expression throughout development (Figures 3.2c

and 3.4c, Me). This staining pattern was no longer visible by Day 5 after birth. The BALB/cBD negative control (MoAb 135-14, Figure 3.2k) shows very little background staining.

The Reproductive System

In the reproductive tract of adult mice, staining for TM was present in both testes and ovaries. In the ovaries , expression was limited to the theca of maturing follicles (Figs 3.1d and 3.3d) TM is also detected in the endometrial stroma, the myometrium (M), and the serosal surface of the adult uterus (Figs 3.1g and 3.3e) In the adult male, less intense TM staining was present in the scant interstitium of the testes (Fig 3.1e, see arrow). Sections of developing embryos displayed intense staining in the interstitium of the developing testes. In Figures 3.2h and 3.4f, the testes are marked with a T. The developing ovaries, which do not contain a large interstitium, express less TM (Figures 3.2b and 3.4b, O).

In the placenta, intense TM staining was identified in the parietal endoderm throughout development as detected by others (Imada *et al.*, 1987). The visceral endoderm was negative for TM expression (Figures 3.2f, 3.2i, and 3.4i) TM was also found in the cells lining large vascular spaces in the maternal placenta.

Other Sites of TM expression

Strong TM staining is seen in several additional regions during fetal development, namely the vibrissae follicles, teeth (Te) (Figure 3.2j), and

mesenchyme that is differentiating into cartilage. In the adult, no TM staining is seen in whisker follicles, teeth, or cartilage (data not shown). Staining for TM protein is visible around the developing whiskers and teeth from Day 15.5 through Day 19.5. Developing cartilage shows strong TM staining in the feet, nasal cavities (Figure 3.4j, Na), tracheal rings, and the ethmoid plate (Ep) (Figure 3.2j). No staining of these sites was noted in the control (Figure 3.2i, foot and tracheal rings not shown)

Discussion

The results of this study concerning the distribution of TM in developing murine tissues indicate that TM may play several different roles in development: one role being clot regulation, as seen in the adult vascular system. The second developmental role for TM is hypothesized to be the proper formation of blood vessels, through protein interactions involving the lectin-like region of TM.

Our results on the distribution of TM in the adult animal was similar to findings in several other species including humans and rabbits (DeBault *et al.*, 1986a; Maruyama *et al.*, 1985; Boffa *et al.*, 1987). We have shown TM to be expressed in major arteries and veins throughout the body, and in the smaller capillaries of many organs, with the lung microvessels containing approximately 70% of the total adult body levels of TM (Ford *et al.*, 1992; Chapter 1). The microvessels of the kidney glomerulus, the hepatic sinusoids, and all of the

vessels in the adult mouse brain displayed little or no stain for TM. TM expression in brain blood vessels is a disputed point. TM staining was reported to be absent in human brain (Ishii *et al.*, 1986) but present in rabbit brain (DeBault *et al.*, 1986a).

In the adult animal, TM is located primarily on the luminal surface of blood vessels (DeBault *et al.*, 1986a). Therefore, the blood vessels of the developing embryo were expected to stain positive for TM where its function is likely that of clot prevention. In fact, TM was first detected in the developing blood vessels at Day 9.5 in the forming aortic arch. Although there are other rudimentary vessels in the embryo at this point, TM staining was not detected in them. TM staining of the aortic arch was still visible at Day 11.5 when strong staining was also detected in the lung-bud epithelium. From Day 11.5 onward, all cross sections of major arteries and veins stain for TM. One finding of interest was the strong staining for TM in the developing heart valves, observed in embryos from Day 13.5 through 18.5 (Fig 3.2e and 3.4e), whereas the atrial and ventricular endocardium did not demonstrate TM staining until very late in development (Day 19.5). This finding suggests that TM is necessary early in development, only in the heart valves where there may be high risk of unwanted clotting.

The intestines and stomach exhibited a very similar pattern of TM staining in the developing animal. In the intestines, TM was first detectable on Day 14.5 in the mucosa stroma; concurrently, strong staining was seen in both the

mucosal and villus vessels of the intestines. The stomach showed submucosal vessel staining starting at Day 15.5, whereas mesothelial staining was first evident on Day 18.5. These tissues demonstrated a pattern of TM expression where MoAb staining was apparent only when the organs were undergoing rapid proliferation, suggesting that TM may be expressed to a marked degree in regions where angiogenesis is underway. The process of angiogenesis may lead to the expression of large amounts of TM. A developing capillary system is composed of short regions of vessel channels that bud off, forming isolated regions that eventually reconnect again (Noden, 1989). These isolated regions of capillaries are likely to contain stagnant blood. In such regions, high amounts of an anti-coagulant, like TM, might be critical to proper formation of the vascular system. The regions of TM expression mentioned thus far suggests TM may function as an endothelial cell specific anti-coagulant during development. However, TM staining was detected in several regions were an organized endothelium was not visible.

TM has been shown to be expressed in the parietal endoderm-like tissue of differentiated F9 embryonal carcinoma cells and in the parietal endoderm of Day 7.5 embryos (Imada *et al.*, 1987). The data presented support this earlier work, showing high amounts of TM expression exclusively in the parietal endoderm, even at later stages of development (Figs 3.2f, 3.2i, and 3.4i). While there are vessels located in this region, the staining seems too intense to be the result of expression on endothelial cells alone.

The developing lung was another area where TM expression was visible in regions lacking mature vessels. In the adult lung, TM staining was most intense in the lung microvessels, whereas, no staining was seen in the epithelial lining of the lung airway mucosa (Figs 3.1a and 3.3a). Therefore, staining in the developing lung was expected, after the formation of the lung vasculature. Surprisingly, the developing lung showed TM expression very early, in the lung bud epithelium (Day 11.5), before the vessles are visible. Even at the later stages of development, TM staining is most pronounced in the epithelial tissue of the airway mucosal layers, with smaller amounts of TM staining in the developing blood vessels (Figs 3.2d, 3.2g, 3.4d, and 3.4g). These results suggest that TM may be needed prior to the formation of lung blood microvessels, which are not identifiable histologically until Day 16.5 (Ten Have-Opbroek,1981). Work by Imada *et al.* (1990) in Day 10.5 embryos detected similar staining in the lung bud epithelium. We have shown that TM is present and continues to be present in large amounts in epithelial tissue of the developing lung from Day 11.5 to birth and following birth (data not shown).

Other regions of undifferentiated tissue demonstrated TM staining during development. By Day 13.5, there was strong staining in the bronchial mucosa (Fig 3.2d) and the developing tracheal mucosa. The nasal submucosa showed strong TM staining as it developed from Day 15.5 onward (Fig 3.4j, Na), although there was relatively little staining in the adult nasal submucosa (data not shown). It is possible that the undifferentiated cells that stain for TM are

those which are destined to form blood vessels, and therefore, are stimulated to produce TM during differentiation. However, as the vascular system has not yet formed, the necessity of an anti-coagulant, like TM, at this time is not clear.

Perhaps the most striking finding in this study was the staining pattern found in the meninges of the developing brain and spinal cord, cartilage, and testes. The first time such staining was evident in the central nervous system was on Day 9.5 (just below the midbrain). From Day 12.5 onward, the meninges surrounding the brain and spinal cord, demonstrated the most intense staining seen in the entire embryo (Figs 3.2c, 3.2j, 3.4c, and 3.4j). Although there are arteries and veins visible in this region by Day 11, the distribution of the stain seems too intense to be the result of only staining of vessels. In contrast to this result, very little staining for TM is visible in the adult mouse brain (Figure 3.1f).

The unusually high expression of TM in the lung-bud epithelium, the meninges, the interstitium of developing testes, differentiating cartilage, and the placental parietal endoderm may indicate a role other than the regulation of coagulation. TM has structural features which indicate it may have multiple functions. It is a sulfated proteoglycan (Bourin, 1991) with a large lectin-like amino-terminal domain for which no specific function has been described. Recently, several other proteoglycans have been implicated in tissue morphogenesis and remodeling (Ekblom *et al.*, 1986; Simons and Fuller, 1985). It may be that TM serves multiple functions in the developing animal. TM may

act as both an anti-coagulant in developing vessels and as an important morphogenic regulator of tissue development. One possible developmental function for for TM might be pre-endothelial cell recognition, through TM binding receptors on separate pre-endothelial cells, leading to proper organization of the endothelium. Whereas in the adult vascular structures its primary function appears to be regulation of coagulation.

It has been previously demonstrated that some strains of mice contained an IAP provirus inserted 5' to the initiation site for TM (see chapter 2). Therefore, it was possible that the provirus was responsible for some of the unusual sites of TM expression during development. BALB/cBD, the animal used for most of our experiments, had the IAP element 5' to the TM gene. An additional strain of mouse, A/J, lacking the IAP element, was also examined for TM expression during development. The expression pattern for developing A/J mice was shown to be identical to that found in BALB/cBD mice which indicates that the expression of TM during is not controlled by the provirus during development.

EPILOGUE

The goal of this dissertation was to examine the function of a previously identified protein, P112, which was found to be located primarily on the vascular surface of lung capillaries (Rorvik *et al.*, 1988; Kennel *et al.*, 1988). Partial sequence analysis of a cDNA clone and amino acid analysis of the purified protein indicated that P112 was similar to another recently sequenced protein, thrombomodulin (TM)(Jackman *et al.,* 1987), which is involved in the regulation of clotting within the vascular system (Esmon, 1987; Dittman and Majerus, 1990). Further analysis using a subtractive monoclonal antibody experiment for P112, followed by Western blotting with a polyclonal antiserum to TM, demonstrated that P112 and TM were identical. In addition, a biochemical assay for protein C activation confirmed these results (see Chapter 1, Table 1.2).

The distribution of TM has been studied in many different systems. Initially, it was thought that TM was present only in vascular tissue. TM was found on the surface of endothelial cells of the blood vessels and lymphatics (DeBault *et al.*, 1986a). A proteolytic fragment of TM was found in both the blood and urine (Ishii and Majerus, 1991). TM has also been found on the epithelial linings of body cavities and the squamous epithelium of the epidermis (Yonesawa *et al.*, 1987). For all animals studied, the lung appeared to be the major site of TM expression (DeBault *et al.*, 1986a). However, it was believed

that the lung contained higher amounts of TM due to the high degree of vascularization in this organ. Northern blot analysis of poly-A containing RNA and MoAb two-site assay analysis for protein levels from eight different murine tissues demonstrated that the lung microvessels did indeed contain much higher amounts of TM per surface area of microvessels. The kidney, which is also highly vascularized but with fenestrated endothelium, contained only one-sixth the amount of TM per cm^2 of vascular surface whereas all other organs tested exhibited even smaller amounts of TM. The liver, which is composed of sinusoidal endothelium, contained minimal amounts of TM as determined by two-site assay. These results suggested that TM may be expressed in large amounts on continuous endothelium, with moderate levels on fenestrated endothelium and minimal amounts on the sinusoidal endothelium. However, the blood vessels in the brain, which are also considered to be continuous endothelium, display minimal amounts of TM (see Chapter 1, Table1.3). This may not be surprising since the vasculature of the brain has been demonstrated to be structurally and functionally unique from all other organ vessels (Palade *et al.*, 1979; Belloni and Tressler, 1990; Belloni and Nicolson, 1988). These results raise the possibility that there may be additional undiscovered mechanisms for the prevention of clotting in organ endothelium other than the lung. It also demonstrates specialized expression of proteins in endothelium of different structure and function throughout the body.

Proteins which are expressed only at specialized sites in the body are

presumed to be regulated by rigorous control mechanisms. Many of the DNA elements that control transcription have been found in the immediate 5' genomic region of the gene (Latchman, 1990; Faisst and Meyer, 1992). Analysis of this region of the TM gene was undertaken to aid in elucidation of the mechanisms that govern the restricted expression of TM. Comparison of the sequence generated from BALB/cBD DNA to a 5' promoter region for TM listed in Genbank (MUSTHROMBO, Accession #M74231) demonstrated 98 % homology with one major exception. The 5'-most region of BALB/cBD sequence contained an inserted intracisternal-A particle (IAP) provirus, which was not present in the Genbank sequence. Examination of ten other mouse strains, using polymerase chain reaction and Southern blot analyses, demonstrated that five of the strains contained the IAP proviral sequence at the same site, while the other five did not. In other systems, the insertion of IAP proviral DNA in the 5' promoter region has lead to changes in transcription levels (Ymer *et al.*, 1985; Canaai *et al.*, 1983; Chang-Yeh *et al.*, 1991; Mold *et al.*,1991); therefore, we were interested in determining whether this IAP proviral sequence had any effect on TM expression in mice. An investigation of the basal levels of both TM protein and mRNA in the lungs of different mouse strains demonstrated differences that could not be correlated with the presence or absence of the IAP provirus (see Chapter 2). Since TM is an intronless gene, which is present in only one copy in both humans and mice (Dittman and Majerus, 1990), it is not possible that these differences are due

to transcription from additional copies of the gene. Thus, the regulation of TM transcription, while independent of the IAP provirus, must be controlled by other elements in the 5' promoter region of the TM gene.

Another method used to gain insight into the function of P112/TM was the analysis of its expression during murine development. TM had previously been demonstrated to be expressed in the head mesenchyme, aorta, lung bud, and neuroepithelium of Day 10.5 embryos (Imada *et al.*, 1990). TM had also been localized to the parietal endoderm of retinoic-acid-and cyclic-AMP-induced F9 embryonal carcinoma cells (Imada *et al.*, 1987). Immunohistochemistry on fetal sections from Day 6.5 post coitum to birth demonstrated that TM is present in developing blood vessels (see Chapter 3). However, TM has also been found in some unexpected sites. As early as Day 6.5, TM was detected in high amounts in the parietal endoderm of the placenta whereas it is absent from the visceral endoderm. Other regions of TM expression during development include developing cartilage, neural meninges, and the interstitium of the developing testes (Chapter 3). IAP elements have been shown to be actively transcribed in the very early stages (2 to 8 cell stage) of murine development (Biczysko *et al.*, 1973; Kuff and Lueders, 1988). BALB/cBD, which contains an IAP provirus, was the first mouse strain studied for developmental expression of TM. We hypothesized that the IAP provirus was hypothesized to be responsible for some of the unusual sites of TM expression. To test this possibility, another strain, A/J, which does not contain the IAP provirus near to

the TM gene, was examined for TM expression during development. Immunohistochemical analysis of developing A/J embryos exhibit the same distribution pattern for TM as found in BALB/cBD embryos. These results demonstrated that the unusual sites of TM expression during development are not due to the presence of an IAP provirus 5' to the TM gene.

Since some of the regions exhibiting TM staining during development (cartilage, meninges, testicular interstitium) are regions that do not contain endothelial cells in large amounts to account for the high level of TM expression, it was concluded that TM plays an important role in development that is different from its role of the regulation of blood clotting in the adult vasculature. Much work has been conducted in order to pinpoint which domains were responsible for the separate functions of TM (Stearns *et al.*, 1989; Zushi *et al.*, 1989; Kurosawa *et al.*, 1988). To date, no function has been identified for the lectin-like domain, which accounts for one third of the protein. It seems possible that this region may have an important role during development that accounts for TM's unusual expression pattern.

Attempts to make mutant transgenic mice that lack a functional TM gene are currently underway at the Massachusetts Institute of Technology. These studies are expected to create mice with a propensity toward thrombosis. However, one can easily visualize the necessity for clot regulation during development. Therefore, it seems equally likely that such a mutant will not survive to birth. Since no known function has yet been attributed to the

lectin-like region of the gene, it would be interesting to determine the phenotype that results from a mouse with a functional TM gene lacking the only amino-terminal lectin-like region. Such experimentation might demonstrate a specific role for TM during development also might be lethal.

Future studies will involve deciphering the possible role of TM during embryogenesis. The current workin hypothesis is that TM aides in pre-endothelial cell/pre-endothlial cell recognitionand attachment, leading to proper organization of blood vessels. This may be accomplished through TM dimerization, mediated by the lectin-like region, or through TM binding to an another protein on the surface of pre-endothelial cells. This type of interaction would be predicted to be specific for the tissues that express TM. Comparison of TM structure at these sites may identify unique carbohydrate modifications which occur only in TM isolated from developing tissues. Identification of a new TM/protein interaction involving the lectin-like region of TM would be a big step in understanding TM expression during tissue development and may point to a general mechanism whereby glycoproteins may serve several different fuctions dependent on when and where they are expressed.

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VITA

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