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GENE TARGETING IN HEMOSTASIS. PROTHROMBIN

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1. ABSTRACT

There have been extensive studies on the structure and function of prothrombin; a protein critical for the coagulation of blood. The biological functions of prothrombin and its activated form, thrombin are discussed, as well as the structure and functional domains of the protein. Prothrombin is expressed in a tissue-specific manner and its gene structure and regulatory elements have been analyzed in detail. In order to learn more about the functions of prothrombin in an *in vivo* context, the gene was ablated in mice. Homozygous deletion of prothrombin results in a partial embryonic lethal phenotype. Approximately half of the homozygous mutant mice die during mid-gestation and the remainder die soon after birth. The cause of death of neonates is due to excessive bleeding, while null embryos have a lack of integrity of the yolk sac membrane resulting in bleeding into the yolk sac cavity. These results are discussed in relation to the phenotypes found for other mice lacking specific coagulation factors.

2. INTRODUCTION

2.1. The biology of prothrombin and thrombin

The critical role that prothrombin/thrombin plays in the blood coagulation pathway was the first identified biological function for this protein. Subsequently, thrombin has been identified to be critical for the regulation of prothrombin activation in the anticoagulant process and to have mitogenic and/or regulatory properties on specific cell types by activation of specific thrombin receptors.

Activation of prothrombin to thrombin is the final enzymatic activation step in the coagulation pathway (1, 2). Prothrombin is proteolytically activated to thrombin by factor Xa in the presence of calcium ions, a phospholipid membrane and its cofactor, factor Va. This activation requires the cleavage of two peptide bonds and subsequent release of the serine protease, thrombin from the amino-terminal portion of the protein. Thrombin is then free to circulate and cleaves soluble fibrinogen such that the resultant fibrin forms the initial stages of the blood clot. Thrombin also activates the transglutaminase factor XIII to factor XIIIa, an enzyme responsible for the cross-linking of fibrin and the stabilization of the fibrin clot.

As an anticoagulant, thrombin binds to the cell surface receptor thrombomodulin. By binding to this receptor, thrombin undergoes a change in its three-dimensional structure such that it has a different substrate specificity; specifically toward protein C rather than toward fibrinogen (3). Thrombin activates protein C, which then proteolytically digests its substrates, factors Va and VIIIa to inactivate them. In this way, thrombin regulates its own synthesis, since factors Va and VIIIa are involved in the pathway leading to blood clot formation.

More recently, thrombin has been found to elicit specific cellular responses in specific cell types (table 1). These responses are mediated through thrombin-activated receptors called PARs (4-8). PARs are G-coupled protein receptors that have seven-transmembrane spanning

Table 1. Properties of Prothrombin

Functions	Substrates or target cells
Coagulation	Fibrinogen
	Factor XIII
	Platelets (PARs and integrin αIIβb3)
Anticoagulation	Protein C
	Factors Va and VIIIa
Inflammation	Monocytes
	Macrophages
Atherosclerosis/restenosis	Vascular smooth muscle cells
	Endothelial cells
CNS functions/response to injury	Neurons
	Astrocytes
	Synapses
Tissue repair	Fibroblasts
	Endothelial cells
Mitogen	Fibroblasts
	Endothelial cells
	Astrocytes
	Vascular smooth muscle cells

domains and are activated in a unique process involving proteolytic cleavage by thrombin in the extracellular domain. The amino-terminal cleaved portion of the receptor stays bound to the remainder of the receptor as a tethered ligand that is then responsible for the activity of the receptor. Thrombin activates PAR-1, PAR-3 and PAR-4. These receptors have been localized to platelets, vascular endothelial cells, the bone marrow, megakaryocytes and the nervous system. Recently, it has been shown that PAR-3 acts as a cofactor for the activation of PAR-4 by thrombin (7).

In platelets, thrombin activates PARs which leads to stimulation of secretory reactions and induction of a shape change that allows the platelets to function in the control of blood loss (8). Prothrombin has also been found to interact with integrin αIIβb3 on platelets, which accelerates platelet activation (9). In other cell types where PARs are expressed, thrombin mediates secretion of proteins required for cell growth, such as cell matrix proteins, growth factors and cytokines.

Numerous *in vitro* studies involving cell culture experiments have implicated thrombin to function in a diverse number of biological processes including inflammation, wound healing, atherosclerosis and astrocyte growth. Thrombin's function in atherosclerosis and restenosis has been inferred from studies involving smooth muscle cell proliferation following vessel injury. Thrombin receptors are normally expressed in the endothelial layer of the artery, while in arteries with atherosclerosis the receptors are expressed in regions rich in smooth muscle cells and macrophages (10-13). When added to smooth muscle cells, thrombin upregulates expression of specific thrombin receptors, stimulates proliferation and increases the growth of the cells and induces mobilization of intracellular calcium (14-16).

As a mitogen, thrombin increases the DNA synthesis of fibroblasts and endothelial cells (17-21). Thrombin also binds to the subendothelial extracellular matrix and thus may be involved in cell invasion and in increasing the metastatic ability of neoplastic cells (22, 23). Prothrombin and its derivatives are able to promote the growth and invasiveness of specific tumor cell lines, including those derived from melanomas (24). In experimental models, anticoagulant therapy suppresses the invasion of cancer cells (25).

Studies have shown that thrombin has chemotactic and mitogenic properties for monocytes and macrophges and therefore may have a role in the inflammatory response (18, 26, 27). Early events that are associated with the stimulation of inflammatory cells by chemotactic agents, such as the rapid association of cytosolic actin with the cytoskeletin and an increase in free calcium in the cytosol occur when thrombin is added to these cells in culture (26, 27). As a mitogen, thrombin stimulates DNA synthesis in macrophages (18). In addition, others have shown that infusion of prothrombin into the caudoputamen of the rat leads to the infiltration of inflammatory cells to this site as well as the proliferation of mesenchymal cells (28).

Thrombin has been found to regulate specific activities of neurons, synapses and astrocytes and may therefore play a role in the development of the central nervous system and its response to injury (29, 30). Prothrombin has been found to be synthesized in the brain, specifically in astrocytes and neurons (31-34). The level of expression in the central nervous system is approximately 1% of that found in the liver (35). In culture, thrombin is a mitogen for astrocytes and is involved in the regulation of the growth of neurites. Thrombin stimulates astrocytes by increasing cell number and DNA synthesis and by stimulating the secretion of nerve growth factor (29, 36, 37). Thrombin also reverses the stellation of astrocytes (29). Neurite outgrowth of neuroblastoma cells and primary neuronal cells is inhibited by thrombin (30). Thrombin may also be required for the viability of neurons and astrocytes (38).

The biological functions of prothrombin are diverse and dependent upon the sites of synthesis of its receptors and the presence of its substrates. The generation of *in vivo* models involving prothrombin expression will be critical for confirming the biological role of this protein in various processes.

2.2. The structure of prothrombin

The major site of synthesis of prothrombin is in the liver (39), although much smaller amounts have been found to be synthesized in the central nervous system, skeletal and smooth muscle cells and the kidney (11-13, 35, 40). Prothrombin is expressed as a single polypeptide chain of 72 kDa (figure 1; 41). The form of prothrombin found circulating in the blood contains 579 amino acids, 8% carbohydrate and 10 γ-carboxyglutamic acid residues (42-44).

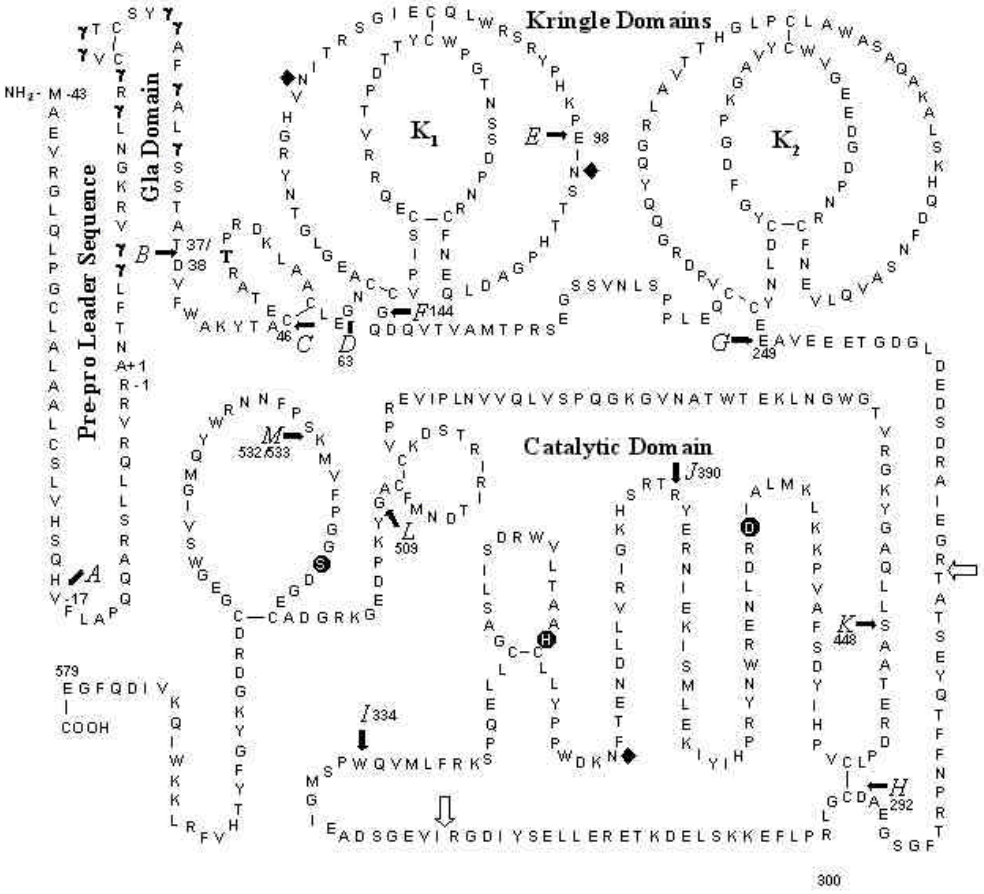

Figure 1. Diagrammatic structure of the amino acid sequence of human prothrombin. The amino acid sequence of human prothrombin is indicated starting with the signal peptide from –43 to –19, the propeptide required for gamma-carboxylation of specific Glu residues (-18 to –1), the Gla domain, two kringle domains (K1 and K2), and the serine protease domain (catalytic domain). The amino-terminal of the protein before secretion is indicated by an NH2 at residue –43 while the amino acid at the amino-terminal end of the mature protein is at +1. The carboxy-terminal is indicated by a COOH at residue 579. Gla residues are indicated by γ, carbohydrate attachment sites are represented by the placement of solid diamonds, active site amino acids are indicated in solid circles and disulfide bonds are shown with lines between the appropriate cysteines. Factor Xa activation sites are indicated by the two open arrows. The placement of the thirteen intervening sequences in the human prothrombin gene with respect to the amino acid sequence of prothrombin are indicated by labelled solid arrows (A-M) including the amino acid(s) interrrupted by this sequence. The catalytic portion of the protein, thrombin which becomes active after cleavage by factor Xa at two sites is composed of two chains; an A chain of 49 amino acids disulfide bonded to a B chain of 259 residues.

The entire amino acid sequence of both human and bovine prothrombin has been determined by traditional protein sequence analysis approaches (42, 45-48). These sequences were confirmed when the cDNAs coding for bovine and human prothrombin were isolated and sequenced (44, 49, 50). Full-length cDNAs coding for human, bovine, mouse and rat prothrombin have been isolated and characterized (44, 50-52). In addition, a parital cDNA has been characterized for rabbit prothrombin (53). The sequence of the B chain of thrombin has been determined for nine vertebrate species (54).

Several post-translational events are required for the appropriate synthesis of a functional prothrombin molecule. A signal peptide (residues -43 to -19 in Figure 1) is required for secretion through the rough endoplasmic reticulum and is removed from the amino-terminal of the protein during this process (44). A propeptide of 18 amino acids (residues -18 to -1 in Figure 1) that is present between the signal peptide and the mature amino-terminal end of the protein is required for recognition by a γ-carboxylase that prothrombin is a substrate for gamma-carboxylation (55). The first ten glutamic acid residues at the amino-terminal end of the mature protein (after removal of the signal and propeptide) are the residues modified to γ-carboxyglutamic acid (Gla). The microsomal γ-carboxylase requires vitamin K as a cofactor and is present in the rough endoplasmic reticulum. When vitamin K is lacking (as is the case with newborn infants) or in the presence of the carboxylase inhibitor, warfarin, prothrombin circulates as its noncarboxylated precursor form and is nonfunctional (56). Several other coagulation proteins, as well as proteins

found in the bone also require vitamin K for their biosynthesis (42, 57). Finally, carbohydrate is added to several sites throughout the protein (Asn78, Asn100 and Asn373) and comprises 8% of the mass of the protein (43).

Prothrombin contains several domains that are responsible for unique functions. These include the Gla domain, the aromatic stack region, two kringle structures, and a serine protease domain. The Gla residues reside within the first 40 amino acids at the amino-terminal end of the protein. The Gla residues are required for prothrombin to bind calcium and for interaction with phospholipids on the cell surface following injury (42, 58, 59). This region is homologous to the Gla domain in other vitamin K-dependent proteins, especially those involved in the coagulation process (factors VII, IX, X and protein C). The aromatic stack region is comprised of a short region of hydrophobic amino acids. The two kringle domains immediately follow the aromatic stack region. These domains were first identified in prothrombin due to the fact that there was an internal duplication identified by amino acid sequence analysis (42). These structures are about 80 amino acids in length, contain three disulfide bonds and are approximately 50% identical to each other, as well as to the kringle structures in other proteins. These structures function autonomously and fold independently (60-62). They function as protein binding domains that interact with cofactors, substrates or receptors required for the regulation and function of the protein (63, 64). Factor Va, a cofactor for the activation of prothrombin to thrombin, binds to the second kringle domain in prothrombin (65, 66). Several other proteins have kringle domains including the coagulation and fibrinolytic proteins-factor XII, plasminogen, urokinase, and tissue plasminogen activator.

The serine protease domain resides at the carboxy-terminal portion of the protein. This region is homologous to other serine proteases such as trypsin. Activation of prothrombin by factor Xa, results in the cleavage of two peptide bonds. The catalytically active portion of the protein, thrombin is released from the amino-terminal end of the protein containing the Gla and kringle domains that are bound to the cell surface. Thrombin is then free to circulate and activate or cleave its substrates (i.e. fibrinogen, protein C, and factors Va, VIIIa and XIII). Thrombin is composed of two polypeptide chains that are held together by a disulfide bond and has a molecular weight of 39 KDa. In the human enzyme there is a 49 residue A chain and 259 residue B chain. The residues required for the enzymatic activity that comprise the active site of thrombin are His-363, Asp-419 and Ser-525. Other residues are required for the limited substrate specificity of thrombin when compared to trypsin.

Because of its pivotal role in the coagulation and anticoagulation process, thrombin binds to many proteins. Activation of prothrombin results in expression of two regulatory domains in thrombin, exosites I and II (67-69). Exosite I is an anion-binding region composed of basic amino acids that reside on the surface of the protein at some distance from the active site (70). This region is responsible for the substrate specificity of thrombin. Substrates that bind to this region include fibrinogen, factors V and Va, thrombomodulin, the PAR family of receptors, and inhibitors such as the activated fibrinolysis inhibitor (TAFI) and hirudin (present in the saliva of leech), heparin cofactor II and the single-stranded aptamer (68, 71-82). Anion-binding exosite II, another positively charged surface, appears to be responsible for binding thrombin to the cell surface while maintaining the accessibility of the active site and anion-binding exosite I for binding its substrates (83-85). This site interacts with glycosaminoglycans and heparin and binds to the second kringle of prothrombin where it is masked until activation to thrombin removes this interaction (86).

The substrate specificity of thrombin is modulated by the binding of sodium at a region near the carboxy-terminal of the protein (87, 88). With sodium present thrombin is more specific towards fibrinogen as its substrate and when no sodium is bound in the sodium-binding loop thrombin becomes an anticoagulant and activates protein C. The binding of sodium to thrombin results in a transition from a slow to a fast form of the enzyme. The binding loop consists of eight amino acid residues which are highly conserved throughout evolution.

Results from characterization of the cDNAs indicated the presence of the pre-pro leader sequence in prothrombin before secretion from the rough endoplasmic reticulum (44). The full-length and partial sequences of cDNAs coding for prothrombin indicate regions that are conserved in all species characterized to date as well as the overall conservation of the protein. While human and mouse prothrombin are approximately 70% identical overall, specific regions are more highly conserved and indicate an absolute requirement for this region in the function of prothrombin/thrombin. The size of the B chain has remained invariant throughout evolution, as well as the sequences surrounding the active site, cysteines and tryptophans have also been conserved in this region of thrombin (54). In addition, extensive site-directed mutagenesis experiments have identified critical amino acid residues in exosites 1 and 2 that confer their unique binding properties (89).

X-Ray diffraction studies have determined the three dimensional structure of thrombin in a complex with either a chloromethylketone or hirudin (74, 90, 91) at approximately 2 A resolution. Compared to trypsin, thrombin has two extended insertions on its surface resulting in loop-like structures. These loops appear to be responsible for the strict substrate specificity of thrombin.

2.3. The gene coding for prothrombin

The gene coding for prothrombin has been localized to human chromosome 11 near the centromere (92) and to mouse chromosome 2 proximal to the catalase locus (51). This region of mouse chromosome 2 is syntenic with the proximal end of human chromosome 11, indicating that it is likely that the prothrombin locus is on this side of the centromere.

Table 2. Comparison of the lengths of exons and introns in the genes coding for human, bovine and mouse prothrombin[1]

Exon	Human amino acids	Human Gene[2] (bp)	Bovine Gene [3] (bp)	Mouse Gene (bp)	Intron	Human Gene[2] (bp)	Bovine Gene [3] (bp)	Mouse Gene (bp)
1	-43 to –17	79+[4]	94+[5]	79+[4]	A	386	342	376
2	-17 to 37	161	164	164	B	659	601+/- 62	579
3	38-46	25	25	25	C	242	227	221
4	46-63	51	51	51	D	2326	1504+/-73	1600
5	63-98	106	106	106	E	96	98	78
6	98-144	137	137	137	F	2338	1381+/-99	2146
7	144-249	315	315	318	G	324	293	242
8	249-292	129	135	114	H	84	75	76
9	292-334	127	127	127	I	1157	1055+/-94	631
10	334-390	168	168	168	J	497	397+/-46	317
11	390-448	174	174	ND	K	542	242	ND
12	448-509	182	182	ND	L	9447	6940+/-255	ND
13	509-532	71	71	ND	M	146	135	128
14	533-polyA	241	266	253				

ND: A portion of the mouse prothrombin gene was not isolated and therefore not characterized; this included exons 11-13 and intervening sequences K and L. [1] All sizes were determined by DNA sequence analysis, except for those in the bovine gene indicated with standard deviations which were determined by heteroduplex analysis with the electron microscope. [2] Reference 93 [3] Reference 95 [4] Due to variability in the transciption start site the size of the exon is indicated as starting at the initiator methionine [5] The length of exon 1 is indicated as the size starting in the 5' flanking region where the longest cDNA started.

The structure of the gene coding for human prothrombin has been completely determined (44, 93). The gene is 20,241 bp in length from the site of initiation of transcription to the site of polyadenylation. Fourteen exons separated by 13 intervening sequences code for prothrombin (table 2). The exons range in size from 25 to 315 bp in length while the introns range from 84 to 9447 bp in length. Sequence has also been determined both upstream and downstream of the gene such that there is 26,929 bp of continuous sequence for this locus (93, 94). For the 5' flanking region, 6544 bp has been characterized while 145 bp have been sequenced in the 3' flanking region. Partial sequences have been obtained for both the mouse and bovine prothrombin genes (95; unpublished). Comparison of the sizes of exons and introns is shown in Table 2. Exons are almost identical in length for the genes from human, bovine and mouse with exon 8 being the most variable. Exon 8 codes for the region following the second kringle and includes the A chain of thrombin which is variable in length between species. Introns are amazingly similar in size indicating some evolutionary constraint. IVS L is the largest intron in both the human and bovine genes while IVS H is the smallest for all three species where the prothrombin gene has been characterized.

The structural domains present within the prothrombin protein are also reflected by the location of intervening sequences within the gene (figure 1; 93). Exons or sets of exons code for the domains. For example, separate exons code for the signal peptide, the Gla domain attached to its pro-peptide, the hydrophobic stack region and the second kringle domain. The first kringle domain is separated into two exons. In the serine protease domain, each of the catalytic amino acids is separated into individual exons. This genomic structure is similar to that found in genes coding for homologous proteins such as tissue plasminogen activator (96, 97) and plasminogen (98).

The human prothrombin gene is unique in that it is composed of approximately 50% repetitive DNA sequences within the introns and in the 5' flanking regions of the gene (93, 94). Alu repetitive sequences are the most common type in the prothrombin gene (99), with 41 copies present. These sequences are approximately 300 bp in length and comprise about 5% of genomic sequences. They are characterized by flanking direct repeats and often occur in tandem arrangements. This has been found to be the case for those in the prothrombin gene. Other repetitive sequences present in the prothrombin gene include two partial Kpn I repeats and several copies of MER middle repetitive sequences (93, 100). In addition, there are two regions in the prothrombin gene that are homologous to regions of the adenosine deaminase gene (100).

The bovine prothrombin gene is approximately 15.4 kb in length and has the same exon/intron structure as the human gene (table 2; 95). The difference in size between the human and bovine genes is likely due to the excessive amount of repetitive sequence in the human gene. There is no equivalent to Alu repetitive sequence in the bovine genome. Since DNA sequence was only determined for approximately 5 kb of the bovine gene it has not been determined whether there is repetitive sequence present or not.

Table 3. Exon-intron junctions in the mouse prothrombin gene[1]

E1	A	T	G	**G**	**T**	A	A	G	G	C	T	G	C	**A**	**G**	T	G	T	E2
E2	A	C	A	**G**	**T**	G	A	G	T	T	T	G	T	**A**	**G**	G	A	C	E3
E3	C	A	G	**G**	**T**	G	A	G	T	T	T	T	T	**A**	**G**	G	A	C	E4
E4	A	A	G	**G**	**T**	G	G	G	G	C	T	G	T	**A**	**G**	G	T	C	E5
E5	T	G	A	**G**	**T**	G	A	G	T	T	T	A	C	**A**	**G**	A	A	T	E6
E6	G	T	G	**G**	**T**	G	A	G	C	C	A	C	C	**A**	**G**	G	C	C	E7
E7	G	C	G	**G**	**T**	G	A	G	C	C	T	A	C	**A**	**G**	A	G	G	E8
E8	C	A	G	**G**	**T**	G	A	G	A	C	T	G	C	**A**	**G**	A	C	T	E9
E9	C	T	G	**G**	**T**	G	C	G	T	C	G	T	T	**A**	**G**	G	C	A	E10
E10	C	A	G	**G**	**T**	A	T	G	A	G	C	A	C	**A**	**G**	A	T	A	E11
E11																			E12
E12																			E13
E13	A	A	G	**G**	**T**	A	T	G	C	C	C	T	C	**A**	**G**	A	G	C	E14

[1]Exons 11 and 12 and the corresponding intervening sequences have not been isolated

Continuous sequence for the mouse prothrombin gene has been determined for the 5' flanking region up to and including exon 10 (101; unpublished data). This sequence includes 3262 bp of 5' flanking region. In general, the exon/intron structure is identical to the human and bovine prothrombin genes (table 2). Repetitive sequences are present in the mouse gene and include three copies of B1 repeats, two copies of B2 repeats and two regions of internal homology of 160 bp in length (102). The sequences at the 5' and 3' ends of the intervening sequences conform to the rule that a GT is present at the 5' splice site, while an AG is present at the 3' splice site (table 3).

The site of initiation of transcription of the human prothrombin gene has been mapped to a region between 3 and 38 bp upstream of the initiator methionine (94, 103). Although there is no canonical TATA and CCAAT box recognition sequences for binding RNA polymerase II, the site that best fits with a consensus transcription start site sequence is 31 bp upstream of the initiator methionine (this site has been labeled as +1; 94). For the bovine gene, the start site has been determined to be 24 bp upstream of the initiator methionine (95).

Genes are regulated by the binding of regulatory proteins to DNA sequences upstream or within genes. These trans-acting protein factors bring DNA and other proteins together in order to promote transcription. Numerous trans-acting factors and associated cis-acting DNA binding sequences have been identified to be involved in tissue-specific, temporal and developmental expression of genes (104, 105). Since the liver is the major site of synthesis of prothrombin (39, 106) studies have been performed to determine the sequences in the regulatory regions of the prothrombin gene that are responsible for this specificity. In HepG2 cells it was determined that sequences between –919 and –849 upstream of the start site were required for liver-specific expression (107). Deletion of this region resulted in no expression in HepG2 cells. Gel mobility shift and DNase I foot printing assays have determined that several proteins bind in the region between –919 and –790 in HepG2 cells (103, 107). The liver-specific factor, hepatocyte nuclear factor-1 (HNF-1) appears to bind to the region between –887 and –875. HNF-1 has been identified to be responsible for the liver-specific expression of several genes (108). Other proteins that bound to the –919 to –790 region of the human prothrombin gene were also identified in HeLa cell extracts, indicating that other non-liver proteins are involved in the regulation of prothrombin expression (107).

When the 5' flanking regions of the human and bovine genes are compared only a few regions of identity exist, indicating that these regions may have been conserved because of their importance in the regulation of expression of the gene. Two regions of identity are between –717 to –581 and –108 to –1 in the human gene that are 69 and 79% identical to –580 to –443 and –110 to –1 in the bovine gene, respectively (94, 95). Since sequence upstream of –580 in the bovine gene has not been determined, it is not known whether the region identified to be required for liver-specific expression in the human gene is conserved. The region between –110 and –1 in both genes is most likely responsible for the basal expression of the gene, while the upstream region of identity has not yet been identified to have a specific function.

Comparison of the 5' flanking region of the mouse and human genes, indicates two regions of identity (101). The HNF-1 binding region between –921 and –844 in the human gene is 70% identical to an upstream region in the mouse gene; although the specific site where HNF-1 has been inferred to bind is the least similar part of this region. The promoter region of both genes (-88 to –15 in the human gene) is 79% identical, similar to that found between the human and bovine genes. The upstream region of identity found between the human and bovine genes is not conserved between the human and mouse genes.

At present, common DNA sequences responsible for the expression of vitamin K-dependent proteins have not been identified. The only common features between genes coding for these proteins is the fact that there is no canonical TATA and CCAAT sequences for RNA polymerase II binding and the fact that most are expressed in a liver-specific manner (109-111).

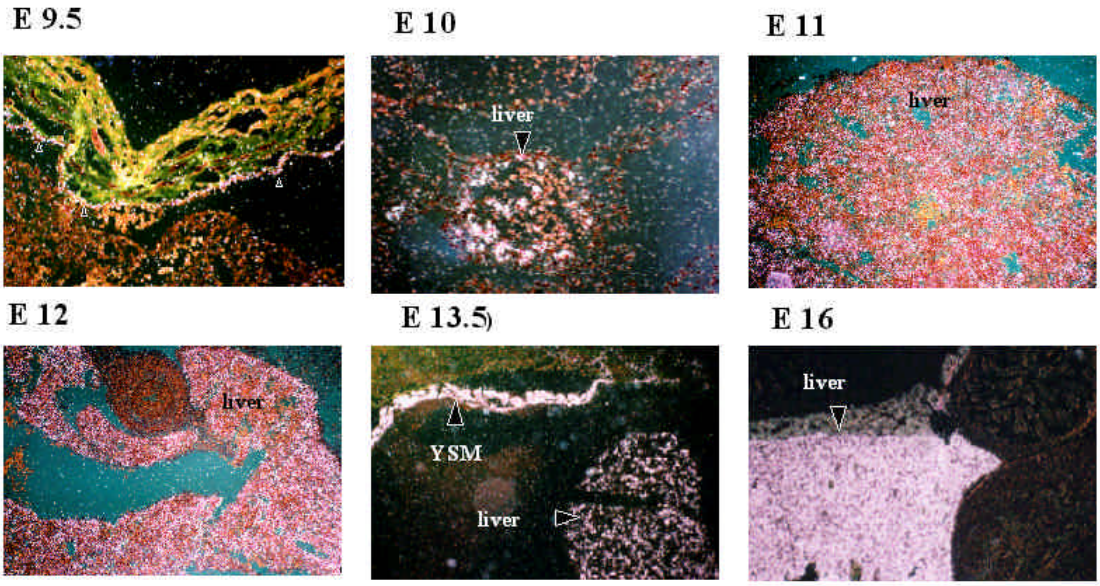

Figure 2. The developmental expression of prothrombin in the mouse (embryonic days 9.5 to 16). *In situ* hybridization was performed to localize the expression of prothrombin during various stages of development of the mouse embryo and surrounding tissue. At E9.5 prothrombin is expressed in the yolk sac membrane (YSM); this expression continues to at least E13.5. Prothrombin is expressed in the developing liver as early as E10.

2.4. The developmental and tissue-specific pattern of expression of prothrombin and the PAR-1 receptor

In all studies to date it has been determined that the major site of synthesis of prothrombin is in the liver of the adult and developing embryo (39, 106). The expression pattern for prothrombin has been determined at various stages of development in the mouse and rat (106, 112, 113). *In situ* hybridization analysis of the developing mouse embryo, shows that prothrombin is expressed in the liver between E9.5 and E16.5 (figure 2; 112) and in the visceral endoderm of the yolk sac as early as E9.5 with continued expression even at E13.5 (113).

The developmental expression of prothrombin has been studied extensively in the rat (106) by northern analysis. The liver-specific expression of prothrombin increases several fold in the embryo during late gestation and reaches its maximum level at about 2 weeks after birth. Although the majority of prothrombin is synthesized in the liver (at least 95%), it is expressed at lower levels in adrenal tissue, diaphragm, intestine, kidney, spleen and stomach tissues during some stages of development. *In situ* hybridization analysis has localized prothrombin and the PAR-1 receptor expression in the mouse and rat brain throughout development. Specific areas include the cortex, the cerebellum, and the olfactory bulb (31, 32).

Maternal expression of prothrombin during pregnancy is detected primarily in the liver, but also in the diaphragm, placenta, stomach and uterus at some time points (106). Expression in the liver increases slightly during pregnancy, but decreases to approximately 50% of these levels immediately following delivery.

Since prothrombin is secreted into the bloodstream, its target tissues reside elsewhere in the body. Localization of the expression of one of the receptors for prothrombin, PAR-1 shows that it is expressed in mesenchymal cell populations such as the heart and blood vessels (112). Expression of both prothrombin and one of its receptors during the time of organogenesis indicates that thrombin may play a role in development. The developmental pattern of expression of PAR-3 and PAR-4 has not yet been determined.

3. RESULTS

3.1. Targeting strategy

Mice deficient in prothrombin were generated in order to study the biology of prothrombin in an *in vivo* model system. Two targeting strategies have been used with similar results (113, 114). The targeting strategy described by Sun and coworkers is shown in figure 3. The mouse prothrombin gene was isolated from a 129/Ola genomic library and portions of the gene were used for construction of the vector. The 711 bp short arm of homology included a small portion of the 5' flanking region upstream of the first exon of the mouse gene. The long arm of homology was 4.2 kb in length and coded for exons 3 to 6 and parts of the flanking intervening sequence. The 6 kb hypoxanthine phosphoribosyl transferase (HPRT) gene was inserted between these two regions of homology, thereby replacing exons 1 and 2 of the prothrombin gene. The HPRT gene was included as a positive selection marker for the growth of embryonic stem cells that have undergone homologous recombination. This gene was cloned in the opposite transcriptional orientation as the mouse prothrombin gene. The herpes simplex virus thymidine kinase gene (HSV-tk) was cloned upstream of the short arm of homology to serve as a negative selection marker to select against random insertion into the mouse genome.

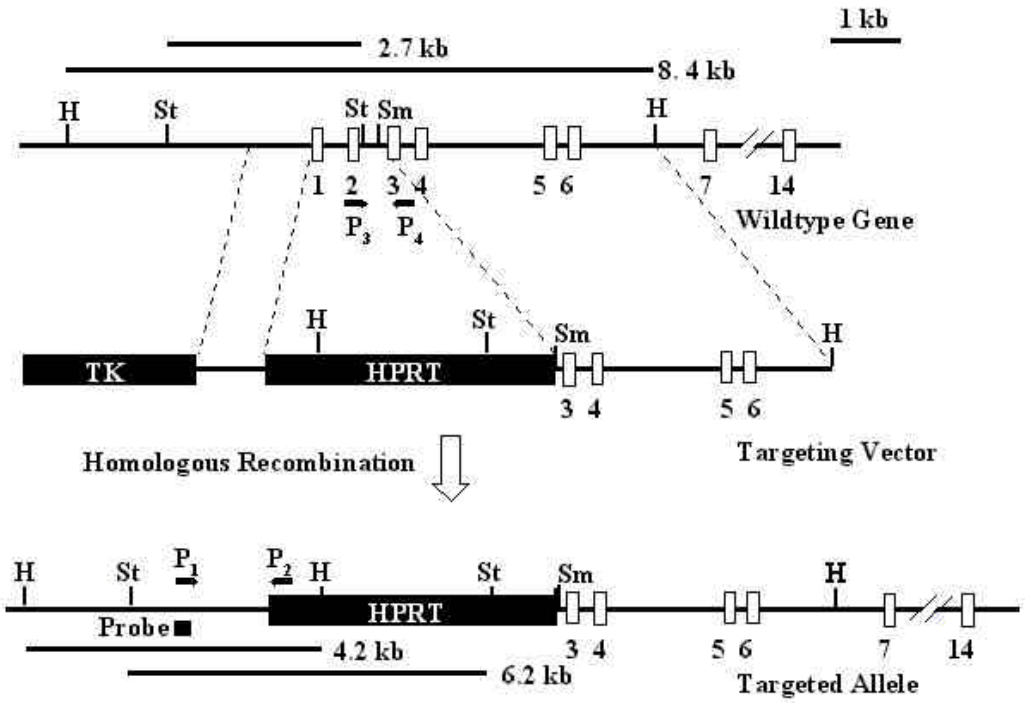

Figure 3. The targeting strategy for the deletion of part of the prothrombin locus in embryonic stem cells by homologous recombination. The endogenous mouse prothrombin gene structure is shown on the top line and is indicated as the wild-type gene. Exons are indicated by open boxes and intervening sequences are indicated as lines in between these boxes. The targeting vector (on the second line) includes two regions of identity to the prothrombin gene (indicated as the regions in between the dotted lines). The short arm includes 5' flanking sequence and the long arm includes sequence coding for exons 3-6 and the appropriate intervening sequences. The hypoxanthine phosphoribosyl transferase (HPRT) gene has been inserted in place of exons 1 and 2 of the prothrombin gene. The thymidine kinase (TK) gene is at the 5' end of the targeting construct. The locus following homologous recombination in ES cells is shown as the targeted allele on the bottom line. The HPRT gene has been inserted in place of exons 1 and 2. Restriction sites important for cloning or for identification of targeted versus endogenous alleles by Southern analysis are indicated. H, Hind III; St, Sst I; Sm, Sma I. The sizes of restriction fragments from Sst I or Hind III digests are indicated for the endogenous allele (2.7 kb for Sst I and 8.4 kb for Hind III digests) and the targeted allele (6.2 kb for Sst I and 4.2 kb for Hind III digests). Primers used for genotyping by PCR are indicated as P1 to P4. The scale is shown by the 1 kb bar at the top right.

If homologous recombination occurs in embryonic stem (ES) cells following electroporation of the targeting vector, the HSV-tk gene will be eliminated and the regions of prothrombin identity including the HPRT gene in the targeting vector will replace the endogenous prothrombin gene spanning the regions of identity and thereby disrupting the structure of the normal gene. In this case, exons 1 and 2 of the endogenous prothrombin gene will be deleted. These exons code for the signal and propeptide, as well as the Gla domain. It is unlikely that prothrombin lacking these regions will be synthesized and even if this does occur the resultant protein should be non-functional. Because of the selection media for growth and selection of ES cells, only those cells that have undergone homologous recombination will survive. The presence of a functional HPRT gene will enable the cells to grow on media containing hypoxanthine, aminopterin and thymidine. The HSV-tk gene is included in the targeting construct to prevent random insertion into the genome. If a random insertion event occurs, the HSV-tk gene would be part of the insertion, while if homologous recombination occurs this gene is not present since it is outside the regions of homology required for homologous recombination. Inclusion of gancyclovir results in selection of cells that do not express thymidine kinase.

3.2. Generation of prothrombin-deficient mice

ES cells are the cells of choice for electroporation of the targeting construct and selection of cells that have undergone homologous recombination since they are pluripotent and their pattern of differentiation is similar to that of normal embryos. ES cells with modified genes will contribute to tissue formation in chimeric mice to varying extents when placed back in an embryonic environment. If the modified gene is included in the genome of germline cells, they will then be carried into the next generations of mice.

The prothrombin targeting vector was electroporated into ES cell line E-14TG2a (113). Following the selection procedure described above, 120 ES cell clones were obtained. Following genotyping by both PCR and Southern analysis, 15 clones were found to have

Table 4. Genotypes of embryos and pups from matings of mice hemizygous for the targeted prothrombin allele (FII+/- x FII+/-)

Prothrombin Genotype						
Stage	**Total**	**Genotyped**	**+/+**	**+/-**	**-/-**	**%[1]**
E8.5	37	34	10	16	8	23.5
E9.5	76	73	15	39	18	25.4
E10.5	104	103	29	58	7	6.8
E11.5	36	32	6	19	3	9.4
E12.5	99	86	26	46	8	9.3
E15.5	53	48	19	24	5	10.4
E18.5	96	95	34	51	10	10.5
Term	135	134	47	77	10	7.4

[1]Viable prothrombin-deficient mice calculated as the percentage of all genotyped mice in the same age group.

Table 5. Coagulation assays on plasma isolated from wild-type mice and mice hemizygous (FII+/-) and homozygous (FII-/-) for the targeted prothrombin allele

Genotype	Prothrombin time (sec)	Thrombin time (sec)
Wild-type	16.1 +/- 1.6	7.3 +/- 1.4
FII+/-	19.9 +/- 14.7	6.2 +/- 0.75
FII-/-	>240	6.0 +/- 0.58
FII+/- + FII-/-	15.4 +/- 2.1	ND

undergone the appropriate homologous recombination. Three of these ES cell lines were introduced by injection into blastocysts that had been obtained post-fertilization from C57BL/6 female mice. Injected balstocysts were transferred to the uterus of pseudo-pregnant C57BL/6 females. Eight male chimeric mice were obtained and two derived from different ES cell line successfully transmitted the targeted gene through their germline. These mice were mated with female NIH Black Swiss mice to generate mice hemizygous for the knock-out allele. Brother/sister matings of hemizygous mice were performed to generate homozygous prothrombin-deficient mice (FII-/-).

The genotypes of pups that were the result of matings of hemizygous prothrombin-deficient mice were determined by PCR analysis (table 4; 113). Based on normal Mendelian patterns of inheritance it was expected that 50% of the mice would be hemizygous for the mutant allele (FII+/-), 25% would be wild-type (FII+/+) and 25% would be homozygous for the mutant prothrombin allele or deficient in prothrombin (FII-/-). Fewer homozygous mice were observed than expected, indicating that a portion of these mice died prior to birth. Of 135 mice born, 10 (7.4%) were homozygous for the mutant allele. Mice generated from both of the original ES cell lines gave the same results.

3.3. Characterization of prothrombin-deficient mice

All homozygous mutant mice were normal in size and had no obvious congential malformations, but they did have a bleeding phenotype and all died within hours of birth (figure 4). These neonates had massive bleeding into the abdominal cavity, bleeding into the intestinal lumen and purpura around the head, back, abdomen and joints. Blood in the abdominal cavity was unclottable.

No prothrombin mRNA was detected by northern analysis in the liver of homozygous mutant mice, while hemizygous mice had approximately half of wild-type levels. Prothrombin protein could not be detected in the plasma of null mice as determined by western analysis, and its activity could not be measured in clotting assays (table 5). Addition of thrombin to the blood isolated from null mice did result in normal clotting activity, indicating that all other components required for coagulation to occur are present and normal. The clotting activity of blood from hemizygous mice was normal. The growth and fertility of hemizygous mice were normal when compared to wild-type littermates.

In order to determine when the remainder of prothrombin-deficient mice died during gestation, embryos were isolated at various times during the gestational period, genotyped and examined phenotypically (table 4). At E8.5, prothrombin-deficient embryos were present at the expected level and were normal in appearance compared to their littermates. Approximately half of the prothrombin-deficient embryos between E9.5 and E12.5 were phenotypically abnormal as indicated by their smaller size and blood pooled in the yolk sac cavity (113). The yolk sac membranes were pale and appeared to be lacking any blood. At E10.5 more than half of the prothrombin-deficient mice were developmentally arrested and their sizes were as low as 15% of normal size. Hemizygous and wild-type littermates were normal in size and phenotype. Following E11.5, prothrombin-deficient mice comprised approximately 10% of the pups, indicating that the defective pups had been lost by this time. No developmental failures were observed past E11.5. Therefore, there are two populations of prothrombin-deficient mice; those that die between E9.5 and E11.5 and those that survive through the gestational period, but die soon after birth due to complications resulting from bleeding.

Several pathologies were observed phenotypically in mice between E9.5 and E11.5 that are

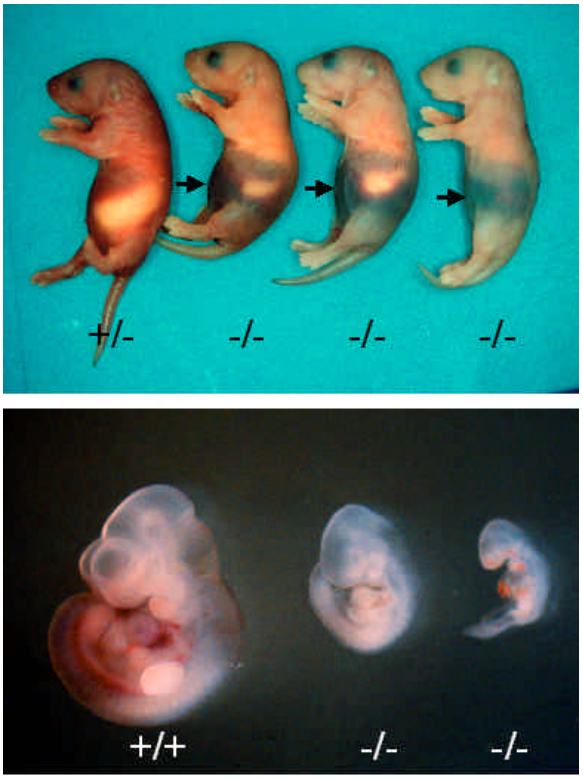

Figure 4. Prothrombin-deficient mice die from spontaneous bleeding events. The top figure shows prothrombin-deficient mice (-/-) within 1-2 days of birth compared to a mouse hemizygous for the targeted allele (+/-). These prothrombin-deficient mice have severe abdominal bleeding (arrows), bleeding in regions of the head, snout, leg joints and beneath the skin has also been observed. Prothrombin-deficient embryos (10.5) are shown in the bottom portion of the figure. Littermates at E10.5 indicate developmental arrest of two prothrombin-deficient embryos (-/-) compared to a wild-type embryo (+/+).

most likely nonspecific to the prothrombin-deficiency since they were not observed uniformly (113). These defects could be associated with a variety of circulatory failures and included distended hearts and enlarged pericardial sacs.

At the histological level, variable degrees of bleeding were found in most prothrombin-deficient embryos between E9.5 and E11.5 (figure 5; 113). Bleeding was most often found in the yolk sac cavity where free blood was found in amounts ranging from minimal to large pools of fetal blood cells. Free blood was occasionally found in the exocoelomic cavity and the pericardial sac, but rarely in tissues. Extraembryonic vessels were well developed, but in some cases were patent but had empty lumens. In addition, the vascular system, including the heart were well developed and showed no structural abnormalities. Necrosis in prothrombin-deficient embryos was frequently observed in embryos at E10.5 when microscopic evidence of bleeding was observed. Analysis of the vasculature of prothrombin-deficient mice by electron microscopy indicated that the structural development of the blood vessels in the yolk sac were normal and therefore not the cause of leakage of blood cells into the yolk sac.

Although spontaneous bleeding was observed in many of the prothrombin-deficient embryos during the mid-gestation period, the embryonic and extraembryonic vasculature was normal in appearance indicating that the loss of vascular integrity was due to a failure in hemostasis. Inability to maintain hemostasis in prothrombin-deficient mice leaves the animals susceptible to blood loss during critical stages of embryonic development and at birth. During midgestation, as the vitelline and chorioallantoic circulation develop, intravascular pressure increases. This may contribute to the observed spontaneous bleeding events in these null embryos.

3.4. Summary of results obtained by others in the generation of prothrombin-deficient mice

The targeting strategy used by Xue and coworkers to generate prothrombin-deficient mice involved the deletion of exons 7 through 12 in the mouse prothrombin gene (114). The neomycin positive selection marker was substituted in place of exons 7 to 12 in the targeting vector. The pgk-tk gene was used for negative selection. The deleted region spans the second kringle structure, the factor Xa activation sites and two of the three active site amino acids. ES cells were grown in G418 and gancyclovir. At least one ES clone was identified to have undergone homologous recombination. Positive ES cells were injected into C57BL/6J female mice. Further crosses were with the same strain of mice.

Hemizygous mice were normal in appearance, viability and fertility with no overt bleeding even following tail biopsy. Following crosses between hemizygous mice, only 3% of neonates were homozygous for the targeted allele. Although the amino-terminal of the protein could be synthesized, no prothrombin protein was detectable in the liver of these neonates as determined by Western analysis. Homozygous newborns had massive intraperitoneal bleeding and died within the first day of birth. Hemorrhage was also observed over the head, in the skin and occasionally intracranially. Analysis of the developmental times where the remainder of homozygous mice were lost indicated that these embryos died at E9.5-10.5 and at about E14.5. All of these mice developed normally and had no tissue hemorrhage or bleeding into the yolk sac. The yolk sacs did show enlargement of capillary vessels and flattening of the visceral endoderm suggesting a defect in the yolk sac integrity or in its formation.

4. PERSPECTIVE

In humans, two types of genetic disorders, hypo- and dys-prothrombinemia, are caused by defects in the prothrombin gene. These disorders are rare and there is always residual prothrombin procoagulant activity detectable (115). The lethal phenotype found in prothrombin-deficient mice suggests that complete prothrombin deficiency in humans may also be lethal.

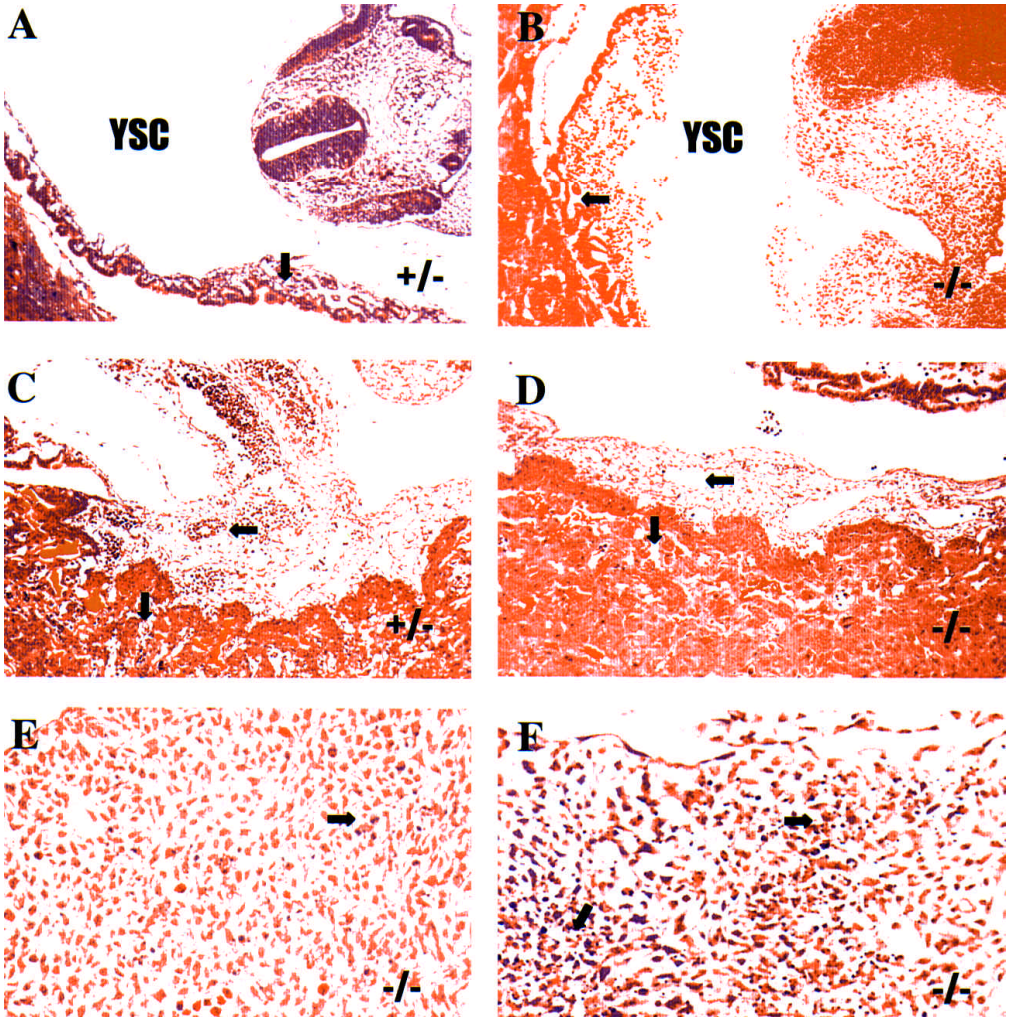

Figure 5.Histological examination of hemizygous and prothrombin-deficient embryos at E9.5-E11. Embryos hemizygous for the targeted prothrombin allele (+/-) and deficient in prothrombin (-/-) were examined histologically. A. Section through a hemizygous embryo at E11 showing a normal yolk sac cavity (YSC) with no evidence of bleeding and a normal yolk sac membrane filled with blood cells (arrow). B. Section through a prothrombin-deficient embryo at E10.5 showing leakage of fetal blood cells into the yolk sac cavity (arrow). C. Section through the placenta of a hemizygous embryo at E11 showing the presence of both maternal and embryonic red blood cells (arrows). D. Section through the placenta of a prothrombin-deficient embryo at E10.5 showing the lack of fetal blood cells in the vasculature (arrows). E. Section through a prothrombin-deficient embryo at E9.5 showing the initiation of necrosis (arrows). F. Section through a prothrombin-deficient embryo at E10.5 showing more extensive necrosis (arrows).

The cause of partial embryonic lethality due to prothrombin-deficiency in mice is still not clear. It may be that the maternal supply of circulating prothrombin, enables a portion of the prothrombin-deficient mice to survive to birth, but that following birth when this source is no longer present the mice die due to excessive bleeding. Interestingly, mice deficient in the noncirculating, membrane-bound thrombin receptor, PAR-1 also have a partial embryonic phenotype (116, 117); an example where there can be no maternal supply of this receptor.

Deficiency in mice of other proteins involved in the extrinsic pathway of prothrombin activation, results in embryonic lethality in the same time frame as observed for prothrombin deficiency. At approximately E9.5, mice deficient in tissue factor, tissue factor pathway inhibitor (TFPI) and factors V and X have spontaneous bleeding phenotype (118-124). Whether the bleeding is due to a defect in blood vessel development or in vascular integrity due to a defect in hemostasis is controversial. Two of three laboratories report normal vasculature development in embryonic and extraembryonic tissues in tissue factor-deficient mice (118, 121, 122), while the other infers that blood vessel development is defective in these mice (119). Factor V-deficient mice apparently have defective blood vessel development, while TFPI-deficient mice have

normal development of these vessels (120, 123). Factor X-deficient mice have no histological defects in their vasculature or yolk sac (124).

In the case of prothrombin, there is no localized expression of prothrombin mRNA in developing vessels. Prothrombin is initially expressed in the yolk sac membrane, the site where blood loss occurs in prothrombin-deficient embryos (113). These results are consistent with lethality due to a defect in vascular integrity rather than in blood vessel development.

Clot formation at the site of a wound requires activation of platelets to form a platelet plug and fibrin formation. Deficiency in either fibrinogen or platelets does not lead to a lethal phenotype, while prothrombin-deficiency does, indicating its critical role in both of these processes. Fibrinogen-deficient mice generally survive gestation, although a small portion die as neonates and as adults, due to spontaneous bleeding (125). Blood in these mice is unclottable. It is likely, that platelet plug formation in fibrinogen-deficient mice is sufficient to control most bleeding events. Mice that have a significantly lower amount of platelets due to deficiency in NF-E2 have a bleeding phenotype that results in many mice dying in the neonatal period, although mice do live to adulthood (126, 127). These mice are able to form fibrin, which enables a proportion of these mice to survive. In prothrombin-deficient mice, fibrin can not form a clot and platelets can not be activated. Therefore, no type of plug can be formed at the site of injury to enable these mice to survive a bleeding event.

Based on the similar phenotypes of mice deficient in prothrombin, tissue factor, TFPI, PAR-1 and factors V and X it can be inferred that the failure of embryos during midgestation is due to a defect in a common pathway shared by these proteins. It is likely that the defect is due to a failure of thrombin-mediated proteolysis and downstream associated events. Assuming that thrombin is the only activator of PAR-1, the common phenotype of these mice suggests multiple pathways that might be compromised in prothrombin-deficient mice. This hypothesis predicts that deficiency in other proteins involved in the extrinsic pathway of blood coagulation would also result in midgestation lethality. Factor VII-deficient embryos do survive to the neonatal period (128), although it cannot be ruled out that a maternal source of factor VII promotes this survival.

It is apparent that prothrombin plays a critical and central role in hemostasis and results suggest that it many also play an important role in mouse development. Future studies are directed toward understanding how the structure of prothrombin relates to its function, as well as the biological importance of the various sites of synthesis of prothrombin. Strategies to bypass the embryonic lethality of prothrombin-deficiency are possible using tissue-inducible ablation techniques, such that prothrombin-deficiency later in development and in adulthood can be studied.

5. ACKNOWLEDGMENTS

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