Functional studies on factor VIII variants containing different lengths of the B-domain AND The construction of a new helper vector for the production of helper-dependent adenovectors

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To my parents, my loving wife and my little sweet kids

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Al-Alami JR, Tayeh MK, Najib DA, **Abu-Rubaiha ZA**, Majeed HA, Al-Khateeb MS, El-Shanti HI. Familial Mediterranean Fever Mutation Frequencies and Carriers Among a Mixed Arabic Population. *Saudi Med J* 2003 Oct 24: 1055-9.

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Abbreviations

Ad5	Adenovirus type 5
AdV	Adenovector
aPTT	Activated partial thromboplastine time
ATCC	American Type Culture Collection
bp	Base pair
BSA	Bovine serum albumin
CHO	Chinese Hamster Ovary cell line
CMV	Cytomegalovirus
COS	monkey kidney cell line
CTL	Cytotoxic T-lymphocytes
ddNTP	Dideoxynucleoside triphosphate
DEPC	Diethyl pyrocarbonate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
dNTP	Deoxynucleoside triphosphate
DPBS	Dulbecco's phosphate buffered saline
DSMZ	Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH
E (1-4)	Early DNA regions in Ad5 genome
EDTA	Ethylenediamintetraacetic acid
FBS	Fetal bovine serum
Fig.	Figure
FVIII:Ag	Factor VIII antigen
FVIII:C	Factor VIII activity
hAAT	Human alpha1 antitrypsin
HEK 293	Human embryonic kidney 293 cells
hr	Hour
HRP	Horseradish peroxidase
HT supplement	Hypoxanthine Thymidine supplement
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	Isopropyl-beta-D-thiogalactopyranoside

ITR	Inverted terminal repeat
IU	International unit
iu	infectious unit
kb	kilo base pair
L (1-5)	Late DNA regions in Ad5 genome
LB broth	Luria-Bertani broth
MCS	Multiple cloning site
min	minute
MLP	Adenovirus major late promoter
MMTV	Mouse Mammary Tumor Virus promoter
MTX	Methotrexate
nt	Nucleotide
OD	Optical density
ori	Origin of replication
pA	Polyadenylation signal
PAGE	Polyacrylamid gel electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
RCA	Replication competent adenovirus
RE	Restriction enzymes
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room temperature
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
SOC	Super Optimal broth with Catabolite repression
TAE	Tris acetate EDTA buffer
TBS	Tris buffered saline
SV40	Simian virus 40
UV	Ultraviolet
V	Volt
vp	viral particle
vWF	von Willebrand factor
wt	Wild type

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1. Introduction

1.1 Factor VIII: Function in blood coagulation

To maintain the integrity of the vascular system which is critical to all higher organisms with closed circulatory systems, a complex, highly regulated system has evolved, consisting of extensive interactions between the endothelial cell lining of the blood vessels, the blood platelet, and an intricate cascade of plasma proteins (Davie *et al.*, 1991; Flier and Underhill, 1992; Broze, 1992; Colman *et al*., 1994 and Schafer, 1994).

The coagulation cascade was one of the first biologic systems associated with human diseases to be studied in detail at the biochemical level (Ratnoff, 1991). It is an ordered cascade consisting of a plasma protease activating an inactive zymogene target to an active protease form, which subsequently acts on the next step in the cascade (Fig. 1). It is traditionally divided into the „intrinsic“ and „extrinsic“ pathways, but the biologic significance of this distinction has been recently questioned. The extrinsic pathway is now viewed as the major mechanism of coagulation under most circumstances, while the intrinsic pathway boost the coagulation.

Coagulation begins almost instantly after an injury to the blood vessel has damaged the endothelium lining the vessel. Exposure of the blood to proteins such as tissue factor initiates changes to blood platelets. Platelets immediately form a plug at the site of injury; this is called *primary hemostasis*. *Secondary hemostasis* occurs simultaneously (Fig. 1): The sequential activation of plasma proteases in the intrinsic pathway of coagulation leading to the localized generation of thrombin, which then converts the fibrinogen to cross-linked fibrin polymers that strengthen the platelet plug (Furie B and Furie BC, 2005). FVIII is proteolytically activated by activated factor X (FXa) and thrombin. FVIII has no enzymatic activity of its own, but once activated, it acts as a cofactor to increase the maximal velocity of FX activation by activated factor IX (FIXa) in an order of 10.000 in the presence of negatively charged phospholipids and calcium (Kaufman 1992). In the next step, FXa acts in the presence of activated factor V (FVa), negatively charged phospholipids, and calcium to activate prothrombin to thrombin. Thrombin then enhances fibrin production and subsequently activated factor XIII (XIIIa) leads to the cross-linking of fibrin monomers.

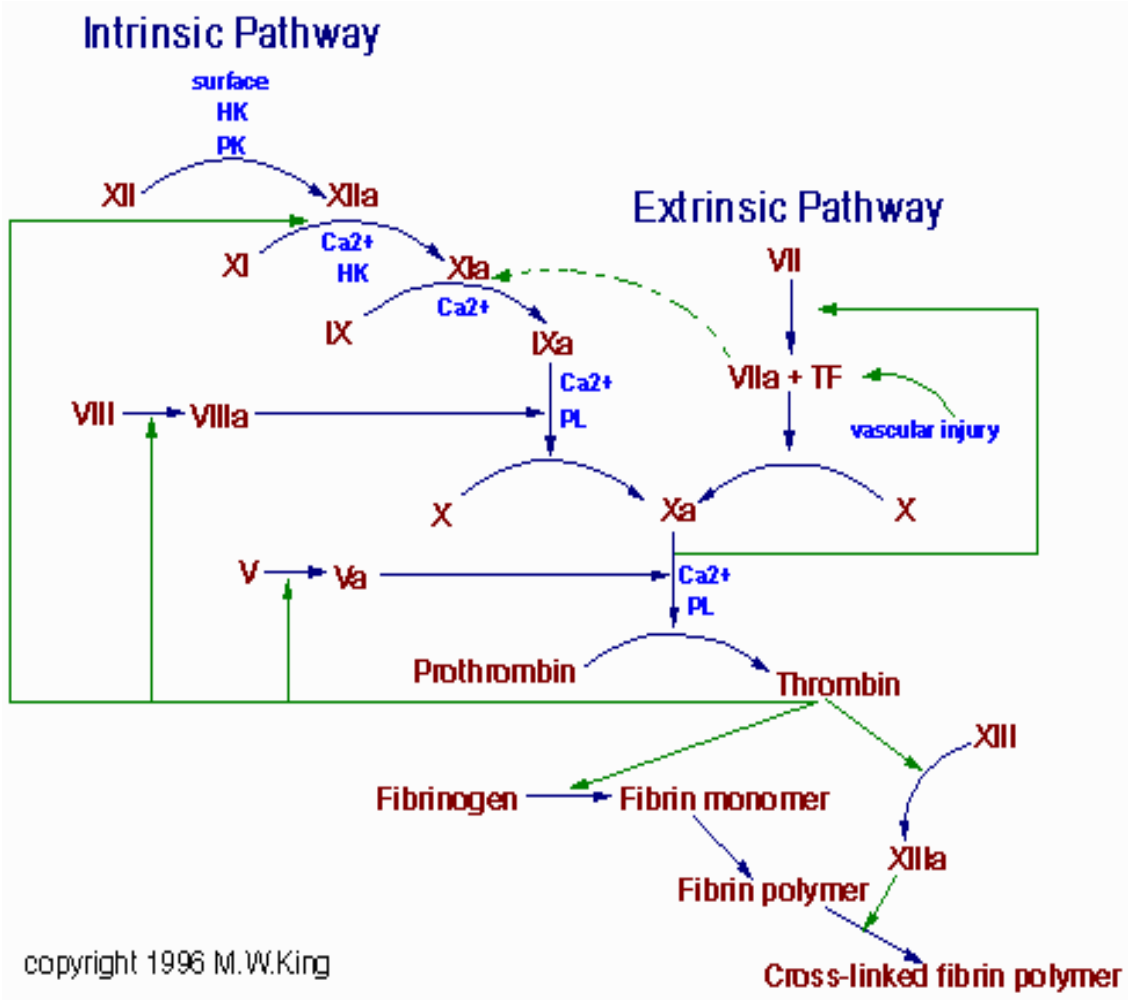


Figure 1: The coagulation cascade. The intrinsic cascade (which has less *in vivo* significance in normal physiological circumstances than the extrinsic cascade) is initiated when blood and exposed negatively charged surfaces comes in contact to each other. The extrinsic pathway is initiated upon vascular injury which leads to exposure of tissue factor, TF (also identified as factor III), a subendothelial cell-surface glycoprotein that binds on phospholipids. The green dotted arrow represents a point of crossover between the extrinsic and intrinsic pathways. The two pathways converge at the activation of factor X to Xa. Active factor Xa hydrolyzes and activates prothrombin to thrombin. Beside this function, Factor Xa has a role in the further activation of factor VII to VIIa as depicted by the green arrow. The role of thrombin is to convert fribrinogen to fibrin and to activate factor XIII to XIIIa. (also termed transglutaminase). However, thrombin can also activate factors XI, VIII and V furthering the cascade. In the last step of coagulation, factor XIIIa cross-links fibrin polymers solidifying the clot. HMWK = high molecular weight kininogen. PK = prekallikrein. PL = phospholipid.

Coagulation factor VIII (FVIII) is one of the key components of blood coagulation cascade (Mann 1999). It circulates in the bloodstream as a complex with von Willebrand factor (vWF), which protects and stabilizes FVIII (Saenko *et al*., 1999). Deficiency of FVIII activity leads to hemophilia A, a congenital bleeding tendency of variable severity that is caused by distinct FVIII gene mutations (Kemball-Cook *et al*., 1998).

1.2 Structure, biosynthesis and life cycle of factor VIII

1.2.1 Factor VIII gene

The FVIII gene has been localized to the distal Giemsa staining band positioned on the long arm of the X chromosome, Xq28 (Poustka *et al*., 1991; Freije and Schlessinger 1992). It is oriented with its 5' untranslated region toward the telomere and thus it is transcribed toward the centromere. It spans over 180 kb, and -as such- is one of the largest genes known. The FVIII gene (Fig. 2) comprises 26 exons, which encode for a polypeptide chain of 2351 amino acids. This includes a signal peptide of 19 amino acids and a mature protein of 2332 amino acids (Wood *et al*., 1984; Vehar *et al*., 1984 and Toole *et al*., 1984).

The coding sequences (26 exons) range from 69 to 262 bp except for exon 26 and exon 14. While exon 26 is nearly 2 kb and primarily contains 3' untranslated sequence, exon 14 is the largest exon (3.1 kb). The FVIII cDNA and mRNAs are approximately 9kb (Thompson 2003).

Introns vary from 0.2 kb for intron 17 to 32 kb for intron 22. Within intron 22, there are two distinct non-FVIII gene elements controlled by a bidirectional promoter (Levinson *et al.*, 1990). The first, is a FVIII-associated A gene (*F8A*), a single exon transcribed in the opposite direction. Homologous recombination between the *F8A* gene and one of two almost identical copies that are localized telomeric of the FVIII gene leads to FVIII gene interruption and accounts for nearly half of the families with severe hemophilia A (Kemball-Cook *et al.*, 1998). The second gene, *F8B*, represents an initial new first exon that is transcribed in the same direction as FVIII and includes FVIII's exons 23 to 26 (Levinson *et al.*, 1992). The first intron of the FVIII gene contains a 1-kb sequence, *int1h-1*, that is repeated in opposite direction outside of the 5' FVIII gene in a distance of 140 kb. (Bagnall *et al*., 2002). These sequences provide additional sites of homologous recombination events, accounting for approximately 5% of families with severe hemophilia A.

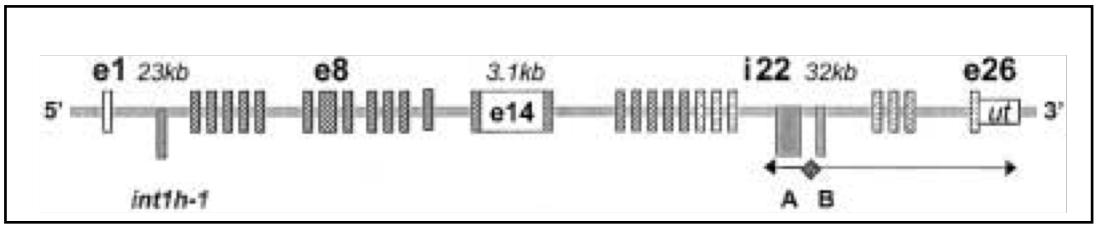


Figure 2: Factor VIII gene. The 26 exons (e) and their intervening sequences (i) are shown. Exon 14 is the largest follwed by exon 26, which is composed primarily of 3' untranslated sequences (ut). Within the large intron 22 are the A and B genes that share a bidirectional promotor (◊ and arrows). (Thompson 2003).

1.2.2 Biosynthesis and processing of factor VIII

Factor VIII mRNA has been demonstrated in a variety of tissues, including spleen, lymph nodes, liver, and kidney (Wion *et al*., 1985; Levinson *et al*., 1992; Elder *et al*., 1993). However, the liver most likely provides the primary source of FVIII (Lenting *et al*., 1998). Studies on factor VIII biosynthesis and secretion have been limited by the lack of human cell lines that properly express significant amounts of FVIII. The best evidence for the intracellular synthetic pathways comes from nonhuman, mammalian cell lines (like COS and CHO cell lines) transfected with FVIII cDNA. These studies showed that, in general, FVIII is poorly expressed (Thompson 2003). Low expression is associated with a low level of steady-state mRNA (Kaufman *et al.*, 1989) and inefficient secretion (Dorner *et al*., 1987).

FVIII is synthesized as a 2351-amino-acid single chain precursor from which a 19-amino-acid signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum (ER). In the ER, high mannose oligosaccharides are added to multiple asparagine (Asn) residues within the FVIII molecule, a process known as glycosylation (Kaufman 1992). Within the ER, factor VIII appears to interact with a number of chaperone proteins, including calreticulin, calnexin, and the Immunoglobulin-binding protein (BiP) (Dorner *et al*., 1989; Marquette *et al*., 1995; Swaroop *et al*., 1997; Pipe *et al*., 1998). Due to the interaction with these chaperone proteins, a significant proportion of the FVIII molecules are retained within the ER, thereby limiting the transport of FVIII to the Golgi apparatus (Lenting *et al*., 1998). Release from Bip is energy dependent and necessary before FVIII can be transported to the Golgi apparatus, basically by another chaperone protein, the endoplasmatic reticulum-Golgi intermediate compartment-53 (ERGIC-53). The interaction with ERGIC-53 is mannose dependent and primarily involves Asn-linked oligosaccharides within the B domain (Moussalli *et al*., 1999; Cunningham *et al*., 2001; Pipe 2009). Within the Golgi, Asn-linked

carbohydrates are modified, O-linked sugars are added to specific Ser and Thr residues within the B domain, disulfide bonding and protein folding occur, and six Tyr residues (within the three acidic peptide sequences) are sulfated (Fig. 3) (Thompson 2003). Sulfation of tyrosine residues increases the procoagulant activity of FVIII and is required for high affinity interaction with vWF (Pittman *et al*., 1992; Leyte *et al*., 1991). In addition, FVIII is among the many proteins that undergo intracelullar proteolysis (Kaufman *et al*., 1988, van de Ven WJM *et al*., 1990, Hutton 1990). FVIII is cleaved after residues 1313 and 1648 within the B domain to generate a 200 kDa amino-terminal-derived heavy chain (A1-a1-A2-a2-B) and an 80 kDa carboxy-terminal-derived light chain (a3-A3-C1-C2), giving rise to the heterodimeric molecule that circulates in plasma (Kaufman 1992) (Fig. 4 A and B).

1.2.3 Factor VIII protein structure

Analysis of the deduced primary structure determined from the cloned FVIII cDNA showed the presence of a discrete domain structure: A1-a1-A2-a2-B-a3-A3-C1-C2 (Wood *et al*., 1984; Vehar *et al*., 1984; Toole *et al*., 1984 and Fay 2006) (Fig. 3). The A domains of FVIII occur twice in the heavy and once in the light chain and have 30% homology to each other. The C domains occur twice in the carboxy terminus of the FVIII light chain and exhibit homology to proteins that bind glycoconjugates and negatively charged phospholipids (Poole *et al*., 1981; Stubbs *et al.*, 1990). The B domain, encoded by a single large exon of 3.1 kb (Exon 14), has no known homology to other proteins, and contains 18 of the 25 potential asparagine *(N)*-linked glycosylation sites within FVIII (Kaufman 1992; Pipe 2009). The A domains are bordered by short spacers (a1, a2, and a3) that contain clusters of Asp and Glu residues, the so-called acidic regions (Lenting *et al*., 1998).

The three homologous A domains bind Ca^{2+} and are essential for catalytic cofactor activity (Lollar *et al*., 1993). The short acidic sequences between A1 and A2 and at the A2-B and B-A3 junctions contain sulfated Tyr residues (Pittmann *et al*., 1992). Most of the large, central B domain can be deleted without losing FVIII activity (Pittman *et al*., 1993; Eaton *et al*., 1986). The acidic sequence connecting B with A3 (Glu^{1649}-Arg^{1689}) contains a major vWF-binding site (Foster *et al*., 1988) that requires sulfation of Tyr^{1680} for vWF binding (Leyte *et al*., 1991). C1 and C2 domains have homologies with FV and a class of lectins (Kane and Davie, 1988). These carboxy-terminal domains contribute to vWF, FIX, FX binding, and also help FVIIIa to

bind to phospholipids (Thompson 2003). In addition, thrombin and FXa appear to bind to a site on the surface of C2 domain (Nogami *et al*., 2000 and 1999).

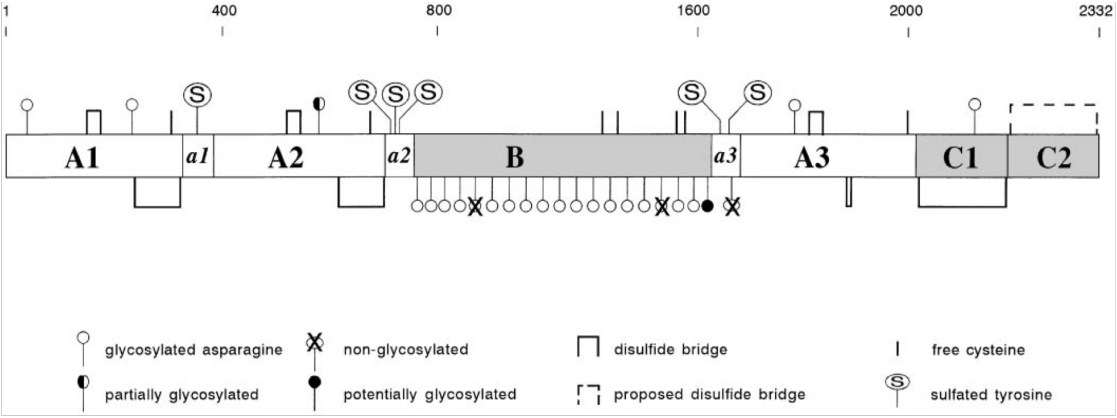


Figure 3: The factor VIII protein. Mature factor VIII consists of 2332 amino acids, which are arranged in a discrete domain structure: A1 (residues 1-336), A2 (373-710), B (741-1648), A3 (1690-2019), C1 (2020-2172) and C2 (2173-2332). The A domains are bordered by acidic regions a1 (337-372), a2 (711-740) and a3 (1649-1689) (Lenting *et al*., 1998).

1.2.4 Factor VIII in circulation:

FVIII circulates at 0.2 ug/ml in plasma as a heterodimer that is processed from a single polypeptide upon secretion from the cell (Kaufman 1992) (Fig. 4 B). It circulates in complex with vWF, an association that not only stabilizes and protects FVIII, but also helps to localize FVIII to sites of vessel wall damage (Fay and Jenkins 2005; Lenting *et al*., 2010). The half-life of FVIII is much shorter than that of vWF. About 95% of FVIII is bound at any time to vWF in vivo with a molar ratio of about 1:50 (Vlot *et al*., 1995).

Recent evidence suggests that the extrinsic pathway may be the most significant physiologic initiator of FVIIIa generation. FVII initiates the FVIII activation and FXa and thrombin generation provide positive feedback to amplify FVIII activation (Kaufman 1992). Activation of FVIII proceeds by limited proteolysis catalyzed by thrombin or factor Xa, with the former likely representing the physiologic activator (Pieters *et al*., 1989; Fay 2004). Thrombin binds to the sulfonated acidic peptides a1, a2, and a3 and cleaves peptide bonds after Arg^{372}, Arg^{740}, Arg^{1689}, respectively (Thompson 2003) (Fig. 4 B and C). In detail: cleavage within the heavy chain after Arg^{740} generates a 90-kDa polypeptide (A1-A2), which is subsequently cleaved after Arg^{372} to generate polypeptides of 50 (A1) and 43 (A2) kDa. Cleavage of the FVIII heavy chain at Arg^{372} is essential to expose a factor IXa-interactive site (Fay *et al*., 2001),

whereas cleavage at Arg^{740} removes the B domain or its fragments from A2 subunit (Fay and Jenkins 2005). Concomitantly, the 80 kDa light chain (A3-C1-C2) is cleaved after Arg^{1689} to generate a 73-kDa polypeptide (Eaton *et al*., 1986). Furthermore, cleavage at 1689 releases FVIII from vWF, relieving the vWF inhibition of FVIII-phospholipid interaction (Pittman and Kaufman, 1989; Hill-Eubanks *et al*., 1989) and permitting the interaction of FVIII with platelets where it interact with FIXa and FX.

Inactivation of the cofactor comprises two distinct pathways: spontaneous dissociation and proteolytic degradation. Activated FVIII may be inactivated through spontaneous dissociation of the A2-domain (43-kDa subunit) (Fig. 4 D) from the rest of the molecule. The association of A2 subunit with the A1/A3–C1–C2 dimer is a weak interaction by affinity forces and dissociation of A2 represents the primary mechanism for the observed lability of FVIIIa and the self-dampening of factor X activity (Eaton *et al.,* 1986). FVIIIa is proteolytically degraded within the heavy chain by various enzymes; such as factor IXa, factor Xa and activated protein C (APC) (Koedam *et al.,* 1990; Rick *et al.,* 1990; O'Brien *et al*., 1992). APC cleaves FVIIIa after residues 336 and 562 and FXa cleaves it after residues 336 and 36 (Eaton *et al*., 1986; Fay *et al*., 1991) (Fig. 4). Cleavage at position 336 in FVIIIa releases a1, the acidic sequence that interconnects the A1 and the A2 domain. Because of this release, the A2 domain dissociates more rapidly from the factor VIIIa heterotrimer (Fay *et al.,* 1993). This acidic spacer has been proposed to comprise a binding site for the substrate factor X (Regan *et al.,* 1996; Lapan and Fay 1997), indicating that release of this site results in impaired substrate binding. Thus, cleavage at Arg^{336} affects both intramolecular (A2 domain dissociation) and intermolecular (FVIII-FX) interactions (Lenting *et al.,* 1998). Inactivation most closely correlates with cleavage at residue 562. Arg^{562}, which is part of the A2 domain sequence that comprises a factor IXa interactive site, is exclusively cleaved by APC (Fay *et al*., 1991). It seems conceivable that loss of cofactor activity due to cleavage at this site reflect the loss of the ability to interact with factor IXa (Lenting *et al.,* 1998). The activation of protein C by thrombin is regulated by thrombomodulin on the endothelial cell surface (Esmon 1989).

FVIII is removed from the blood circulation at least in part by binding to low-density lipoprotein-related receptor protein (LRP) on the surface of hepatic endothelial cells (Schwartz et al., 2000). This binding is facilitated by cell surface heparans (Sarafanov *et al*., 2001).

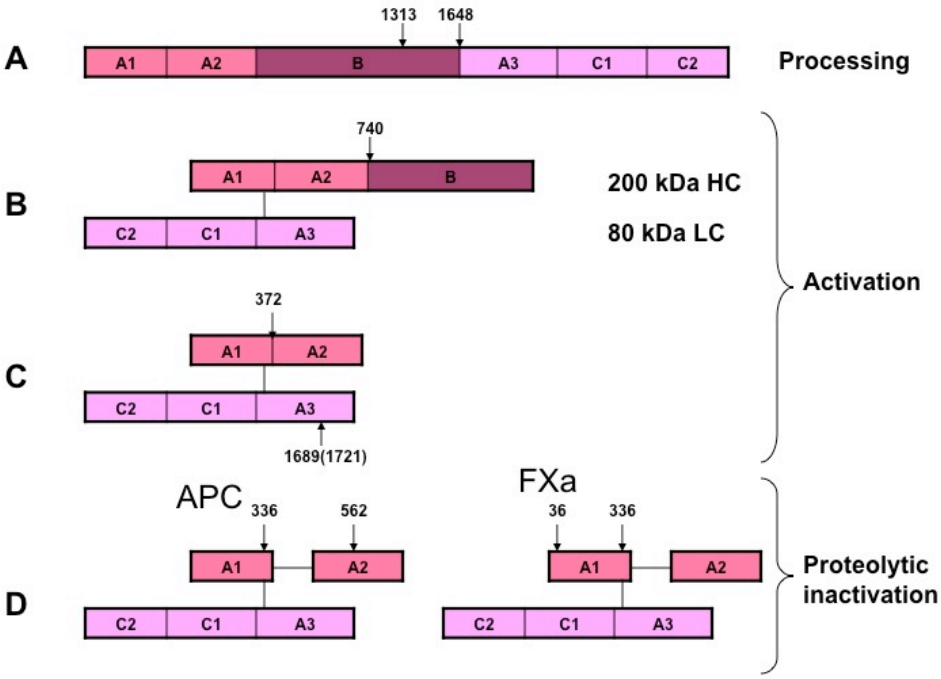


Figure 4: Scheme of proteolytic conversions of factor VIII in the course of processing, activation and inactivation. HC: heavy chain; LC: light chain; APC, activated protein C; FXa, activated factor X. (modified of Khrenov *et al*., 2006).

1.3 Hemophilia A

1.3.1 Phenotype

Hemophilia A (OMIM 306700) is the most common inherited severe bleeding disorder. Its X-linked pattern of inheritance was first recognized by Jewish scholars in the second century AD (Ingram, 1976; Ratnoff, 1991). Hemophilia A is a heterogeneous disorder resulting from defects in the FVIII gene that lead to a reduction in the circulating levels of functional FVIII. The reduction in activity can be due to a decreased amount of FVIII protein, the presence of a functionally abnormal protein, or a combination of both. In patients with hemophilia, clot formation is delayed because thrombin generation is markedly decreased. The clot that is formed is friable and easily dislodged, thus resulting in excessive bleeding and poor wound healing (Christiansen *et al.,* 2010; Williams Hematology, 5th edition, 1995).

The clinical manifestations of hemophilia A vary considerably and range from severe bleedings already visible shortly after birth until very mild phenotypes that are only diagnosed very late in life and during operations (Jones and Ratnoff, 1991; Hoyer, 1994; Furie *et al*.,

1994). The severity of the disease can be predicted quite accurately from the level of residual FVIII activity. Patients with very low or no FVIII activity (<1%) are affected with severe hemophilia A, whereas those with FVIII levels of 1-5% have moderate and those with 5-25% FVIII activity have mild hemophilia. FVIII levels above 25% are generally associated with a normal phenotype.

Classic (severe) hemophilia is characterized by excessive bleeding into various parts of the body. Soft tissue hematomas and hemarthroses are highly characteristic of the disease. Severely affected patients with hemophilia may experience bleeding without known trauma. Hemarthroses become frequent at about the time the patient begins to walk. Without effective treatment, recurrent hemarthroses, resulting in chronic hemophilic arthropathy, occur by young adulthood and are highly characteristic of the severe form of the disorder. Moderately affected patients with hemophilia have occasional hematomas and hemarthroses, usually, but not always, associated with known trauma. Although hemarthroses occur in moderately affected patients, hemarthropathy is less disabling than that occurring in severely affected patients. Mildly affected patients have infrequent bleeding episodes, and the disease may go undiagnosed for years, only to be discovered because of excessive hemorrhage postoperatively, following trauma, or after the toss and tumble of contact sports. Female carriers of the disease have roughly 50% FVIII activity and experience no bleeding difficulty, even with surgical procedures (Williams Hematology, 5th edition, 1995).

A broad spectrum of genetic defects within the FVIII gene has been identified, resulting in a range of hemophilia phenotypes determined by the amount of residual FVIII activity (Tuddenham *et al*., 1994; Hoyer, 1994; Antonarakis *et al*., 1995a). The frequency of hemophilia A in the population is estimated to be approximately 1:10 000-1:5000 of all born males (Soucie *et al*., 1998). As predicted by the Haldane hypothesis, about one-third of cases appear to be new mutations. A large number of hemophiliacs have been studied at the DNA level (Tuddenham *et al*., 1994) and a regularly update database of known mutations can be assessed at: http://hadb.org.uk/

1.3.2 Assessment

Patients with severe hemophilia A characteristically have a prolonged activated partial thrompoplastin time (APTT) (see section 2.4.2.1) and a prolonged whole blood clotting time

Different combinations of APTT reagents and instrumentation exhibit widely varying sensitivities to FVIII levels. Functional FVIII activity (FVIII: C) is measured by clotting assays. FVIII protein is measured by immunologic assays of FVIII antigen (FVIII: Ag), which will detect both normal and abnormal FVIII molecules. If the FVIII antigen level is normal, but the clotting activity is reduced, the patient has a dysfunctional FVIII molecule. FVIII activity is expressed as percent of normal (normal: 100% FVIII: C) or as units per milliliter of plasma (normal range: 1U/ml). One unit FVIII per ml is 100 percent of normal (William's Hematology, 5th edition, 1995).

1.3.3 Current therapy and side effects

Treatment of hemophilia A rests on replacement of the deficient FVIII activity. Hemophilia A was initially treated with unpurified plasma, which contains 1 U/mL of FVIII activity. The cryoprecipitate fraction of the plasma was subsequently shown to be enriched for FVIII activity, thus permitting administration of smaller volume. Over the past three decades, highly purified FVIII concentrates have become the main therapy for hemophilia A. Standard commercial concentrates are prepared from plasma that has been generally pooled from 2000-200,000 donors. Unfortunately, the purification process used until the mid-1980s failed to inactivate common viral contaminants, including hepatitis B and C, cytomegalovirus (CMV) and HIV. As a result, all these infections were nearly uniform among patients heavily treated during the late 1970s and early 1980s. To prevent possible viral infections, purification methods were developed to inactivate all these viruses by detergent or heat treatment. Monoclonal antibody purification procedures further improved the quality of these products. As a result, all currently available FVIII concentrates are thought to be free of viral hazard (Brettler and Levine, 1989; Hoyer 1994; Furie *et al*., 1994).

With the advent of recombinant biotechnology, cloning of the FVIII gene led to the development and production of the first generation of commercially available recombinant FVIII (rFVIII) products, heralding a new era of viral safety in the treatment of hemophilia A (Tuddenham 2003).

Newly diagnosed and previously untreated patients are generally managed with recombinant FVIII or highly purified plasma-products, whereas most clinicians prefer the former. Many hemophiliacs are followed in comprehensive clinics, where they receive multidisciplinary

care. A dose of FVIII of approximately 50 U/kg will generally raise the level from 0% to 100%. The half-life of FVIII is approximately eight hours, requiring repeated doses in case of severe hemophilia (2-3 times a week) or treatment by continuous infusion. Therapy is monitored by following the FVIII activity level. A FVIII level of 50% or more should achieve normal homeostasis, even under extreme conditions.

Approximately 10-20% of patients develop inhibitor antibodies to FVIII that dramatically complicate therapy. These antibodies can reach extremely high titers, and infusions of sufficient FVIII to overwhelm the inhibitor may often be impractical. A variety of strategies have been used to treat these inhibitors, including immunosuppression and various regimens to induce tolerance (Hoyer, 1994; Furie *et al*., 1994). The most commonly used protocol to eradicate inhibitors is still the 'Bonn protocol' (Brackmann *et al.,* 1996).

1.4 Recombinant factor VIII molecules

The first generation of rFVIII molecules was produced by insertion of a full-sequence human FVIII cDNA into expression vectors, yielding what is typically referred to as 'full-length' rFVIII (FL-rFVIII), which is biochemically and functionally similar to plasma-derived FVIII (pdFVIII) (Eaton *et al*., 1987). In 1992, the company Baxter in conjunction with the Genetics Institute introduced the world's first genetically engineered factor VIII under the name of 'Recombinate'. Then in February 1993, Genetech produced second recombinant factor VIII product named 'Kogenate'. The 'first generation' of recombinant products used animal products in the culture medium and contained human albumin added as a stabilizer.

As the B-domain is not required for FVIII procoagulant activity (Andersson *et al*., 1986), bioengineering of the FVIII molecule by removing the B-domain was found to dramatically improve the yield of rFVIII (Dorner *et al*., 1987), enhancing expression of rFVIII as much as 20-fold (Pittman *et al*., 1993). The removal of the B-domain coding sequence brought several advantages compared to the full-length molecule. The shorter cDNA led to a large increase of FVIII mRNA amounts (Pittman *et al*., 1993; Pipe *et al*., 1998; Pittman *et al*., 1994) that resulted in an improved production of a 170 kDa FVIII protein, secreted as an association of heavy chain (A1-A2; 90 kDa) and light chain (A3-C1-C2; 80 kDa) (Pittman *et al.*, 1993). Such advantages rendered the BDD-FVIII more attractive for producing recombinant FVIII compared to the FL-rFVIII (Sandberg *et al.*, 2001). In addition, the limited size of the cDNA

construct allowed its insertion in most viral vectors that were used for gene therapy studies (Chao *et al*., 2000; Connelly *et al*., 1996; Gnatenko *et al*., 1999; VandenDriessche *et al*., 1999).

Recombinant FVIII with the B-domain deleted (BDD-rFVIII) (moroctocog alfa; ReFacto® [Wyeth]) was developed with an albumin-free formulation to decrease the risk of pathogen transmission (second-generation rFVIII products). Since the licensure of ReFacto, the portfolio of FVIII concentrates has expanded to include additional FL-rFVIII preparations, Kogenate® FS (Kogenate® Bayer in the USA) [octocog alfa (Bayer)] and Advate® (octocog alfa [Baxter]). Both have been produced using animal-derived materials in the culture medium, but instead of albumin, they used sucrose or other non-human derived material as a stabilizer. 'Third generation' products have no human or animal protein during the production process. Advate and newly ReFacto AF made by Wyeth are examples (Franchini and Lippi, 2010).

While BDD-rFVIII is effectively used in the treatment of haemophilia A patients, recent experimental and clinical studies revealed a number of issues related to its equivalency to plasma-derived-FVIII (Gruppo *et al.,* 2003; Lollar 2003). In particular, it was shown that the activity of BDD-rFVIII determined by one-stage clotting assay is up to 50% lower than that determined by chromogenic assay, thus complicating determination of BDD-rFVIII activity in hemophilia A patient blood (Barrowcliffe *et al*., 2002). The differences in activity determined in these assays may indicate that while BDD-rFVIII can effectively replace plasma-derived-FVIII in the X-ase complex (as seen by the chromogenic assay), it differs from plasma-derived-FVIII in its interaction with other components of the coagulation cascade (as shown by the clotting assay). Some biochemical differences between plasma-derived-FVIII and BDD-rFVIII were previously observed at the early stages of BDD-rFVIII development, such as a five-fold more rapid thrombin cleavage of the BDD-rFVIII light chain at Arg1689 (Eaton *et al*., 1986) and a higher affinity of inactivated BDD-rFVIII to activated platelets in comparison with plasma-derived-FVIII (Li and Gabriel, 1997). The meta-analysis of the data accumulated during long-term clinical usage of ReFacto revealed its lower efficiency in prophylaxis, giving a 2.5-fold higher incidence of bleeding in comparison with full-length FVIII (Gruppo *et al*., 2003 and 2004).

1.5 Gene therapy for hemophilia A

1.5.1 Overview

The ambitious objective of gene therapy is to edit a defective gene sequence in situ to achieve complete reversion of a disease phenotype for the lifetime of the patient. In spite of recent successes in site-specific correction of defective gene sequences, the focus of most gene therapy strategies to date is on gene addition rather than gene replacement (Urnov *et al*., 2005). This simplified approach relies on a delivery mechanism to provide a correct copy of the defective gene in somatic cells without removal of the error-containing genomic sequence. Hemophilia A and B are among the most extensively researched diseases in the field of gene therapy (Murphy and High 2008). Virtually every emerging gene delivery strategy, whether in vivo or ex vivo, viral or non-viral, has been tested in the hemophilia animal models (Kaufman 1999). Gene transfer approaches for hemophilia are attractive because available clotting factor concentrates have not completely (1) allay the fears of possible viral contamination, specially plasma-derived concentrates, (2) alleviate chronic debilitating joint disease, or (3) resolve the extreme expense of replacement therapy (Azzi *et al*., 2001; Zakrzewska *et al*., Fischer *et al*., 2001). Additional important factors driving interest in gene transfer approaches in hemophilia are (1) the detailed understanding of factor VIII gene, as well as structure, function, and biology of the factor VIII protein (Kaufman 1999); (2) the beneficial clinical effects expected to occur with even modest degrees of correction (1–5% of normal levels) (Nilsson *et al*., 1992; Ljung 1999); (3) the availability of standardized coagulation assays to monitor factor expression; (4) the capacity of multiple target organs to secrete and perform the required post-translational modifications of FVIII (Yao *et al*., 1991; Arruda *et al*., 2001; Fakharzadeh *et al*., 2000; Rosenberg *et al*., 2000); and (5) the availability of canine and mouse hemophilia models that phenotypically mimic the human disease (Bi *et al*., 1995; Lin *et al*., 1997; Evans *et al*., 1989; Hough *et al*., 2002).

Ex vivo and *in vivo* approaches are being pursued for hemophilia gene therapy. *Ex vivo* gene therapy involves the isolation of cells from the patient followed by expansion and genetic modification in culture with vectors expressing FVIII and subsequent re-administration of engineered cells to the patient. Successful *ex vivo* gene therapy requires efficient engraftment of the engineered cells leading to sustained FVIII production in circulation (Chuah *et al.*

2001). Different cell types have been considered as potential targets for *ex vivo* hemophilia gene therapy (Hortelano *et al*., 1996; Page and Brownlee 1997; Fakherzadeh *et al*., 2000). Roth *et al.*, 2001 have used *ex vivo*-transduced fibroblasts to transfer factor VIII in patients with severe hemophilia A. Most *ex vivo* gene therapy strategies have relied on the use of retroviral vectors and to a more limited extent on non-viral gene transfer systems. Alternatively, *in vivo* gene therapy involves the administration of a gene transfer vector encoding FVIII directly to the patient leading to *in situ* genetic modification of the target cells. Intravenous administration of a FVIII vector would be a more cost-effective treatment than *ex vivo* protocols. However, the main disadvantage of *in vivo* gene therapy based on viral vectors is that a host immune response towards the viral vector would preclude vector re-administration, if more than one injection would be required to achieve therapeutic FVIII levels (Chuah *et al*., 2001).

Hemophilia gene therapy requires the use of a gene delivery system that is efficient, safe, non-immunogenic and allows long-term gene expression. Both viral vectors as well as non-viral vectors have been considered for the development of hemophilia gene therapy (High 2003). In general, viral-vector mediated gene transfer is far more efficient than non-viral gene transfer and is therefore the method of choice. The viral vectors are of retroviral, lentiviral, adenoviral and adeno-associated viral (AAV) origin, each with their own advantages and limitations (Chuah *et al*., 2001).

Five different gene therapy Phase I clinical trials were initiated between 1998 and 2001 for the treatment of hemophilia using different gene delivery systems (Kay *et al.,* 2000; Roth *et al.,* 2001; Manno *et al.,* 2003 and Powell *et al.,* 2003). Three of them dealt with hemophilia A. The first was initiated by injecting a Moloney murine leukemia virus-based retroviral vector encoding B-domain deleted factor VIII. Doses ranging from $2.7 * 10^7$ transducing units (TU)/kg to $4.4 * 10^8$ TU/kg were tested. Factor VIII levels above 1% were sporadically detected but didn't correlate with the dose administered; thus the signs of clinical improvement following vector infusion were modest at best (Powell *et al.,* 2003). These findings were consistent with animal studies suggesting that efficient retroviral transduction of hepatocytes would require higher doses of viral vectors and a stimulation of cell division of hepatocytes.

A second Phase I clinical trial was initiated to test the safety of a high capacity (gutless)

adenoviral vector encoding human factor VIII. Because of a priori concerns regarding immunogenicity, the trial was constructed to monitor carefully changes of liver function tests or platelet count. The first subject, enrolled at the lowest dose of $4.3 * 10^{10}$ vector particles (vp)/kg, experienced inflammation, fever and myalgia upon vector infusion; these symptoms are commonly observed with infusion of adenoviral vectors. The subject also experienced thrombocytopenia and an elevation in serum transaminases. The experiment resulted in having 1% of normal factor VIII levels for several months (Chuah *et al.,* 2004). Due to safety concerns and disappointing results, no additional subjects were enrolled in this trial.

One non-viral delivery method was also tested as a clinical trial (Roth *et al.,* 2001) consisting of transplantation of factor VIII-transduced autologous fibroblasts. After isolation from skin biopsy, patient cells were transfected with a plasmid encoding a human factor VIII cDNA ex vivo and stable transfectants were selected. Single clones were expanded and tested for factor VIII expression level, as well as tumorigenicity and microbial safety. After reimplantation into the omentum, only a modest and temporary positive effect could be observed. The treatment was well tolerated and leaves the possibility of future attempts open using more potent expression systems for the ex vivo transduction and selection process.

1.5.2 Adenoviruses as vectors for hemophilia A gene therapy

Adenoviruses have several attractive features that make them particularly well suited for vector development and gene therapy. The genome has a size of 36 kb, which after (partial) deletion leaves a relatively large packaging capacity. Adenoviruses can infect and express genes at high levels in many different cell types and host cell replication is not required for expression, so nondividing cells such as hepatocytes are readily infected. There are at least 50 different human serotypes, sub grouped A to G, which are commonly associated with mild diseases. Most adenoviral vectors currently used are derived from serotypes 2 and 5, which are endemic and cause upper respiratory tract infection (Chuah *et al.,* 2001; Vorburger and Hunt, 2002). No known malignant tumors have resulted from adenovirus infection. Given that they have been used in live virus vaccine studies for many years without problems, they have a long-standing safety record (Thorrez *et al*., 2004).

The difference between an adenovirus and its cognate vector is that the parental virus can replicate and infect other cells after infection, whereas adenoviral vectors can enter a cell, but

cannot use the cell to replicate. This impaired replication is due to the lack of at least one of the essential viral regulatory genes, which is replaced by the gene of interest, in our case FVIII. Although adenoviral vectors cannot replicate, they do retain all of the other essential properties, including the tropism of the parental viruses from which they are derived. Adenoviral vectors are relatively easy to manufacture at high concentrations (>10^{10} infectious units [iu] / mL) (Thorrez *et al.,* 2004). They are well suited for hepatic gene delivery, but their main disadvantage is that the host immune response limits the duration of transgene expression and the ability to re-administer the vector (Schagen *et al.,* 2004). A second inherent limitation to the duration of transgene expression is that the adenoviral DNA is not integrated into the host genome but is maintained episomally. Hence, dividing cells will gradually lose the adenoviral vector along with its potentially therapeutic gene. However, this also implies that there is virtually no risk of neoplastic transformation caused by insertional mutagenesis (Thorrez *et al*., 2004).

The first-generation adenoviral vectors that were developed contained a deletion of the entire E1A and part of the E1B regions of the adenoviral genome (Kozarsky and Wilson 1993; Krougliak 1995) (Fig. 5A). This deletion allowed the insertion of an expression cassette up to 8 kb. The E1 and other missing genes necessary for vector assembly are complemented in trans using appropriate packaging cells that express these genes (Berkner 1988).

Acute and chronic toxic and lethal effects were observed frequently in nonhuman primates and other animals (including mice, rabbits and dogs) that receive high doses of replication-deficient adenoviral vectors (Connelly *et al*., 1996; Lozier *et al*., 1999). Several clinical trials based on adenoviral vectors had to be discontinued because of acute inflammatory responses and severe morbidity and mortality in some patients (National Institutes of Health Recombinant DNA Advisory Committee Assessment of adenoviral vector safety and toxicity, 2002).

This cellular immune response could be attenuated by further reducing viral gene expression. This was initially accomplished by alteration or deletion of additional early viral genes such as E2 or E4 (Armentano *et al*., 1995; Gao *et al*., 1996; Yeh *et al*., 1996) (Fig. 5B/C). Despite the fact that those second-generation vectors did not reveal any detectable de novo vector DNA synthesis or de novo viral gene expression in transduced cells, there was no significant difference in gene transfer and expression compared to first-generation adenoviral vectors

(Gorziglia *et al*., 1996). Further attenuation of the adenoviral vector backbone by removal of E1/E2/E3/E4 diminished vector toxicity further; however, the duration of transgene expression was reduced (Fig. 5D) (Andrews *et al*., 2001).

High-capacity (HC) adenoviral vectors are the ultimate adenoviral vector modification which comprises a vector containing only the cis-acting elements necessary for replication and packaging, but lacking all adenoviral genes (Morral *et al*., 1999) (Fig. 5E). HC adenoviral vectors, also called gutted or gutless, fully-deleted (FD) and helper-dependent (HD) adenovectors can theoretically accommodate up to 37 kb of insert (Kochanek 1999; Schiedner *et al*., 1998; Parks and Graham 1997). Production of HC adenoviral vectors depends on the use of helper viruses that provide all missing functions in trans but cannot be packaged because of mutations in their packaging signal (Kochanek *et al*., 1996, Mitani *et al*., 1995; Clemens *et al*., 1996). As expected, High-capacity vectors give rise to prolonged transgene expression (Morsy *et al*., 1998; Schiedner *et al*., 1998; Balague *et al*., 2000; Zou *et al*., 2000) showing a significantly reduced acute and chronic hepatotoxicity as well as reduced inflammatory responses compared with first-generation adenoviral vectors (Schiedner *et al*., 1998), even in large animal models such as primates or dogs (Morral *et al*., 1999; Chuah *et al*., 2003), which are more susceptible to the toxic side effects of adenoviral vectors than mice (Gallo-Pen *et al*., 2001; Schnell *et al*., 2001).

The positive effect of HC vectors could be seen when comparing mice treated with HC vectors to mice treated with early generation vectors. HC vector-treated mice displayed 10-fold higher FVIII expression levels that were sustained for at least 9 months (Reddy *et al*., 2002). It is not certain whether these vectors could give rise to life-long transgene expression because several studies demonstrated a slow but significant decline in transgene expression (Balague *et al.,* 2000; Reddy *et al.,* 2002). This may be due to the low turnover rate of hepatocytes leading to the gradual loss of the high- capacity adenoviral vector. The complete absence of de novo expression of adenoviral gene expression in the cells that are transduced with the HC adenoviral vectors is expected to prevent the induction of cytotoxic T lymphocytes (CTL), provided the transgene itself is non immunogenic.

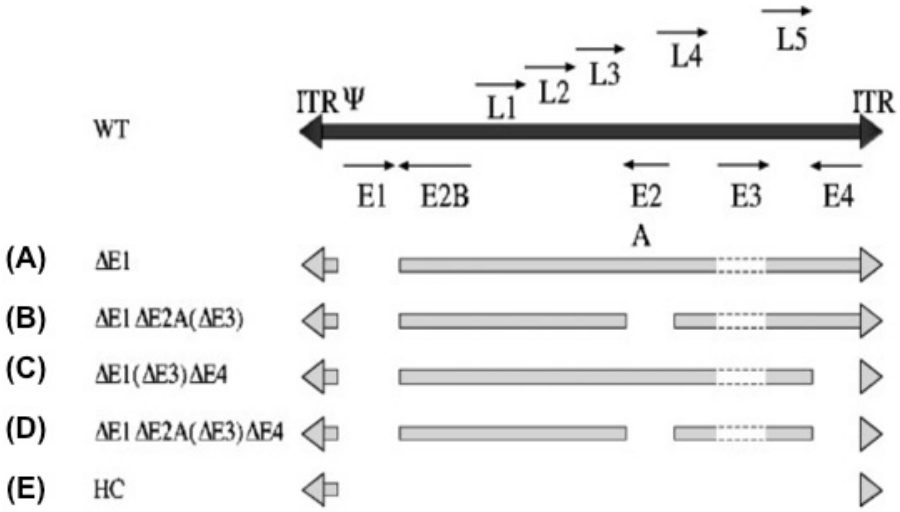


Figure 5: Comparison of deletions in the adenoviral genome for different generations of adenoviral vectors. WT, wild type; HC, high capacity and ITR, inverted terminal repeats (Thorrez *et al.,* 2004).

1.5.3 Recombinant baculoviruses as efficient gene delivery systems

Baculoviruses comprise a diverse group of arthropod viruses (Miller 1997). The best-studied member of this family, Autographa californica nuclear polyhedrosis virus (AcMNPV) is a large enveloped virus with a double-stranded, circular DNA genome of around 130 kb. Baculoviruses usually possess a rod shaped capsid (40 to 50 nm in diameter and 200 to 400 nm in length). Within the protein capsid, the nucleoprotein core contains the condensed DNA genome (O'Reilly *et al.,* 1992). The complete sequence of the viral genome has been determined (Ayres *et al*., 1994). The application of recombinant baculoviruses for the expression of recombinant proteins in insect cells was first described in the early 1980s (Smith *et al*., 1983; Pennock *et al*., 1984). Since these initial reports, the baculovirus insect cell expression system has been extensively developed and used for the production of numerous recombinant proteins in insect cells (O'Reilly *et al*., 1994; Kost *et al*., 2000). The most commonly used insect host cell lines are the Sf9 and Sf21AE lines originally derived from *Spodoptera frugiperda* pupal ovarian tissue (Vaughn *et al*., 1977) and the BTI-Tn-5B1-4 line, also known as 'High 5 cells', derived from *Trichoplusia ni* egg cell homogenates (Granados *et al*., 1994).

Besides expression of proteins, the baculovirus AcMNPV has been recently reported to work as a functional vector for gene delivery in mammalian cells of various origins (Kost and Condreay 2002; Pieroni and La Monica, 2001; Sandig and Strauss, 1996). Several years ago,

baculovirus was shown to infect hepatic cell lines and express foreign genes under the control of mammalian promoters (Boyce *et al*., 1996; Hofmann *et al*., 1995). Subsequently, recombinant baculoviruses has been shown to transduce foreign genes into additional cell lines, including those of non-hepatic origin (Shoji *et al*., 1997).

The use of recombinant baculovirus vectors for gene delivery in mammalian cells has been widely documented over the last few years. Baculovirus has been demonstrated to be a very efficient vector in a variety of cell lines due to a general non-tissue-specific uptake mechanism, and transcription machinery regulated intracellularly by epigenetic factors. Its large insert capacity, the ease of production at high titer and the lack of cytotoxicity are the basic feature of this vector. This vector has been successfully used for therapeutic studies in vitro (e.g. the testing of antiviral compounds) or ex vivo (e.g. anticancer therapy) but has also been developed as a safe and efficient vector suitable for in vivo gene therapy. The most recent examples of gene delivery in vivo in liver, skeletal muscle and the central nervous system of mice and rats shows that it is worth to further develop baculovirus-derived vectors capable to prolong in vivo gene expression. Baculoviral vectors are suitable for the treatment of acquired or inherited diseases in humans (Pieroni and La Monica, 2001). Since baculovirus promoters are silent in mammalian cells, no virus replication occurs. In addition, no cytotoxic effects mediated by this virus in mammalian cells have been reported (Sollerbrant *et al*., 2001).

1.6 Objectives of the study

1.6.1 Functional studies on factor VIII protein variants containing different lengths of the B-domain

The aim of this part of the study is to evaluate the importance and the effect of the B domain region on factor VIII (FVIII) expression and activity and to generate a B-domain deleted recombinant FVIII (BDD-rFVIII) containing the minimal B-domain sequence required for optimal thrombin binding and FVIII activation. This BDD-rFVIII should show the minimal (or no) discrepancy between the clotting assay and the chromogenic activity assay.

For this purpose, several FVIII expression plasmids containing various lengths of the B-domain (approximately 100, 200, 300, 400 amino acids starting from the amino terminus of the B domain) as well as a B-domain deleted construct will be constructed and expressed transiently and stably. FVIII activity should be assessed in culture medium and from purified products, whereas FVIII antigen should be assessed from culture medium and cellular lysates. Purified FVIII products will also be cleaved by thrombin and analysed by western blotting.

FVIII constructs should be expressed at the beginning in three mammalian cell lines (COS-7, HEK 293T and CHO) and the cell line that shows the highest expression level in terms of FVIII activity level in culture medium should then be used for further expression and functional analysis of the constructs. Additionally, the constructs should also be expressed stably in CHO-DHFR[-] cell line.

To evaluate the importance of the B-domain region, the different FVIII constructs (containing different lengths of the B-domain) should be compared to the BDD construct in terms of: 1) Activity level using clotting and chromogenic assays, 2) Assay discrepancy of both activity assays, 3) Expression level in terms of the total amount of FVIII produced; in medium as well as trapped inside cells), 4) Functionality in terms of FVIII specific activity and 5) FVIII activation by thrombin.

1.6.2 Construction of a new helper vector for the production of helper-dependent adenovectors for the use in hemophilia gene therapy

The aim of this part of the study is to design a novel helper system for the production of a helper-dependent adenovector (HD-AdV) carrying a B-domain-deleted factor VIII-cDNA without any contamination with a helper virus. For this, a packaging deficient, replication-competent adenovirus Helper genome will be delivered to the HD-AdVs containing cells by a recombinant Baculovirus / Adenovirus hybrid. This Helper genome should provide (in *trans*) all of the functions necessary for the propagation of HD-AdVs.

In designing our novel helper genome, we will use only Ad5 genes (nts 4000 to 35832) necessary for replication and packaging of HD-AdV. The helper genome will be deleted of the 5'ITR (nts 1-190), the packaging signal (nts 191-358), the 3'ITR (nts 35833-35935) and the E1/E3 region, thus rendering it replication incompetent and packaging incompetent. As the replication of the Ad5 helper genome will result in HD-AdV production, its replication will be achieved by fusing the AAV2-ITRs (5'ITR; 3'ITR) into the helper genome construct. AAV2-ITRs can then serve as origins of replication given that the AAV2-rep78/68 genes are provided in *trans*.

For augmentation of this DNA construct, (AAV2[5'ITR] /Ad5/ AAV2[3'ITR]), it will be integrated into the Baculovirus genome. This hybrid helper vector "Bac-AAV2-Ad5" can then be propagated to high titers in Sf9 insect cells.

Once the Bac-AAV2-Ad5 helper virus is produced, it can be used to rescue the HD-AdV in HEK 293 cells. For this, HEK 293 cells, already transfected with the HD-AdV, will be infected with Bac-AAV-Ad5 and Bac-AAV2-Rep (a baculoviral construct carrying the Rep78/68 genes) viruses. Inside the cells, the AAV2-ITRs should function as an origin of replication and as a termination signal supported by the Rep78/68 protein synthesized by Bac-AAV2-Rep virus. Additionally, the Ad5 genome of the helper vector will provide all Ad5 proteins necessary for replication and packaging of HD-AdV.

2. Materials and methods:

2.1 Materials

2.1.1 Chemicals and reagents

Chemicals and reagents used in this study are listed below in alphabetical order after the supplier's name. All chemicals were of analytical grade.

Applied Biosystems (Weiterstadt)	BigDye terminator; Template suppression reagent
Bio-Rad (Muenchen)	PVDF membrane; Precision protein standards; (10-250Kda); 7.5% SDS-PAGE gels
Biozym (Hess. Oldendorf)	Agarose LE
Dade Behring (Marburg)	Human FVIII-deficient plasma; Lyophilized standard human plasma
Diagnostica Stago (France)	Asserachrom VIII:CAg
Fermantas (St.Leon-Rot)	1 Kb DNA ladders
Fluka (Neu Ulm)	Chloroform:isoamylalcohol (24:1); chloroform; Formamide; Formaldehyde; SDS; Ethidium bromide (10 mg/ml); glycine; Tris base; Nonidet-P40; Luminol
GE Healthcare (Freiburg)	FVIII-select; PD-10 columns; PD-10 buffer reservoir
Invitrogen (Leek, Niederland)	All tissue culture media; EDTA-Trypsin; D-PBS; Phenol:chloroform:isoamylalcohol (25:24:1); Platinum Taq DNA polymerase; EDTA; Ethanol; Methanol; Isopropanol; Glycerol; Sodium chloride; Sodium acetate; Sodium citrate; Lipofectamine 2000; Cellfectin; Penicillin/ streptomycin solution (100x) DH5α competent cells; HT supplement (50x); Trizol; SuperScript III Reverse Transcriptase

Millipore (Ireland)	Ultracel-30K Amicon ultra centrifugal filters
New England Biolabs (Schwalbach/Taunus)	All restriction enzymes, 1 Kb DNA ladder
Nunc Nalgene (Wiesbaden)	Tissue culture dishes, plates, flasks and pipettes
PeqLab (Erlangen)	Pwo DNA polymerase
Qiagen (Hilden)	Mini- and Maxi-Plasmid kits; DNeasy kit; Gel Extraction kit
Roche (Mannheim)	dNTP mix (10 mM); Expand long template PCR Kit; Proteinase K; BSA (20 mg/ml); DNA molecular weight marker X ; T4 DNA ligase; SAP; Klenow enzyme; CompleteTM protease inhibitor cocktail
Sartorius stedim biotech (Gottingen)	Vivaspin 20 columns (30,000 MWCO)
Sigma (Deisenhofen)	Cesium chloride; DMSO; Tween 20; LB broth and Agar; Ampicillin; p-Coumaric acid; Methotrexate; Dialyzed FBS; Insect cell culture tested FBS; DEPC
Stratagene (Heidelberg)	Competent cells (XL10-Gold, BJ5183)
Thermoscientific (USA)	Pierce centrifuge columns (~30μm pore size)

2.1.2 Buffers and solutions

Buffer A	20 mM Imidazol 1 M Sodium chloride 20 mM Calcium chloride 200 ppm Tween-80
Buffer B	20 mM Imidazol 1.5 M Sodium chloride 50% Ethylene glycol 20 mM Calcium chloride 200 ppm Tween-80

Cell lysis buffer	20 mM Tris-HCL, pH 8.0 150 mM Sodium chloride 1 mM EDTA 0.5 % Nonidet-40 CompleteTM protease inhibitor cocktail(added before Use)
DNA gel loading buffer	0.25 % Bromophenol blue 40 % (w/v) Sucrose in water
Laemmli sample buffer	62.5 mM Tris-HCL, pH 6.8 25 % Glycerol 2 % SDS 0.01% Bromophenol blue Add 50 µl of β-Mercaptoethanol per 950 µl buffer Before use
Protein electrode running buffer (10x)	250 mM Tris base, pH 8.3 1.92 M Glycine 1 % SDS
Solution I	15 mM Tris-HCL, pH 8.0 10 mM EDTA 100 µg/ml RNase A
Solution II	0.2 N Sodium hydroxide 1% SDS
Solution A	0.1 M Tris-HCl, pH 8.6, 200 ml 50 mg Luminol
Solution B	DMSO, 10 ml 11 mg para-Hydroxycoumaric acid
TAE (50x)	2 M Tris-base (pH 8.0) 1 M Glacial acetic acid 0.05 M EDTA (pH 8.0)
TBS (10x)	200 mM Tris base, pH 7.6 1.379 M Sodium citrate
Thrombin buffer (10x)	200 mM Tris-HCl. pH 8.4 1.5 M NaCl 25 mM CaCl2
TE buffer	10 mM Tris (pH 8.0) 1 mM EDTA

Western blocking buffer	1x TBS 0.1 % Tween-20 5 % Skimmed milk
Towbin (Transfer) buffer	25 mM Tris base, pH 8.3 192 mM Glycine 20 % Methanol

2.1.3 Primers

All primers are in 5'-3' –orientation. Sense primers end up with (s) while antisense ones with (a).

2.1.3.1 Primers used in the construction of B-domain deleted expression plasmids

Primer name	Primer sequence
pMTF8-6085Ns	CAT CGA CGT ACT TCG CGA CTG TGC TCT CAA AAC CCA CC
pMTF8-6068SNs	GCT CAG CAA CTA GTT GCC ATC GAC GTA CTT CGC GAC TG
pMTF8-7310Aa	ATC CGG AAT AAT GAA GTC TGG CCA GC
pMTF8-3369Na	CTA TGG ACT TCG CGA GAA GCT TCT TGG TTC AAT GGC
pMTF8-3671Na	GTA TAC TCT TCG CGA GTG ATG GAG CTG TGG CCT GAA G
pMTF8-3973Na	GAC GAC GCT TCG CGA ATC ATT ATT TTC TTC ACT CAA G
pMTF8-4243Na	GAC GAC GCT TCG CGA TTC TAA TAT ATT TTG CCA GAC TG
pMTF8-4551Na	CAG GAC TCT TCG CGA TTT TTC TGG TCC TAA GGA TAC
pMTF8-1689s	CTT GAA TTC AGG CCT CAT TGG AGC C

Table 1: Primers used in the construction of B-domain deleted expression plasmids.

2.1.3.2 Primers used in verifying the constructed B-domain deleted expression plasmids

Primer name	Primer sequence
FVIII-210a	AAG GCA CAG AAA GAA GCA GG
FVIII-172s	ATG CAA ATA GAG CTC TCC ACC
FVIII-681s	ACT GTG CCT TAC CTA CTC AT
FVIII-1203s	AGT AGA CAG CTG TCC AGA GG
FVIII-1770s	AGT GAC TGT AGA AGA TGG GC
FVIII-2311s	TCA GAC TTT CGG AAC AGA GG
FVIII-2821s	ACA ACT GCA GCA ACA GAG TTG
FVIII-3297s	GAA TAG TCC ATC AGT CTG GC
FVIII-3862s	CCT CAG ATA CAT ACA GTG AC
FVIII-4381s	AGA TCT CCA TTA CCC ATT GC
FVIII-4906s	AAG CTA TTG GAT CCT CTT GC
FVIII-5406s	TGT CCC TCA GTT CAA GAA AG
FVIII-5941s	TCC CTG CAA TAT CCA GAT GG
FVIII-6501s	GAA GTT CTC CAG CCT CTA CA
FVIII-7001s	AGG AGT TCC TCA TCT CCA GC
pMT2-487s	TCC ACT CGC TCC AGG GTG
pMT2-962s	GAG GTG TGG CAG GCT TGA GAT CTG
pMT2-1131a	CCG TCA AGT TTG GCG CGA AAT C
pMT2-1564a	CTG GTT GAT TCA TGG CTT C
pMT2-1480s	GAA CAA CCG GAA TTG GCA AG
pMT2-2369s	GCT CCA TGG TCG GGA CGC TCT G
pMT2-5109s	CGA CGG CCA GTG CCA AGC

Table 2: Primers used in verifying the constructed B-domain deleted expression plasmids.

2.1.3.3 Primers used for construction and verifying the plasmid, bacmid and viral constructs of Bac-AAV2-Ad5, Bac-AAV2-Rep and Bac-EGFP

Primer name	Primer sequence
AAV2-321s	ATG CCG GGG TTT TAC GAG
AAV2-1382s	CGG GTG CGT AAA CTG GAC
AAV2-289MLs	GGT CTC AAC ACG CGT TTG AAG CGG GAG GTT TGA ACG
AAV2-2310NHa	GGA TAT CAA GCT AGC CTC TGC GGG CTT TGG TGG TGG
Ad5-4231a	CTC TGC AGT GGT GCT ACC
Ad5-35823a	GGC GGA GTA ACT TGT ATG TG
Ad5-35832Bga	CGG CTG ACA GAT CTG TTT TAG GGC GGA GTA ACT TGT
Ad5-34943s	TGG GCG GCG ATA TAA AAT GC
Ad5-4000Bgs	CGT TGA CTA GAT CTG AAG GCT TCC TCC CCT CCC AAT GC
CMV-1Ecos	CGT CGC GAA TTC TTA GTT ATT AAT AGT AAT CAA TTA C
GFP-1329Ha	CTT GTG AAG CTT TTA CTT GTA CAG CTC GTC CAT GCC
MMTV-591Bsps	GAC GCG CTG TCC GGA GTC CCT AGA AGT AAA AAA GGG
MMTV-1347Bama	GTA TCA TAT GGA TCC CCG ACC TGA GGG TGA CCG GGA
BGH-2532NMBs	GCG CGG ATC CAC GCG TCG TAT GCT AGC TAG CCT GTG CCT TCT AGT
BGH-2916EcoRa	GCA GCG CCT GAA TTC CAA GGG ACA TCT TCC CAT TC
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC

Table 3: Primers used in the construction and verification of plasmid and bacmid baculovirus recombinant vectors.

2.1.4 Cell lines and culture media

Cells	Culture Medium*	Source of cells
HEK 293T	DMEM high glucose	AG Prof. W Poller (Freie Universität, Berlin)
COS-7	DMEM high glucose	DSMZ (Braunschweig)
CHO-DHFR[-]	IMDM + HT supplement	DSMZ (Braunschweig)
Sf9	Grace's supplemented	ATTC (Manassas, VA, USA)

Table 4: Cell lines and culture media * All media contain Glutamax and were obtained from Invitrogen.

2.1.5 Plasmids and Vectors

Name	Source
pMT2-FVIII	Dr Rainer Schwaab (Srour *et al.,* 2008)
pFastBac-1	Invitrogen (Leek, Niederland)
pShuttle-CMV	Stratagene (Heidelberg)
pAdEasy-1	Stratagene (Heidelberg)
pTRUF5	Prof. Kleinschmidt (DKFZ, Heidelberg)
Mini-Ad5	Dr Stefan Kochanek (Uniklinik Ulm)
pDG	Prof. Kleinschmidt (DKFZ, Heidelberg)
pTRE-Luc-Ad5	BD Clontech (Palo Alto, USA)
pEGFP-C1	BD Clontech (Palo Alto, USA)

Table 5: Plasmids and vectors.

2.2 Molecular biology methods

2.2.1 Isolation of plasmid DNA, bacmid DNA and extra chromosomal DNA

Plasmid DNA was isolated from *E.coli* cultures (DH5α strain; Invitrogen and BJ5183; Stratagene) grown in LB broth at 37°C by shaking at 225 rpm for 16 hours. Ampicillin (50μg/ml) was used as a selection agent. Mini- and Maxi-plasmid preparations were performed depending on further use of the plasmid. For plasmid analysis by PCR, restriction mapping or DNA sequencing the QIAprep Spin Miniprep kit (Qiagen) was used. If plasmid was isolated for use in cell culture transfection experiments, the QIAGEN Plasmid Maxi kit (Qiagen) was used. Plasmid isolation was performed according to the instructions of the kit supplier.

Bacmid DNA was isolated from E.coli cultures (DH10Bac strain; Invitrogen) grown in LB broth at 37°C by shaking at 225 rpm for 16 hours. Kanamycin (50 μg/ml), gentamicin (7 μg/ml) and tetracycline (10 μg/ml) were used as selection agents. Bacmid isolation was performed according to the Bac-to-Bac Baculovirus Expression System manual (Invitrogen). Briefly, 1.5 ml bacterial culture was centrifuged at 14.000 x g for 1 min. The cell pellet was resuspended in 300 μl of Solution I, gently mixed and a 300 μl Solution II was then added, gently mixed and incubated at RT for 5 min. Slowly and while mixing gently 0.3 ml of 3 M

K+-acetate (pH 5.5) was added and the mixture was cooled on ice for 10 min. After 10 min centrifugation at 14.000 x g, the supernatant was transferred to a new microcentrifuge tube containing 0.8 ml isopropanol, gently inverted several times and cooled on ice for 10 min. The sample was then centrifuged at 14.000 x g for 15 min. and the pellet was washed with 0.5 ml 70% ethanol, and centrifuged for 5 min at 14.000 x g. The DNA pellet was then air dried at RT for 5 to 10 min and dissolved in 40 µl of TE buffer.

Extra chromosomal DNA was isolated from mammalian cells using a modified Hirt procedure (Arad, 1998). Trypsinized cells (106-107) were suspended in 250 µl 50 mM Tris-HCL (pH 7.5), 10 mM EDTA containing 100 µg/ml RNase A and lysed by the addition of 250 µl 1.2% SDS. The suspension was gently mixed and allowed to stand at RT for 5 min. Cellular debris and chromosomal DNA were precipitated by the addition of 350 µl of 3 M CsCl, 1 M K+- acetate and 0.67 M acetic acid. The suspension was then gently mixed and incubated on ice for 15 min. After centrifugation at 14.000 x *g*, the supernatant was loaded onto a QIAprep Spin Column (Qiagen). The column was washed with 750 µl 80 mM K+-acetate, 10 mM Tris- HCL (pH 7.5), 40 µM EDTA and 60% ethanol. DNA was then eluted with 50µl TE buffer or ddH2O.

To gain a very pure DNA, samples were extracted with phenol/chloroform for the removal of proteins (e.g. enzymes) or to reduce salt concentration. DNA samples were suspended in 200 µl ddH2O and extracted once with one volume of phenol: chloroform: isoamylalcohol (ratio 25:24:1) followed by centrifugation at 3.000 rpm for 5 min. The upper layer containing the DNA was precipitated by adding 0.1 volume of 3M Na+-acetate (pH 4.8) and two volumes of prechilled ethanol, and allowed to stay at -80°C for at least 30 min. After centrifugation at 14.000 rpm for 15 min, the DNA pellet was washed once with 1 ml of 70 % prechilled ethanol, vacuum dried and dissolved in TE buffer or ddH2O.

2.2.2 Isolation of total RNA from cells grown in monolayers

Total RNA was prepared from monolayer cell cultures using Trizol reagent according to the supplier recommendations. Cells were washed with ice cold PBS then lysed by adding Trizol reagent (1 ml per 10 cm2) and scraped with cell scraper. Cell lysate was then passed several times through a pipette and vortexed thoroughly. Homogenized samples were incubated 5 min at room temperature and centrifuged to remove cell debris. Supernatants were then mixed

with chloroform (0.2 ml per 1.0 ml Trizol), vortexed vigorously for 15 seconds and then incubated at room temperature for 2 to 3 min. After centrifugation at 12,000 xg at 4 °C for 15 min, the upper aqueous phase containing RNA were moved carefully into a fresh tube. RNA was then precipitated by mixing with isopropanol (0.5 ml per 1.0 ml Trizol), incubating at room temperature for 10 min and centrifugation at 12000 xg at 4 °C for 10 min. RNA pellet was then washed once with 75% ethanol and air or vacuum dried and dissolved in DEPC-treated water.

2.2.3 Measurement of isolated DNA/RNA concentrations

DNA and RNA concentration was determined by measuring OD at 260 nm, where 1.0 OD represents 50 μg DNA/ml and 40 μg RNA/ml (Sambrook and Russell, 2001).

2.2.4 Agarose gel electrophoresis

Nucleic acids were separated according to their size in agarose gel matrix by electrophoresis. The concentration of agarose varied (0.6-2%) depending on the size of the DNA fragments expected. Ethidium bromide (0.5 μg/ml) was included in the agarose gel for visualization of the DNA fragments under UV (λ 302 nm) light (Sambrook *et al*., 1989). Samples were mixed with gel loading buffer before loading in the gel. The gels were run at 100-150 V in 1x TEA buffer. DNA molecular weight markers or ladders (marker X [Roche] and 1 kb ladders [Fermentas and New England Biolabs]) were used to determine the size of DNA fragments.

2.2.5 PCR

An analytical and preparative polymerase chain reaction (PCR) was used. Anaytical PCR was performed using Platinum Taq DNA polymerase (Invitrogen) and was used to analyze plasmids, bacmids and baculovirus plaques. Preparative PCRs were performed using the proof reading Pwo DNA polymerase (PeqLab) and generated fragments were used for cloning. For DNA fragments longer than 2 kb, a preparative long distance (LD) PCR was performed using a mixture of Pfu (a proof reading polymerase) and Taq DNA polymerase (Roche). The fidelity of Pfu and Pwo is 12x and 10x more effective than Taq, respectively.

Typical PCR reactions are shown in following table:

Components	**Final concentrations**		
PCR type	**Taq**	**Pwo**	**LD (Pfu + Taq)**
10x buffer, -Mg^{2+}	1x	1x	1x
$MgCl_2$ 50 mM	1.5 mM	--	2.25 mM
$MgSO_4$ 25mM	--	4 mM	--
dNTPs 10 mM	200 µM	400 µM	500 µM
Sense primer* 100 µM	1 µM	1 µM	0.33 µM
Antisense primer* 100 µM	1 µM	1 µM	0.33 µM
Polymerase	1 U	1 U	2.5 U
Template	50-200 ng	50-200 ng	10-30 ng
ddH_2O	Up to 25 µl	Up to 50 µl	Up to 50 µl

Table 6: Constituents of PCR reactions using different amplification enzymes.
*All primers used are listed in section II.1.3.

Thermal cycling of PCR using Taq and Pwo were as follows: 5 min initial denaturation at 94°C followed by 35 cycles [each cycle consisted of denaturation at 94°C for 30 sec, annealing at 50-60°C for 30 sec, and extension at 72°C for 1min/kb (with a time increment of 20 sec/cycle in the last 20 cycles for Pwo)]; and a final extension step at 72°C for 10 min.

The LD PCR was performed as follows: initial denaturation at 94°C for 2 min. followed by 30 cycles [each consisted of denaturation 94°C for 10 sec, annealing at 60-62°C for 30 sec with time decrement of 0.2°C/cycle, and extension at 68°C for 1 min/kb (with a time increment of 20 sec/cycle for the last 20 cycles)] and a final extension at 68°C for 7 min. The annealing temperature used depends on the melting temperature of the primers. PCRs were performed using GeneAmp PCR system 9700, Perkin Elmer.

For purification, the PCR products were subjected to agarose gel electrophoresis as described in section II.2.3 and the target DNA band was then excised and purified using the QIAquik Gel extraction kit (Qiagen) according to the instructions of the kit supplier.

2.2.6 DNA sequencing

DNA sequencing was performed to analyze plasmids, bacmids, baculoviruses and to verify DNA fragments before and after cloning. A cycle sequencing reaction was performed by mixing DNA template (100-200 ng PCR product or 200-500 ng plasmid DNA) with 20 pmol of sense or antisense primer, 2 µl of BigDye terminator (contains fluorescent labeled ddNTPs, dNTPs, AmpliTaq DNA polymerase FS and Taq buffer) from Applied Biosystems and ddH2O to a total volume of 10 µl. The thermal cycling was performed using GeneAmp PCR system 9700 (Perkin Elmer) as follows: initial denaturation at 96°C for 2 min followed by 25 cycles (each consist of 10 sec denaturation at 96°C, 5 sec annealing at 50-55°C and 4 min extension at 60°C).
Cycle sequencing products were purified by mixing them with 90 µl ddH2O, 250 µl ethanol and 10 µl of 3 M Na+-acetate (pH 4.6), followed by precipitation at 14.000 rpm for 15 min. The pellet was then washed once with 70% ethanol, vacuum dried, resuspended in 20 µl Template Suppression Reagent (TSR; Applied Biosystems), denatured at 90°C for 2 min, chilled on ice and subjected to sequencing using the ABI Prism 310 Genetic Analyzer (Perkin Elmer).

2.2.7 DNA restriction and ligation

DNA fragments, intended for cloning, were generated by PCR (section II.2.4) and included restriction site linkers that were created and inserted into the 5'-end of the respective primers. The DNA fragment (insert) and the plasmid (to be cloned in it) were restricted with restriction enzymes (RE). The restriction reaction which included 0.2-1.0 µg of DNA, 5-10 units of RE and 1x RE buffer was incubated at 37°C for 1-2 hours. RE and their buffers were obtained from New England Biolabs. The RE reaction was scaled up according to the amount of DNA needed. The restricted DNA fragments were then purified by phenol/chloroform or agarose gel electrophoresis purification. Restricted fragments were blunt ended when needed with Klenow fragment (Roche). Before ligation, restricted DNA fragments with blunt ends or complementary cohesive ends were dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent self-ligation. Dephosphorylation was performed using 0.5 unit of SAP (New England Biolabs) per 1 µg DNA at 37°C for one hour. Fifty to 100 ng of each restricted insert and plasmid (insert to plasmid ratio is $\geq$ 3:1) were ligated using T4 DNA ligase (Roche) at 15-25°C for 15 min.

2.2.8 Transformation and electroporation of *E.coli* cells

Competent *E.coli* cells (DH5α strain; Invitrogen and XL10-Gold strain; Stratagene) were transformed with 20-50 ng of ligation products using heat-shock. These bacterial cells were used for routine cloning during construction or propagation of plasmids. For the construction of large plasmids by homologous recombination (He *et al.*, 1998), the electroporation competent RecA+ *E.coli* strain (BJ5183 strain; Stratagene) was used. Briefly, 1 µg of linearized and dephosphorylated transfer shuttle plasmid and a 100 ng of supercoiled acceptor plasmid (plasmid containing the Ad5 genome) were mixed with competent BJ5183 cells on ice and then subjected to electroporation using the Cell-Electroporator *E.coli* system (Gibco-Invitrogen). The electroporation parameters were set at 400 Volt, 330 µF, low-charge, low Ω and voltage booster at 4 kΩ.

Transformed or electroporated cells were grown for one hour in LB broth at 37°C while shaking at 225rpm. Then a 100-250 µl of bacterial culture were plated on LB-agar containing ampicillin or gentamicin depending on the type of the antibiotic resistance gene in the plasmid used. LB-agar plates were incubated for 14-16 hours. Clones were picked and cultured in LB
broth. After propagation of bacteria, plasmids were isolated by mini-preparation. Because the BJ5183 strain is unstable and its plasmid yield is very low, selected clones from this strain were transformed in XL10-Gold cells (suited for propagation of large plasmids) for further analysis and propagation of the plasmid.

2.2.9 Construction of factor VIII expression plasmids with various truncated B- domain regions

The pMT2-FVIII plasmid (Srour *et al.*, 2008) containing the full-length factor VIII cDNA (nt # 1111 to 8386) was used as starting material (figure 6). First, the pMT2-FVIII plasmid was cut with ApaI (cuts at 4411 and 7302) and SpeI (cuts at 1695) and the large fragment (7548 bp) representing the plasmid backbone and the rest of FVIII cDNA was gel extracted (Fig. 7, step 1). Then a Pwo-PCR fragment was created by two round PCR using the pMT2-FVIII as a template, first using the primer pair pMTF8-6085Ns / pMTF8-7310Aa that includes an NruI restriction site at the 5'-end and second, using the primers pMT2F8-6068SNS / pMTF8-7310Aa which adds an SpeI restriction site 5' to the NruI site (Fig. 7, step 2). The PCR

product was then cut with SpeI and ApaI, gel extracted and cloned into plasmid pMT2-FVIII which was cut with SpeI /ApaI (large fragment) to generate pMT2-F8-SNA plasmid (8808 bp) (Fig. 7, step 3). Cloning was verified by DNA sequencing and digestion with SalI (gives two fragments one represents FVIII cDNA (~3 Kb in pMT2-F8-SNA) and one represents the pMT2 plasmid (~5.8 Kb)).

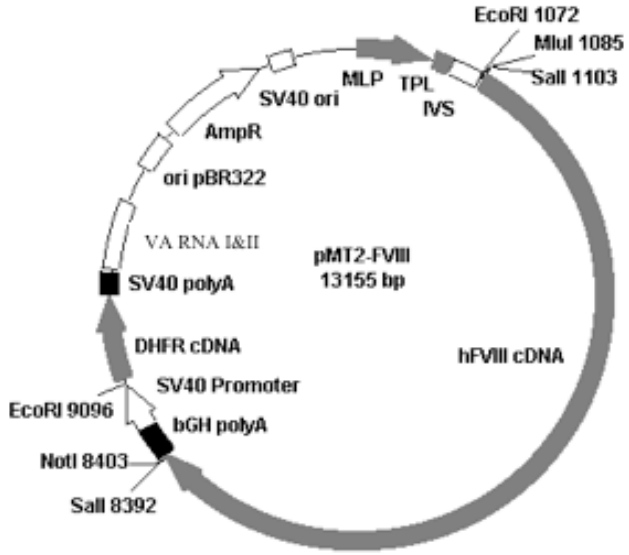


Figure 6: Schematic representation of pMT2-FVIII expression plasmid containing the full length FVIII cDNA (7056 bp plus 214 bp of 3'UTR; GeneBank accession # M14113; nts # 172-7227 + 3'UTR nts # 7228-7440).

The pMT2-F8-SNA plasmid was then cut with SpeI and NruI and the large fragment (~8790 bp) was gel extracted (Fig. 7, step 4). Fragments (AàE) containing various lengths of the B-domain region were then amplified (Fig. 7, step 5) using the pMT2-FVIII plasmid as a template by Pwo-PCR or LD-PCR according to table 6 where the antisense primers hold an NruI restriction site. The amplified fragments were then cut with SpeI and NruI (Fig. 7, step 6), gel extracted and cloned separately into plasmid pMT2-F8-SNA which has been cut with SpeI / NruI to generate the plasmids pMT2-F8-BDD (aa 743/1634), pMT2-F8-101 (aa 844/1634), pMT2-F8-202 (aa 945/1634), pMT2-F8-292 (aa 1043/1634) and pMT2-F8-394 (aa 1137/1634) (see table 7). The plasmids were then verified by DNA sequencing and Sal I digestion.

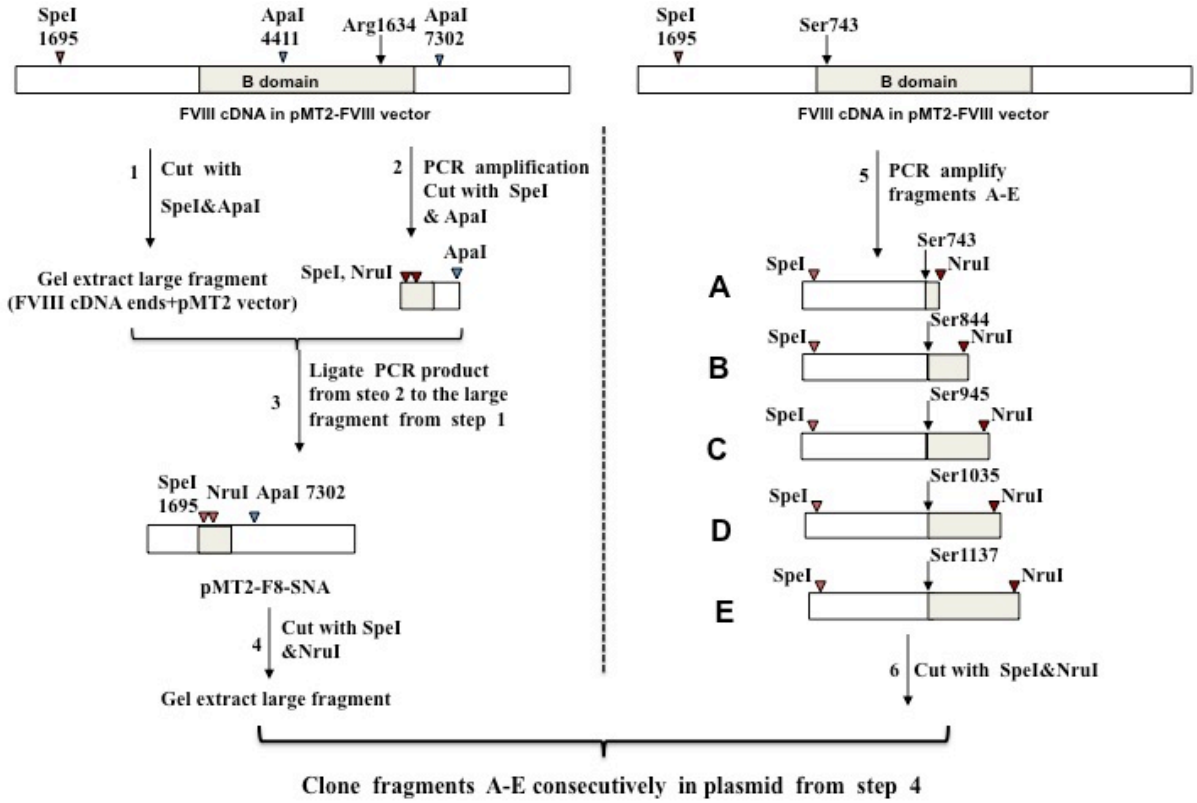


Figure 7: Schematic representation of the construction of various expression plasmids containing different lengths of the B domain. Steps 1,2 and 5 were performed using the pMT2-FVIII vector containing the full length FVIII cDNA (nts # 1111 to 8386). The B domain spans nts # 3388 to 6112 (aa 740 to 1648).

Product	Primer pair	Product size	End plasmid Size	End plasmid name
A	pMTF8-1689s / pMTF8-3369Na	~1694 bp	10485 bp (BDD)	pMT2-F8-BDD
B	pMTF8-1689s / pMTF8-3671Na	~2000 bp	10788 bp (101 aa/B)	pMT2-F8-101
C	pMTF8-1689s / pMTF8-3973Na	~2303 bp	11091 bp (202 aa/B)	pMT2-F8-202
D	pMTF8-1689s / pMTF8-4243Na	~2573 bp	11361 bp (292 aa/B)	pMT2-F8-292
E	pMTF8-1689s / pMTF8-4551Na	~2879 bp	11667 bp (394 aa/B)	pMT2-F8-394

Table 7: Different amplified fragments and the corresponding end plasmids.

2.2.10 Construction of recombinant bacmid DNA containing the adenovirus helper genome

The plasmid pFastBac1 (Invitrogen) (Fig. 8 A) was used as a basic plasmid for the following cloning steps. The DNA fragments Ad5-L and Ad5-R (2707 bp; L corresponds to Ad5 nts 4000 to 5781 plus Pme I & Eco RI restriction sites, while R corresponds to Ad5 nts 43935 to 35832; GeneBank# NC_001406) were amplified by LD-PCR from pShuttle-CMV (Stratagene) using the primer pairs Ad5-4000Bgs / Ad5-3832Bga, cut with Bg1II, gel extracted and cloned between the AAV2 ITRs of plasmid pTRUF5 (obtained from Prof. Kleinschmidt, Heidelberg University; the plasmid contains the AAV2 5'- and 3'-ITRs corresponding to nts 1 to 145 & 4530 to 4675, respectively on AAV2 genome; GeneBank # AF043303) which was cut before with Bg1II and gel extracted to generate pTRUF5-Ad5LR. The fragment AAV2-5'ITR/Ad5LR/AAV2-3'ITR was cut from pTRUF5-Ad5LR with BspHI, blunt ended and cloned into pFastBac1, cut with NotI and blunt ended to generate the shuttle plasmid pBac-AAV2-Ad5LR (Fig. 8 B).

The Ad5 genome was then cloned from pAdEasy-1 (Stratagene; Fig. 8 C) into the shuttle plasmid pBac-AAV2-Ad5LR (Fig. 8 B) by homologous recombination in bacterial cells (section 2.2.7). For this, the shuttle plasmid was linearized with PmeI (cuts between the two Ad5 homologous sequences), purified by phenol/chloroform purification and dephosphorylated with SAP. One µg linearized plasmid was mixed with 100 ng pAdEasy-1 and added to 40 µl of BJ8153 electro competent bacterial cells. The DNA-bacteria mixture was then transferred into a 0.15 cm gap cuvette and electroporated to generate the plasmid pBac-AAV2-Ad5 (35,824 bp) containing the Ad5 genome (Fig. 8 D). The pBac-AAV2-Ad5 selected clones were verified by DNA sequencing using the primers Ad5-4231a (covers the 5'-ITR region and confirms the presence of the 5’ end of the Ad5 genome) and Ad5-35687s (covers the 3'-ITR region and confirms the presence of the 3’ end of the Ad5 genome), by Taq-PCR using the primers Ad5-4000xs / Ad5-7000a (confirms the presence of part of the Ad5 genome which reflect an intact construct) and by digestion with SmaI (gives the following fragments: 6447, 11, 195 [5'-ITR], 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230, 652, 2278, 2262, 550 [3'-ITR] and 11 bp).

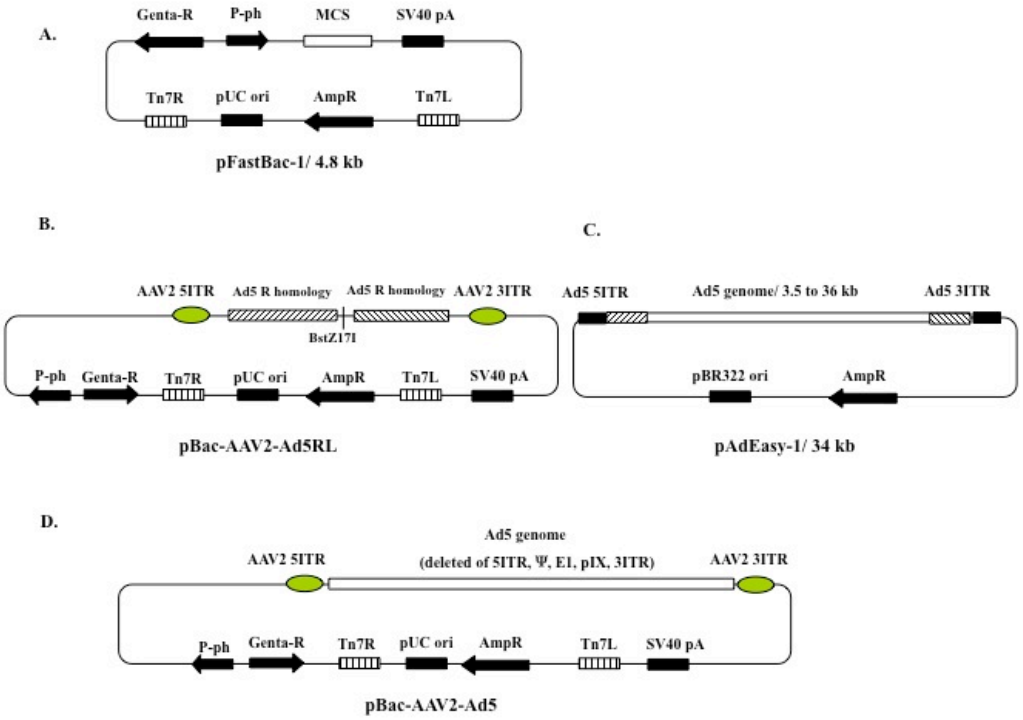


Figure 8: Construction of the pBac-AAV2-Ad5.

To support the AAV-ITRs replication in trans, a Rep-protein is needed. For this, The MMTV-LTR promoter (757 bp; nts 591 to 1347 on MMTV genome; GeneBank#V01175) was amplified from pDG plasmid using primers MMTV-591Bsps / MMTV-1347Bama and cloned at BspE1 / BamHI sites in pFastBac1 plasmid to generate pBac-MMTV (~5387 bp; digestion of pFastBac1 with BspE1 / BamHI removes the insect PH promoter). The bGH polyA signal (258 bp; most of it matches nt# 75 to 299 according to GeneBank # AF117346) plus 126 bp from the 5' end of ß-globin polyA were amplified from pTRE2-Luc-Ad5 (Clontech) using primers BGH-2532NMBs / BGH-2916EcoRa and cloned into BamHI / EcoRI sites of pFastBac1-MMTV to generate pBac-MMTV-bGH (~5780). pBac-AAV2-Rep (~7802 bp) was then constructed by amplifying the AAV2-Rep genes (2022 bp; nt 289 to 2310; GeneBank# AF043303) from pDG plasmid using primers AAV2-289MLs / AAV2-2310NHa and cloning them at MluI / NheI sites in pBac-MMTV-bGH (Fig. 9).

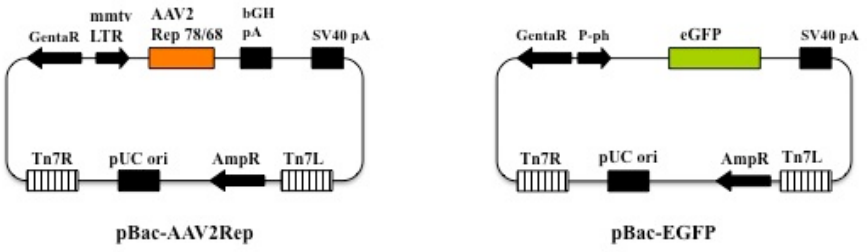


Figure 9: Schematic representation of pBac-AAV2-Rep and pBac-EGFP.

Expression of the AAV2-Rep gene was tested in both Sf9 and 293T cells. SF9 cells were transfected with plasmid pBac-AAV2-Rep using Cellfectin reagent, incubated for 48 hours and then used for total RNA preparation using Trizol reagent (see section 2.2.2). 293T cells were transfected with pBac-AAV2-Rep and pDG (contains the Rep gene expression cassette that were used for pBac-AAV2-Rep cloning) plasmids using Lipofectamin 2000, incubated for 48 hours and then used for total RNA preparation using Trizol reagent. Total RNA was subject to RT PCR using Superscript III reverse transcriptase followed by Taq-PCR using the primer pairs AAV2-2310a / AAV2-1380s (which give a product of 1 kb) and AAV2-2310a / AAV2-321s (which give a PCR product of 2 kb).

As a positive control for transfecting insect cells, the plasmid pBac-EGFP was constructed (Fig. 9). The CMV-EGFP was amplified from pEGFP-C1 using the primer pair CMV-1Ecos/GFP-1329Ha. The resulting amplification product was digested with EcoRI/HindIII and cloned in EcoRI/HindIII digested pFastBac1.

Recombinant bacmids were constructed by site-specific transposition in DH10Bac bacterial cells [contains the baculovirus shuttle vector (bacmid) bMON14272 (136 kb); Invitrogen] (Fig. 10). Twenty five µl of DH10Bac were mixed with 0.25 ng of shuttle plasmid pBac-AAV2-Ad5 in a 15 ml round-bottom polypropylene tube and incubated on ice for 30 min. Cells were heat shocked at 42°C for 45 seconds, incubated on ice for 2 min and a 250 µl SOC medium was then added to them. Transformed cells were incubated 4 hours at 37°C while shaking at 225 rpm and then diluted 1:10 and 1:100 with SOC medium. One hundred µl of each dilution were plated on LB agar plates containing 50 µg /ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal and 40 µg/ml IPTG. Plates were incubated at 37°C for 48 hours. Ten white colonies were picked, restreaked on new LB agar containing the above mentioned selection agents and incubated for 24 hours. One white colony from each plate were picked and cultured in 2.5 ml LB broth containing the three antibiotics and incubated at 37°C overnight.

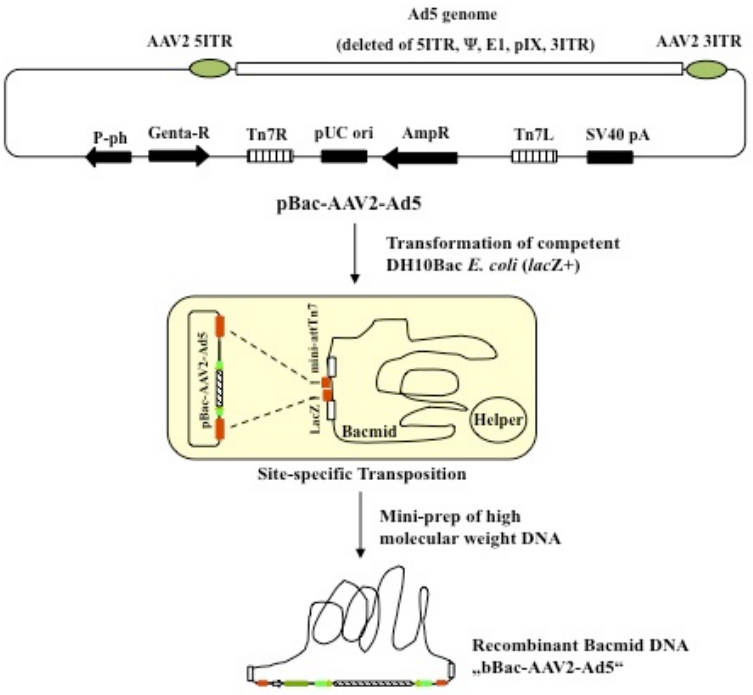


Figure 10: Generation of recombinant bacmid DNA "bBac-AAV2-Ad5".

Recombinant bacmid DNA (bBac-AAV2-Ad5; bBac-EGFP; bBac-AAV2-Rep) was isolated according to section 2.2.1. Recombinant bacmid (bBac-AAV2-Ad5) was analyzed by Taq-PCR using M13 forward / M13 reverse primers (confirms the presence of the plasmid insert: an empty bacmid gives a ~270 bp PCR product, a transposed bacmid gives no product as Taq polymerase cannot amplify such large insert >35 kb), M13 forward /Ad5-4231a primers (covers the 5'-ITR region, confirms the presence of the 5'end of the Ad5 genome and gives ~3.1 kb PCR product), M13 reverse / Ad5-35687s primers (covers the 3'-ITR region, confirms the presence of the 3'end of the Ad5 genome and gives ~1.55 kb PCR product) and Ad5- 4000xs / Ad5-7000a primers (confirms the presence of Ad5 genome and gives ~3.0 kb PCR product). bBac-EGFP was characterized by Taq PCR using the M13 forward and M13 reverse primers. The presence of the EGFP gene resulted in a 3.6 kb fragment.

bBac-AAV2-Rep was analyzed by Taq-PCR using M13 forward / M13 reverse primer pair (confirms the presence of the plasmid insert: an empty bacmid gives a ~270 bp PCR product, a transposed bacmid gives a ~5.3 kb PCR product), M13 forward / MMTV-1347a primers (covers the 5' AAV2-Rep construct end and gives a ~2.3 kb PCR product) and AAV2-321s / M13 reverse primers (covers the 3' AAV2-Rep construct end and gives a ~2.8 kb PCR product).

2.3 Cell culture

2.3.1 Cultivation and storage of cells

Cell lines (HEK 293T, COS-1 and CHO-DHFR[-]) were grown in their respective media (section II.1.4) supplied with 10% fetal bovine serum (FBS), 100 U/ml of Penicillin-G and 100 µg/ml of Streptomycin, and 5% CO2 at 37°C (Freshney, 2000).

Cells were grown in different tissue culture dishes or plates (Nunc) of different sizes depending on the type of the experiment. For cell expansion, cells were washed with Dulbecco's-PBS, trypsinized with Trypsin-EDTA (Invitrogen) and seeded at the appropriate ratio in new dishes. Stocks of cell lines were prepared in freezing medium (70% culture medium, 20% FBS and 10% DMSO) and maintained in liquid nitrogen (Freshney, 2000).

2.3.2 Transient expression of B-domain deleted constructs in mammalian cell lines

2.3.2.1 Transfection

Transfection of expression plasmids in the three animal cell lines was performed on liposomal basis using the transfection reagent Lipofectamine 2000 (Invitrogen) according to the instructions of the supplier. Transient transfections were done in 6-well plates with cells grown to ~90% confluence in an antibiotic free medium. First, 4 µg of each expression plasmid was diluted in 250 µl Opti-MEM I (reduced serum medium; Invitrogen) while a 10 µl Lipofectamine 2000 (1 mg/ml) was diluted in 250 µl Opti-MEM I, separately. After 5 min incubation at RT, both mixtures were combined, mixed gently and incubated 20 min at RT. 500 µl of DNA-Lipofectamine 2000 complexes were then added to the respective well containing cells and medium and mixed gently by rocking the plate back and forth. The plate was then incubated at 37°C and 5% CO2 for 4 hours. Then the medium was replaced by the respective normal medium. The efficiency of transfection was controlled by the transfection of an eGFP expression plasmid pEGFP-C1.

2.3.2.2 Expression studies

Expression experiments were done in three mammalian cell lines: HEK 293, COS-7 and CHO-DHFR[-]. One day before each expression experiment, 1 * 10^6 cells in 2 ml growth medium were plated per well so that they reached ~90% confluency at the time of transfection. The cell culture media used were without phenol red as it interferes with FVIII activity measurement. Expression experiments were performed using the five plasmids containing the B-domain deleted constructs along with pMT2-FVIII (contains the full length factor VIII cDNA) and pMT2-FVIII-BDD (Bonn-BDD; codes for a truncated factor VIII protein in which amino acid 852 is joined to amino acid 1524; Srour *et al*., 2008; Fig. 11). All the expression experiments were performed using the same DNA and Lipofectamine 2000 quantities. Twenty-four hours after transfection, growth medium was replaced with a fresh medium, collected 24 hours later (48 hours after transfection), and then again a fresh medium was added and collected 24 hours later (72 hours after transfection). The two medium collections were centrifuged at 700 x g at 4°C for 5 min, aliquoted and stored at -80°C.

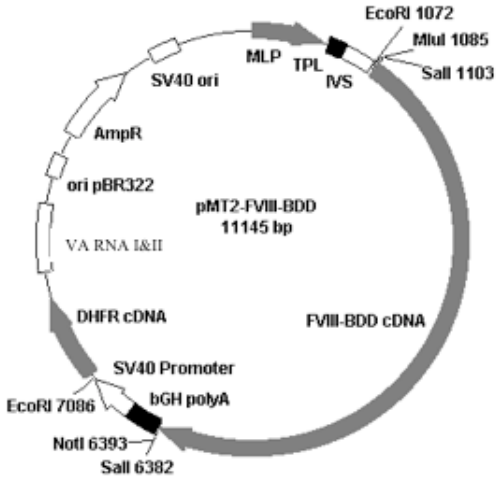


Figure 11: Schematic representation of pMT2-FVIII-BDD (BONN-BDD) expression plasmid containing the FVIII-B domain deleted (FVIII-BDD) cDNA, which is the same as FVIII full length except that most of the B-domain is deleted from nts 2795 to 4804 (nucleotides order is according to Gene Bank # M14113).

Aliquots were used to measure factor VIII activity using the clotting and the chromogenic assays (section 2.4.2). Factor VIII antigen (in culture medium) was quantified by ELISA (section 2.4.3) for the cell line that showed the highest factor VIII activity. The specific factor VIII activity was then calculated. In each expression experiment, the expression plasmids were transfected in triplicates (3 wells for each expression plasmid) along with two negative control wells (no DNA) and one positive control well (pEGFP-C1).

2.3.3 Generation of stable cell lines expressing various factor VIII protein variants in CHO-DHFR[-] cells

2.3.3.1 Transfection, selection and amplification of CHO-DHFR[-]

Transfection. 20 µg of ethanol precipitated linearized (cut with MluI) DNA of each expression plasmid (pMT2-F8-BDD, pMT2-F8-202, pMT2-F8-394 and pMT2-FVIII) and 50 µl Lipofectamine 2000 was used to transfect CHO-DHFR[-] cells which were grown in IMDM (containing hypoxanthine thymidin reagent; Invitrogen) to 90% confluence in 10 cm ø dishes. DNA-Lipofectamine complexes were incubated with the cells for 4 hours. Growth medium containing the complexes was then removed and 3 ml of 10% glycerin were added to the cells for 2 min to facilitate the DNA uptake by cells (Sambrook and Russell, 2001). Cells were then washed by PBS and 10 ml of fresh growth medium was added.

Selection. After 48 hours, transfected cells were splitted into five 10 cm ø dishes at 1:15 ratio using a 10 ml selection medium (IMDM containing dialyzed FBS [Sigma] and deficient in hypoxanthine and thymidin). To each dish a different Methotrexate (MTX; a selection and amplification agent; Sigma) quantity was added (20 nM, 50 nM, 100 nM, 200 nM and 400 nM). The selection medium did not contain nucleosides so that only cells that have the expression plasmid (containing the DHFR gene) could grow. The selection medium and the respective MTX quantity was changed every two days until a well-defined new colonies appear (2-3 weeks). Colonies were then pooled and tested for productivity by assaying factor VIII activity in culture medium. Productive dishes were passaged (splitted and let grow) 4 times and the productivity was assessed each time. Dishes that kept the productivity over passaging were expanded to several dishes for storage, amplification and cloning.

Amplification. Productive dishes were amplified by splitting the cells in a higher MTX concentration steps (5x, 10x, 20x and 50x for example). In each new MTX concentration step the cells were maintained until confluency, passaged 4 times and then the productivity was assessed. Cells that showed higher productivity were expanded for storage, cloning and a further amplification step.

2.3.3.2 Limited dilution cloning and sub cloning

Productive pooled clones were cloned by serial dilution in 96 well plates (Corning protocol, MA, USA) (Fig. 12). Cells were trypsinized and 2 * 10^4 cells/ml cell suspension was prepared. In a 96 well plate, a 100 µl selective medium was added in all wells except well A1 (see diagram below), then 200 µl of the cell suspension was added to well A1. Using a single channel pipettor, 100 µl were quickly transferred from A1 to B1 and mixed by gently pipetting. Using the same tip, this 1:2 dilution was repeated down the entire column, ending by discarding 100 µl from H1. Using an 8-channel micropipettor, 100 µl additional medium was added to each well in column 1, mixed by gentle pipetting and then 100 µl was transferred quickly from column 1 (A1 through H1) to column 2 (A2 through H2). Using the same tips, these 1:2 dilutions were repeated across the entire plate, discarding 100 µl from each of the wells of the last column (A12 through H12) so that all the wells ended up with 100 µl of cell suspension. The final volume in all wells was then brought to 200 µl by adding 100 µl medium to each well. Cells were allowed to settle down at 37°C for one hour and wells were then checked for the presence of single cells using an inverted microscope. Wells containing single cells were marked and followed until they had formed detectable clones (approximately 2 weeks incubation at 37°C in a CO2 incubator). Single colonies were then sub-cultured into a larger vessel (24-well plate) and the productivity (FVIII activity) was then assessed upon confluency. Highly productive clones were then propagated into 6-well plates followed by 10 cm ø dishes for storage, amplification and sub-cloning. Sub-cloning increases the likelihood that the cells originated from a single cell and possibly gives higher productive clones. It was performed using the cloning protocol.

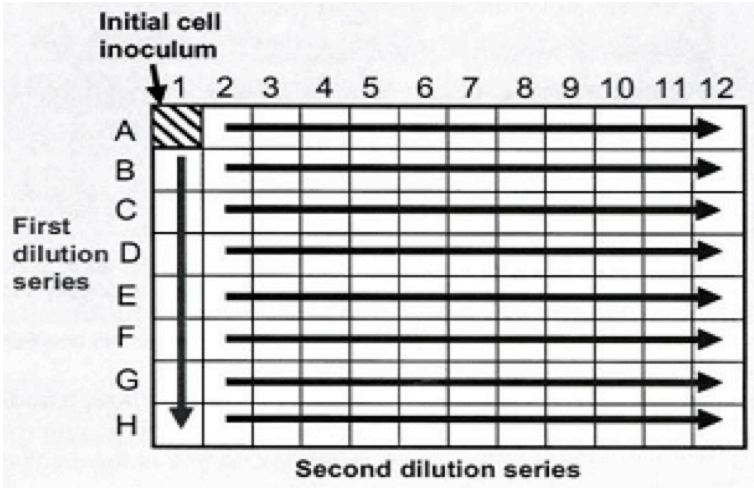


Figure 12: Schematic representation of serial dilutions in 96-well plate.

2.3.4 Generation of helper recombinant baculovirus in insect cells: were performed according to the Bac-to-Bac Baculovirus Expression System manual instructions (Invitrogen).

2.3.4.1 Transfection of Sf9 cells

Insect cells (Sf9 cell line, ATCC # CRL-1711) were grown in supplemented Grace's Insect Cell Culture Medium (Invitrogen) at 27°C supplied with 10% Insect Cell Culture Tested FBS (Sigma), 50 U/ml penicillin and 50 µg/ml streptomycin. Sf9 cells were seeded at 9*105 cells per well in a 6-well plate and allowed to attach at 27°C for one hour prior to transfection. For each transfection sample, 1 µg of purified bacmid DNA (section II.2.1) were diluted in 100 µl of unsupplemented Grace's medium (Invitrogen), and a 6 µl well mixed Cellfectin reagent (Invitrogen) were diluted in a 100 µl unsupplemented Grace's medium, separately. Both mixtures were then combined, mixed gently and incubated at RT for 15 to 45 min. During this incubation time, media was removed from cells and washed once with 2 ml unsupplemented Grace's medium. To each DNA: lipid complex, 0.8 ml unsupplemented Grace's medium was added, mixed gently and the complexes were added then to each well containing the cells. After 5 hours incubation at 27°C, DNA: lipid complexes were removed and a 2 ml supplemented Grace's medium was added to the cells (Fig. 13).

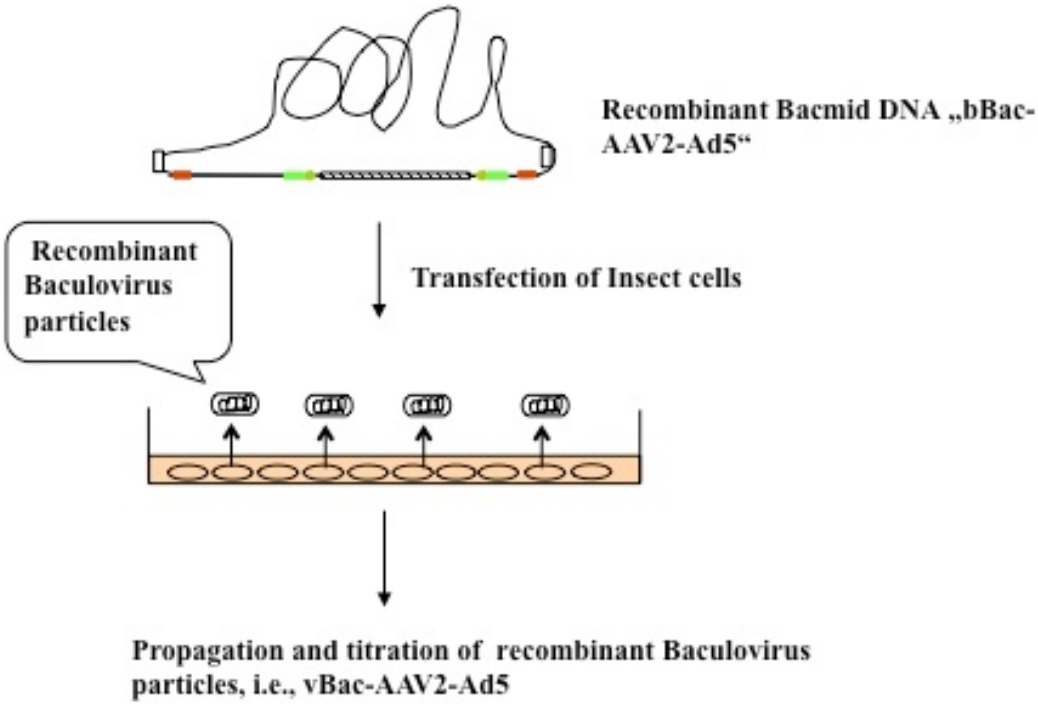


Figure 13: Production of recombinant Baculovirus particles “Bac-AAV2-Ad5”.

Budded viruses should be released into the medium 72 hours after transfection. Virally-infected insect cells typically show the following characteristics: Increased cell diameter and size of cell nuclei (first 24 hours), cessation of cell growth, granular appearance as a sign of viral budding, detachment (24-72 hours) and cell lysis (>72 hours). Once the cells appear infected, the virus was harvested from cell culture medium by collecting the medium in a sterile 15 ml snap-cap tube, and subsequent centrifugation at 500 xg for 5 min. The clarified supernatant containing the virus was then transferred into a fresh snap-cap tube and labeled as the P1 viral stock.

2.3.4.2 Amplification of P1 viral stock

The P1 viral stock is a small-scale, low-titer stock (generally ranges from $1*10^6$ to $1*10^7$ plaque forming units (pfu)/ml). Amplification of P1 allows the production of a P2 viral stock with a titer ranging from $1*10^7$ to $1*10^8$ pfu/ml. To amplify the P1 viral stock, cells ($2*10^6$/well in 6-well plate) were infected at a multiplicity of infection (MOI) ranging from 0.05 to 0.1. MOI is defined as the number of virus particles per cell.

To calculate how much viral stock must be added to obtain a specific MOI, the following formula was used:

$$\text{Inoculum required (ml)} = \frac{\text{MOI(pfu/ml)} * \text{number of cells}}{\text{Titer of viral stock (pfu/ml)}}$$

Assuming a titer of $5*10^6$ pfu/ml and $2*10^6$ cells/well (2 ml culture) which has to be infected by an MOI of 0.1 then:

$$\text{Inoculum required (ml)} = \frac{0.1 \text{ pfu/ml} * 2 * 10^6 \text{ cells}}{5* 10^6 \text{ pfu/ml}} = 0.04 \text{ ml}$$

So, 0.04 ml of P1 viral stock was used to infect Sf9 cells (seeded one hour before infection in 6-well plates at $2*10^6$ cells /well). Forty eight hours post-infection, medium containing virus was collected in 15 ml snap-cap tubes, centrifuged at 500 xg for 5 min, and then the supernatant was transferred to a fresh tube labeled P2 viral stock.

2.3.4.3 Plaque assay

To determine the titer of the baculoviral stock and to plaque purify the virus, plaque assay was performed. The clarified baculoviral stock P2 was diluted in supplemented Grace's Insect Medium without FBS to an 8-log serial dilution (10^{-1} to 10^{-8}). Sf9 cells were seeded into 6-well plates at 1 * 10^6 cells /well, allowed to settle at RT for 1.0 hour and then checked using an inverted microscope for attachment and for 50% confluence. Medium was then removed and 1.0 ml of each viral dilution (10^{-3} to 10^{-8}) was added to the cells and incubated at RT for 1.0 hour. The medium containing the virus was then removed starting from the highest dilution to the lowest. Then a 2 ml plaquing medium (Supplemented Grace's Insect Medium containing 10% FBS and 2% agarose; Invitrogen) was added to the cells, and the agarose overlaying the cells was allowed to harden at RT for 20 min. Now, cells were incubated in a 27°C humidified incubator for 7-10 days until plaques were visible and ready to count. The titer of the viral stock was then calculated according to the following formula:

$$\text{Titer (pfu/ml)} = \text{number of plaques} * \text{dilution factor} * \frac{1}{\text{ml of inoculum / well}}$$

2.3.4.4 Virus preparation

A viral stock from a single viral clone was generated by plaque purifying the baculovirus. For this, Sf9 cells were seeded into 6-well plate at 1 * 10^6 cells /well and let be settled at RT for 1.0 hour. Using a sterile Pasteur pipette and bulb, a clear plaque was picked and the agarose plug (containing the virus) was transferred into a 1.5 ml microcentrifuge tube containing 500 µl supplemented Grace's medium, and then mixed well by vortexing. 100 µl of the agarose plug solution was then added to each well. Cells were incubated in a 27°C humidified incubator for 72 hours. Medium containing the virus was then collected in a sterile 15 ml snap-cap tubes, centrifuged at 500 x g for 5 min and clarified supernatant (the plaque-purified viral stock) was then transferred to fresh snap-cap tubes. This viral stock was then amplified according to section 2.3.4.2.

2.3.4.5 Verifying the purified virus by PCR analysis

Purified viral DNA was extracted from viral stock using the extra chromosomal DNA isolation protocol (section 2.2.1). Viral DNA (vBac-AAV2-Ad5 and vBac-AAV2-Rep) was verified by Taq-PCR using the same approach followed in verifying Bacmid DNA (section 2.2.10).

2.3.5 Rescuing the helper dependant adenovirus in HEK 293T cells (Figure 14)

The ability of the baculovirus to infect HEK 293 cells were tested by infecting 90 % confluent cultures with 200 µl vBac-EGFP P2 viral stock in 6-well plates once at 37 °C for one hour and once at 28 °C for 4 hours.

To rescue the helper dependant adenovirus in HEK 293 cells, cells were splitted into 6-well plates (8 wells total) one day before transfection at 1 * 10^6 cells/well. On 90% confluence, cells were transfected (section 2.3.2.1) with PmeI linearized mini-Ad5 helper dependent vector (pAd5-AAT-hf8) (6 wells) by lipofection along with a negative control well (no DNA) and a positive control (of transfection) well (pBac-EGFP). One day after transfection, HEK 293 cells were infected with different concentrations of Bac-AAV2-Ad5 particles (using different MOI) in combination with vBac-AAV2-Rep according to table 8 with the last well (well#8) infected with vBac-AAV2-Rep only as a negative control for the helper dependent adenovirus production.

Table 8: Infection mixtures used to rescue the helper dependent adenovirus in HEK 293 cells.

Well#1: Negative control	**Well#3:** vBac-AAV2-Ad5 Plaque 9 at 2000 MOI + vBac-Rep Plaque 1 at 200 MOI	**Well#5:** vBac-AAV2-Ad5 Plaque 16 at 2000 MOI + vBac-Rep Plaque 1 at 200 MOI	**Well#7:** vBac-AAV2-Ad5 Plaque 5 at 2000 MOI + vBac-Rep Plaque 1 at 200 MOI
Well#2: Positive control (pBac-EGFP)	**Well#4:** vBac-AAV2-Ad5 Plaque 9 at 500 MOI + vBac-AAV2-Rep Plaque 1 at 200 MOI	**Well#6:** vBac-AAV2-Ad5 Plaque 16 at 500 MOI + vBac-AAV2-Rep Plaque 1 at 200 MOI	**Well#8:** vBac-AAV2-Rep Plaque at 200 MOI

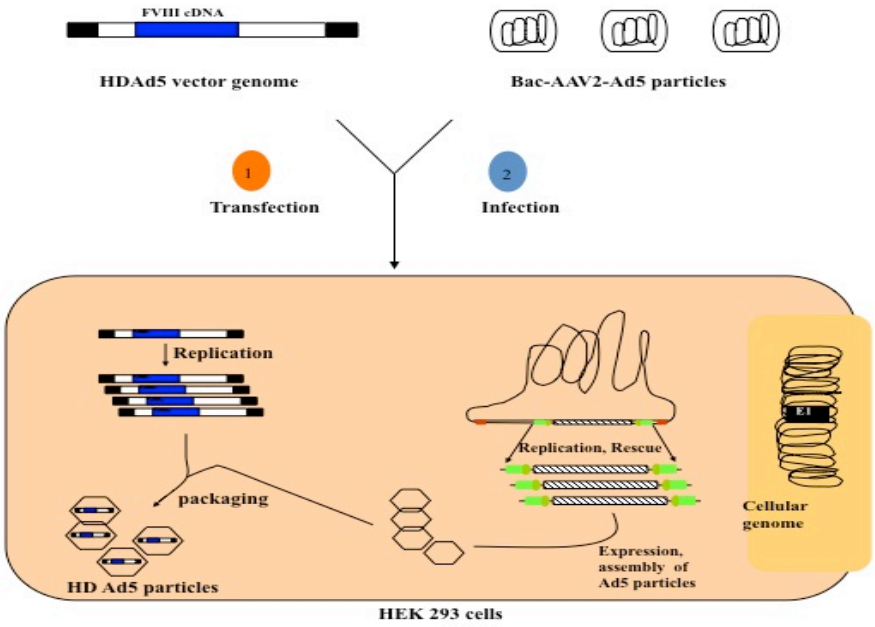


Figure 14: Rescuing the helper dependant adenovirus in HEK 293 cells.

The detailed infection protocol of HEK 293 cells was according to Kenoutis *et al.* 2006. For this, HEK 293 cells were washed with D-PBS and the viral mixtures prepared in 0.5 ml D-PBS were added to each corresponding well. After 4-6 hours incubation at 28 °C, mixtures were removed and 2 ml complete medium (DMEM) were added. Cytopathic effects and signs of viral production were followed over five days. Culture medium was collected from corresponding wells and used for FVIII: Ag ELISA after 10x concentration. Cells were trypsinized and lysed in PBS by 4 rounds of freeze /thaw cycles (freezing for 5 min in dry ice / ethanol bath and thawing at 37°C). Lysates (200 µl) were used for extra chromosomal DNA isolation and /or used for a second round of infection in which fresh (new) 90% confluent HEK 293 cells in 6-well plates were infected with 200 µl lysates at 37°C for 45 min after being infected with the Helper vector for one hour at RT. Signs of viral production were followed over 4 days. Cells were then freezed and thawed as mentioned above and lysates were used for extra chromosomal DNA isolation (section 2.2.1) and for a third round of infection using the same protocol mentioned above for the second round of infection.

FVIII: Ag was assessed in medium and extra chromosomal DNA was digested by DpnI to test whether it is the original transfected DNA or a newly produced DNA in HEK 293 cells, which was under the support of our designed hybrid helper vector.

2.4 Biochemical methods

2.4.1 Purification of factor VIII protein expressed in culture medium

FVIII containing medium (300 ml) collected from stable cell line cultures was concentrated 10x using Vivaspin 20 columns (30,000 MWCO). Concentrated medium was then mixed with 0.5 ml FVIII-Select beads (monoclonal anti-FVIII antibody beads) and shaken at 4°C for 2 hours. Beads were then pelleted (2 min centrifugation at 2000 rpm), resuspended in buffer A, loaded onto a 2 ml pierce column and washed 4 times with buffer A (after each addition of buffer A, the column was centrifuged at 200 rpm for 2 min). FVIII was then eluted with buffer B and 8 batches were collected (beads were suspended 8 times with 0.5 ml buffer B and centrifuged for 5 min at 200 rpm, each time the eluate was collected in a new tube). Batches were assayed for FVIII activity and the productive batches were pooled and buffer exchanged on a PD-10 desalting columns with buffer A. Eluates were then concentrated using Amicon Ultracel (30K) filters and concentrates were then tested for FVIII-activity and FVIII-antigen. For activity assays (see section 2.4.2), purified FVIII was diluted in human FVIII-deficient plasma and for antigen quantitation (section 2.4.3); purified FVIII was diluted in buffer A. For testing the purity of the purified FVIII, approximately 1 U FVIII (measured by FVIII: Ag ELISA) were digested with human alpha thrombin and detected by western blotting (see section 2.4.4).

2.4.2 Factor VIII activity assays

FVIII activity (FVIII: C) was measured in conditioned medium using the clotting assay which is based on the measurement of activated Partial Thromboplastin Time (aPTT) and / or the chromogenic assay that is based on the activation of a chromogenic substrate. In both assays, conditioned medium was treated as a plasma sample.

2.4.2.1 Clotting assay

Clotting assays are based on the measurement of aPTT (Turi and Peerschke, 1986; Ray and Hawson, 1989; Van den Besselaar *et al*., 1993) using ACLTM-analysis systems (Instrumentation Laboratories) that work on centrifugational analyzer principle. Sample to be tested was mixed with FVIII-deficient plasma and then the intrinsic coagulation pathway was

activated by the addition of bovine cephaline and microcrystalline kieselguhr. Clot formation was initiated by adding Ca^{+2} and the machine starts to measure the change in light scattering. When the maximum scattering was attained, time (reflects aPTT measurement) was stopped. Time of measured samples was converted into % using a standard curve that the machine plots automatically from three standard plasma dilutions. A 100% value corresponds to the average FVIII: C in normal pooled plasma. Samples with activities higher than 100% were diluted. Samples with low activity values were evaluated by an additional standard curve for low values. ACLTM-analysis system was operated according the manufacturer specifications. The pipetting of reagents and samples was done automatically. In each measurement, standard plasma was included as a positive control and a conditioned medium from cells that do not secret FVIII was included as a negative control.

2.4.2.2 Chromogenic assay

In the chromogenic assay (van Dieijen *et al*., 1981), FVIII: C was measured by the generation of factor Xa using "Factor VIII Chromogenic assay" kit (Dade Behring, Marburg). In this assay, FVIII is activated by the addition of thrombin. Activated FVIII (FVIIIa) works as a cofactor with FIXa, Phospholipids and Ca^{+2} in the activation of FX to FXa. FXa activity is measured through the hydrolysis of FXa-specific p-nitroaniline substrate. The initial rate of release of p-nitroanaline measured spectrophotometrically at 405 nm is proportional to FXa activity that reflects FVIII: C in the sample. The assay was performed automatically on MLA Electra 900C coagulation analyzer. Samples were diluted 1:41 using 0.9% sodium chloride and results were calculated according to an automatically plotted standard curve. In each measurement, two control plasmas were run: Control Plasma N (normal range) and Control Plasma P (pathologic range).

2.4.3 Factor VIII antigen assay

FVIII antigen concentration (FVIII:Ag) was measured using FVIII:Ag-ELISA (Asserachrom VIII:Ag; Diagnostica Stago; Girma *et al*., 1998). Two monoclonal antibodies (mAb) directed against distinct epitopes of the human FVIII light chain have been used in this sandwich-ELISA. The first mAb was immobilized on wells of microtiter plates. FVIII-containing samples were incubated at 20°C for 2 hours. Following washing, the second antibody, coupled to peroxidase, was incubated at 20°C for 2 hours. Bound antibody was measured by

incubating with tetramethybenzidin for 5 minutes, blocking the reaction with 50 µl of 1 M H2SO4 and reading absorbance at 450 nm. Control plasma, containing 1 U/ml FVIII: C as estimated by comparing with the International standard, was used as a reference (100%). The ELISA was done according to the manufacturer recommendations. The standard curve was made by diluting the control plasma. The detection limit of this assay was 0.5% of FVIII.

2.4.4 Purified factor VIII cleavage by thrombin and western blot analysis

For western blot analysis of purified FVIII products (F8-BDD, F8-202, F8-394 and F8-Full), approximately 1 U containing aliqoutes (as measured by FVIII:Ag, section II.4.3) were mixed with Laemmli buffer, and heat denatured at 95°C for 5 min. For complete thrombin digestion, aliquotes containing 1 U FVIII were digested with 0.5 nM human alpha thrombin (FIIa) in 1x FIIa buffer at 37°C for 4 min. The reaction was then stopped by the addition of laemmli buffer and heat denaturing. For the A2 domain cleavage by thrombin, a master mix for 10 reactions (1 U FVIII, 0.5 nM FIIa and 1x FIIa buffer per reaction) was prepared at 37°C. Aliquots were taken after the addition of FIIa every 15 seconds and reactions were stopped directly by mixing with laemmli buffer. Samples were then heat denatured at 95°C for 5 min. Heat denatured proteins were then separated by electrophoresis over SDS-polyacrylamide gels (7.5%). The proteins were transferred onto a PVDF membrane using a tank blotting apparatus (BioRad). The FVIII proteins were visualized by incubating the membrane with monoclonal anti-human FVIII A2 domain antibody (Green Mountain Antibodies; USA) using a 1:800 dilution, followed by incubation with a peroxidase conjugated anti-mouse IgG (1:4000 dilution). The final detection was achieved by adding 4 ml solution A, 400 µl solution B and 1.2 µl H_2O_2. Evaluation of bands was carried out using a chemillumiscence documentation system (Alpha Innotech).

3. Results:

3.1 Functional studies on factor VIII protein variants containing different lengths of the B-domain:

3.1.1 Construction (cloning) of expression plasmids containing different lengths of the B-domain

The expression plasmids containing different lengths of the factor VIII B-domain region were constructed according to the cloning strategy that is detailed in section II.2.8 and documented in figure 15. The pMT2-F8-SNA intermediate vector (8808 bp; containing the **S**peI / **N**ruI / **A**paI sites) was constructed by digesting pMT2-FVIII with ApaI and SpeI which will remove most of the middle part of FVIII cDNA (Fig. 7 step 1 and Fig. 15 B, lane 1). In the next step, a PCR fragment covering the end part of the B domain and part of the light chain (from position 6068 to position 7310 in pMT2-FVIII sequence) was created by two PCR rounds, the first PCR resulted in 1.25 kb fragment and introduced an NruI restriction site at the 5'-end, the second PCR resulted in a 1.27 kb fragment and introduced an SpeI restriction site 5' to the NruI site (Fig. 7 step 2 and Fig. 15 A lane 1 / 2 and B lane 2). This fragment was then cloned into the large fragment that resulted from step 1. The large fragment contains the pMT2 vector backbone and the beginning as well as the end of FVIII cDNA (Fig. 7 step 3). The selected pMT2-F8-SNA clones were confirmed to be correct by SalI digestion. This digestion resulted in a 5.8 kb fragment representing the vector backbone and a 3.0 kb factor VIII fragment (Fig. 15C). Clones were further confirmed by DNA sequencing.

The different DNA fragments (A→E) containing various lengths of the B-domain region were amplified by PCR using the primer combinations listed in table 7 (Fig. 7 step 5 and Fig. 15 D and E). Those fragments were cloned into pMT2-F8-SNA which has been digested with SpeI and NruI (Fig. 7 step 4 and Fig. 15 F) to generate the plasmids pMT2-F8-BDD (10485 bp), pMT2-F8-101 (10788 bp), pMT2-F8-202 (11091), pMT2-F8-292 (11361 bp) and pMT2-F8-394 (11667 bp) (see appendix). Several clones were picked for each plasmid and tested to have the correct plasmid size by linearizing them with MluI (Fig.15 G) and by digesting them with SalI, which removes the FVIII cDNA from the vector (Fig.15 H).

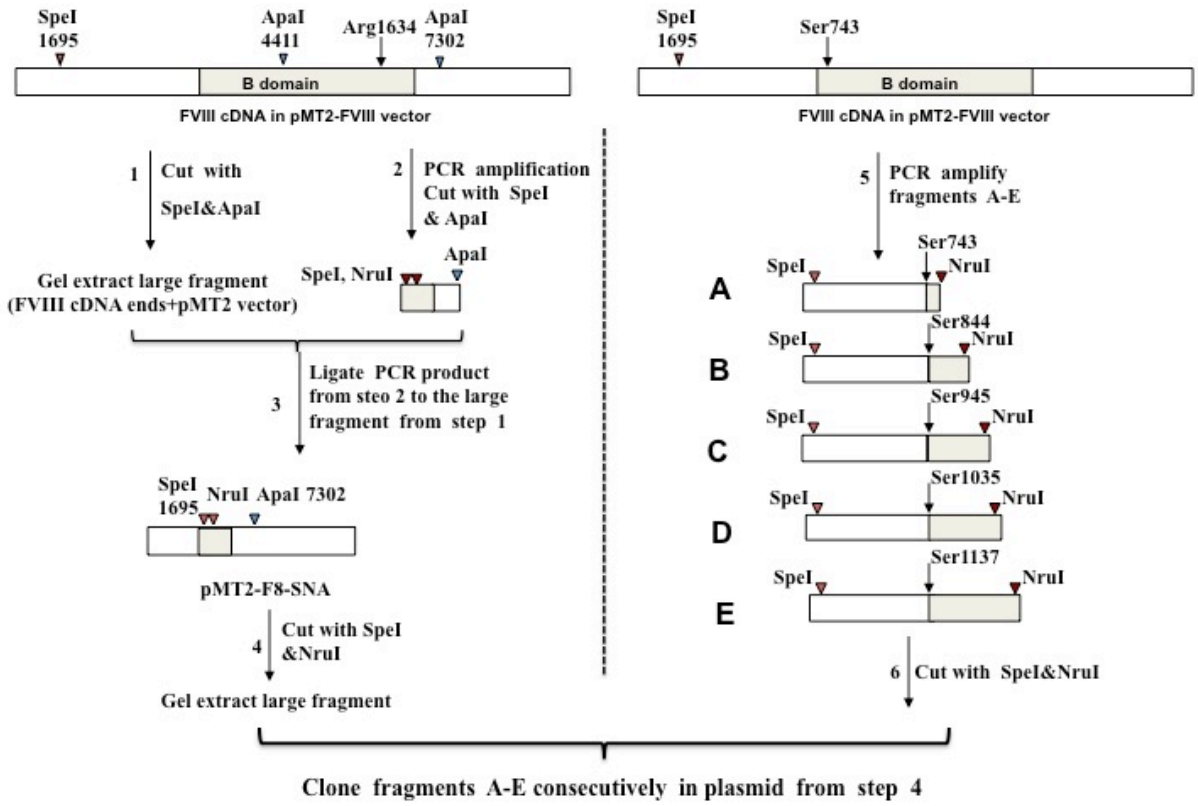
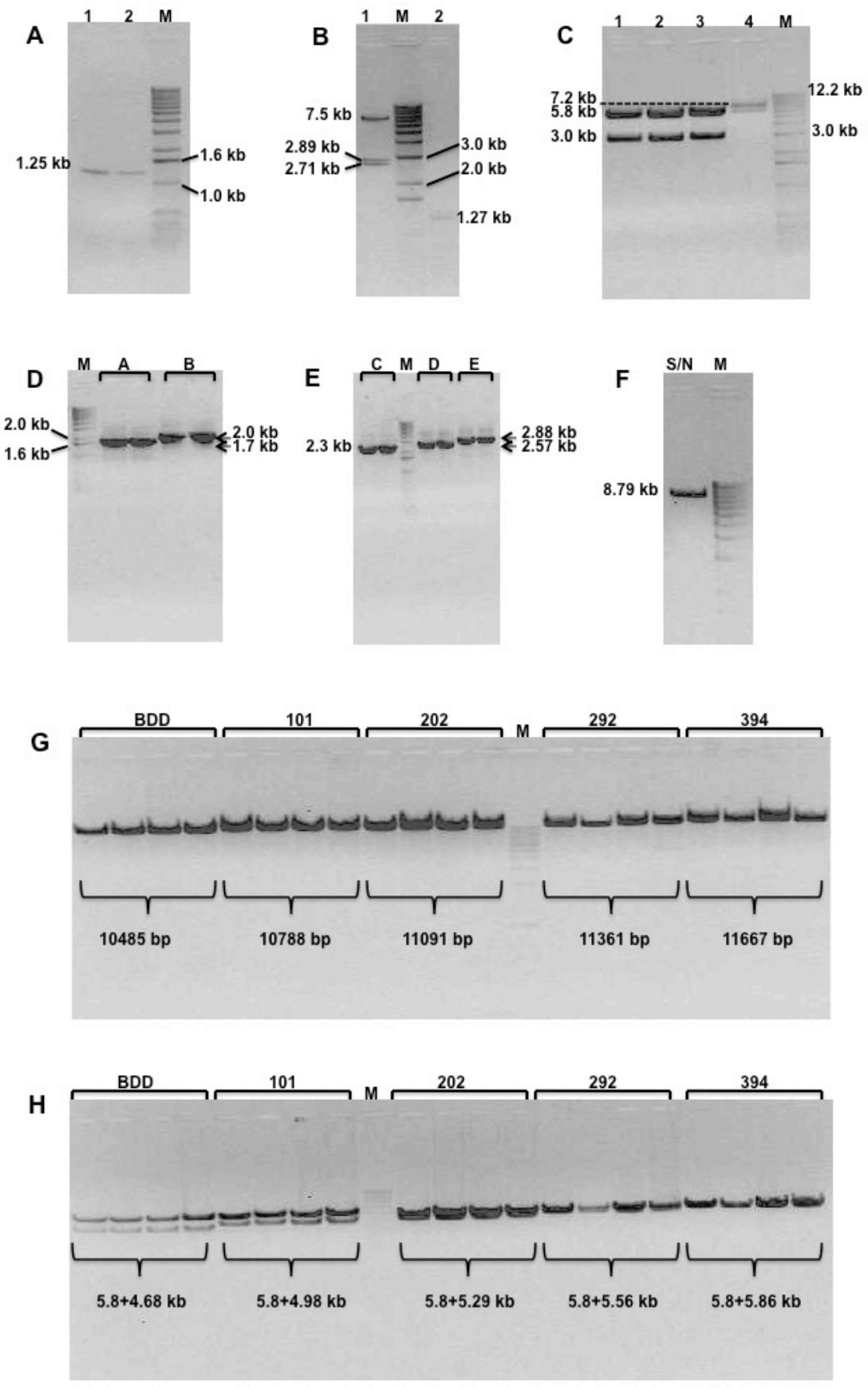


Figure 7 (repeat): Schematic representation of the construction of various expression plasmids containing different lengths of the B domain. Steps 1,2 and 5 were performed using the pMT2-FVIII vector containing the full length FVIII cDNA (nts # 1111 to 8386). The B domain spans nts # 3388 to 6112 (aa 740 to 1648).

A schematic representation of the various B domain deleted constructs on the protein level in comparison to the commercially available recombinant BDD-FVIII (ReFacto) and to the full length FVIII protein is shown in figure 16. The level of the B-domain glycosylation (the number of glycosylated positions) is also shown in the same figure.

Figure 15 (next page): Overview of the various cloning steps used to construct the B-domain deleted expression plasmids. (A) The first PCR product that resulted in 1.25 kb fragment and the introduction of an NruI restriction site at the 5'-end. (B) Lane 2 shows the second PCR product which resulted in a 1.27 kb fragment and the introduction of an SpeI restriction site 5' to the NruI site; Lane 1 shows the pMT2-FVIII plasmid digested with SpeI and ApaI. (C) Lane 1-3 represents SalI digestion (which releases the FVIII-cDNA from the pMT2 vector) of three pMT2-F8-SNA clones; Lane 4 represent SalI digestion of pMT2-FVIII containing the full length FVIII-cDNA. (D and E): The amplified different DNA fragments (AàE) containing various lengths of the B-domain. (F) SpeI/NruI (S/N) digestion of pMT2-F8-SNA (see Fig. 7; step 4). (G) MluI digestion of the different B-domain deleted plasmids. (H) SalI digestion of the different B-domain deleted plasmids. M: DNA molecular weight marker X (0.07 – 12.2 kb; Roche).


A
1
2
M
1.25 kb
1.6 kb
1.0 kb
B
1
M
2
7.5 kb
2.89 kb
2.71 kb
3.0 kb
2.0 kb
1.27 kb
C
1
2
3
4
M
7.2 kb
5.8 kb
3.0 kb
12.2 kb
3.0 kb
D
M
A
B
2.0 kb
1.6 kb
2.0 kb
1.7 kb
E
C
M
D
E
2.3 kb
2.88 kb
2.57 kb
F
S/N
M
8.79 kb
G
BDD
101
202
M
292
394
10485 bp
10788 bp
11091 bp
11361 bp
11667 bp
H
BDD
101
M
202
292
394
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5.8+4.98 kb
5.8+5.29 kb
5.8+5.56 kb
5.8+5.86 kb

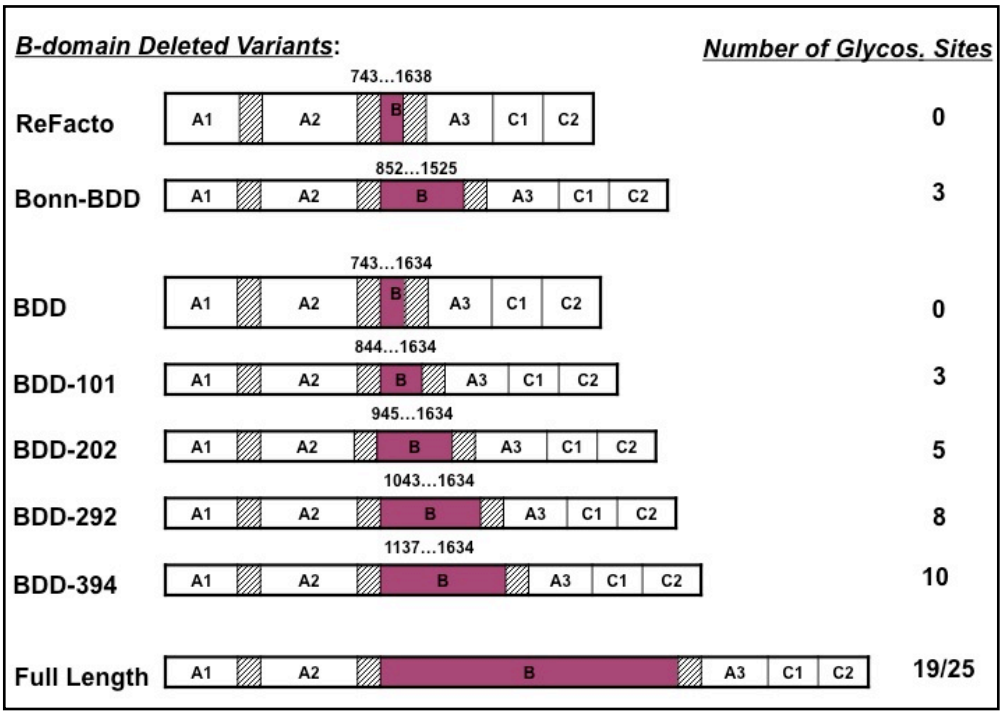

Figure 16: Schematic representation of the various B-domain deleted constructs on the protein level and the corresponding number of glycosylation sites within the B domain.

One correct clone from each plasmid was amplified by maxi culture to be used in expression experiments. All maxi prepared clones were re-tested by MluI and SalI digestion (Fig. 17) and confirmed to be correct by DNA sequencing. Figure 18 shows the DNA sequence of different B-domain deleted constructs across the deleted region. Maxi-prepared DNA confirmed by restriction analysis and DNA sequencing was used for expression studies.

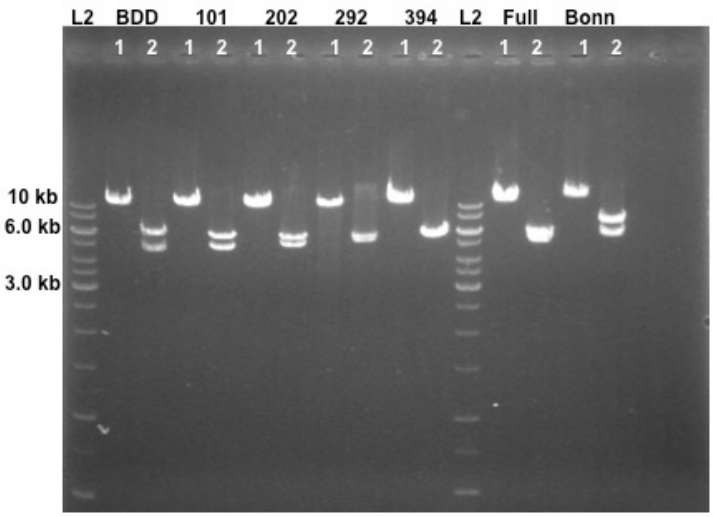

Figure 17: Maxi prepared plasmid DNA of various B-domain deleted expression plasmids. Lane 1: MluI digested plasmids, Lane 2: SalI digested plasmids. L2: Gene Ruler 1kb DNA ladder (0.25 – 10 kb; Fermantas).

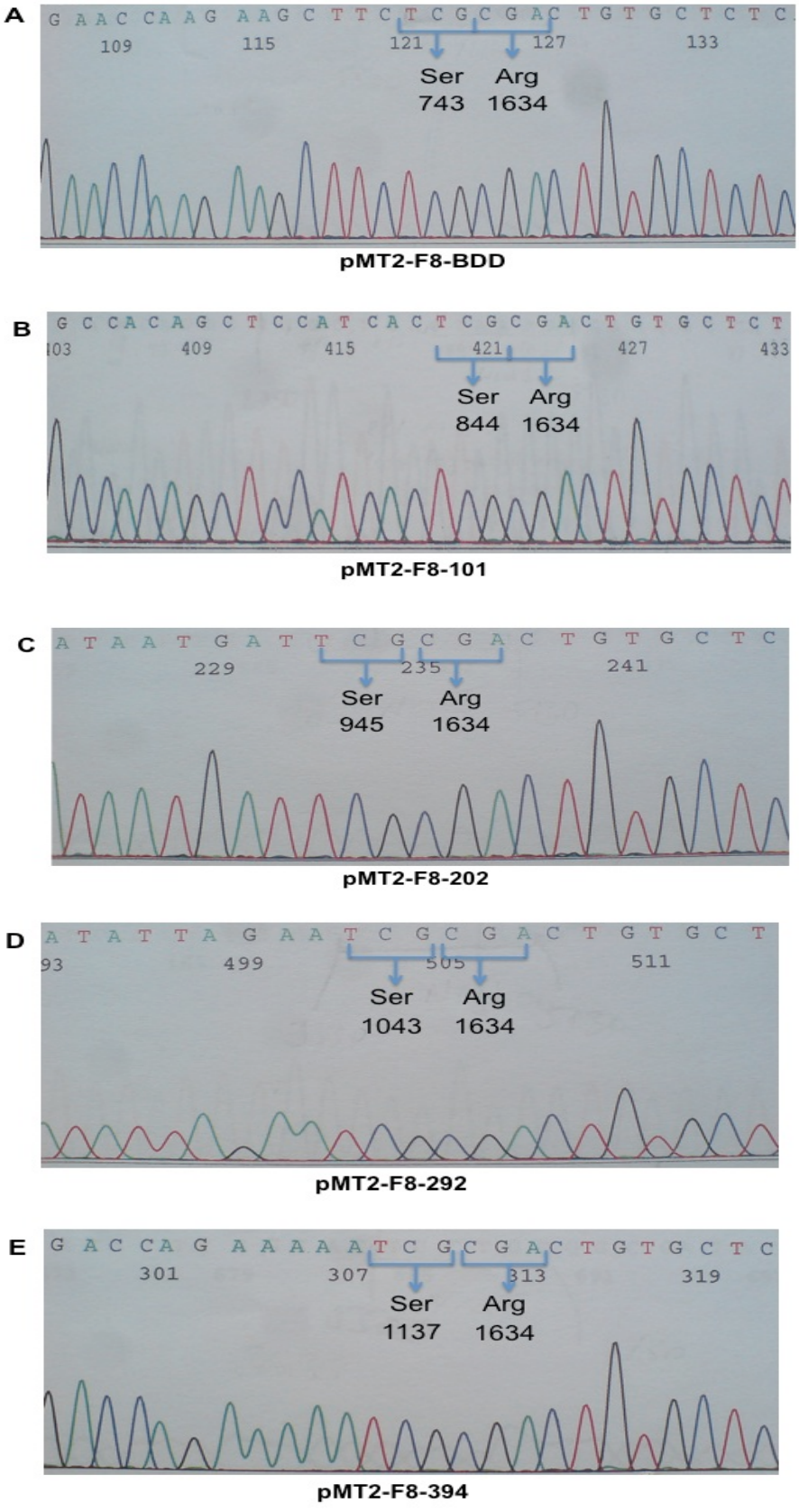

Figure 18: Representative DNA sequences of the different plasmids showing the DNA sequence across the deleted part of the B-domain region in each plasmid. A: pMT2-F8-BDD, B: pMT2-F8-101, C: pMT2-F8-202, D: pMT2-F8-292 and E: pMT2-F8-394.

3.1.2 Transient expression of different B-domain deleted plasmids in three mammalian cell lines

Transfection. The three cell lines were transfected by lipofection at 90% confluency. The transfection efficiency by Lipofectamine 2000 (Invitrogen) was checked by observing the intensity of the eGFP fluorescence 24 hours after transfection. The transfection with the eGFP containing plasmid (pEGFP-C1) was done in parallel along with the transfection of different B-domain deleted plasmids in the three cell lines (Fig. 19).

FVIII activity. Two one-day (24 hours) medium collections were performed: 48 and 72 hours after transfection. The media were clarified by centrifugation and assayed for factor VIII activity using the clotting assay (section 2.4.2.1).

Factor VIII activity (FVIII: C), given in percentage is shown for each cell line in figure 20 A, B and C. Within the two medium collections, the COS-7 cell line showed the highest expression level with an activity range of 20 to 56 % among all expressed plasmids, followed by HEK 293T with a range of 11.2 to 41.1 % FVIII: C and the CHO cell line with a range of 1.7 to 7.5 % FVIII: C. Negative controls (no DNA in the transfection mixtures) showed no factor VIII activity.

The first medium collection (48 hours after transfection) showed higher activity in HEK 293T and COS-7 cells for all the expressed plasmids while the second medium collection (72 hours after transfection) showed higher activity in CHO cells.

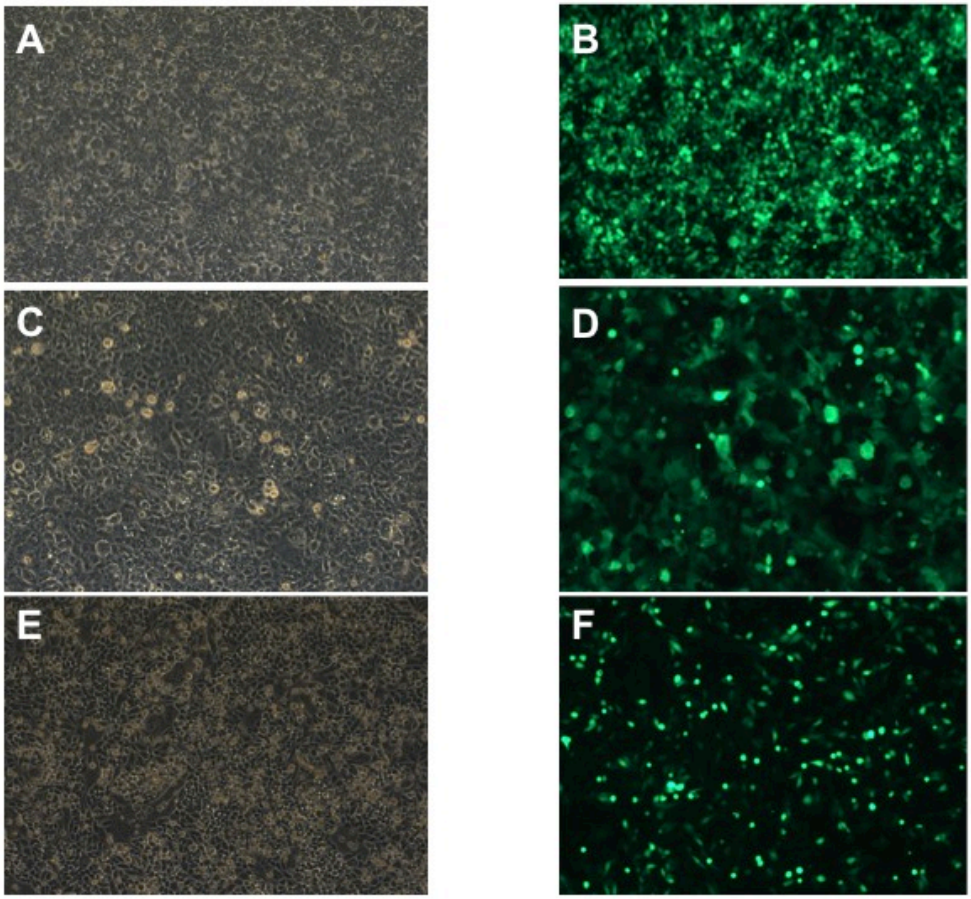

Figure 19: Lipofection efficiency as a transfection method in various cell lines used. A, C and E: Non-transfected negative control cultures of HEK 293T, COS-7 and CHO cell lines respectively. B, D and F: 24 hours pEGFP-C1 transfected cultures of HEK 293T, COS-7 and CHO cell lines respectively.

Activity levels for different constructs in different cell lines were compared in each medium collection (Fig. 20 D and E). In comparison to the BDD construct, the data showed that the FVIII activity levels are generally higher in the constructs containing added lengths of the B-domain in COS-7 and HEK 293T cells. Additionally, with the exception of the 292 construct, the different constructs showed an in increase in the FVIII: C with the increase in the B-domain length in both cell lines (COS-7 and HEK 293T).

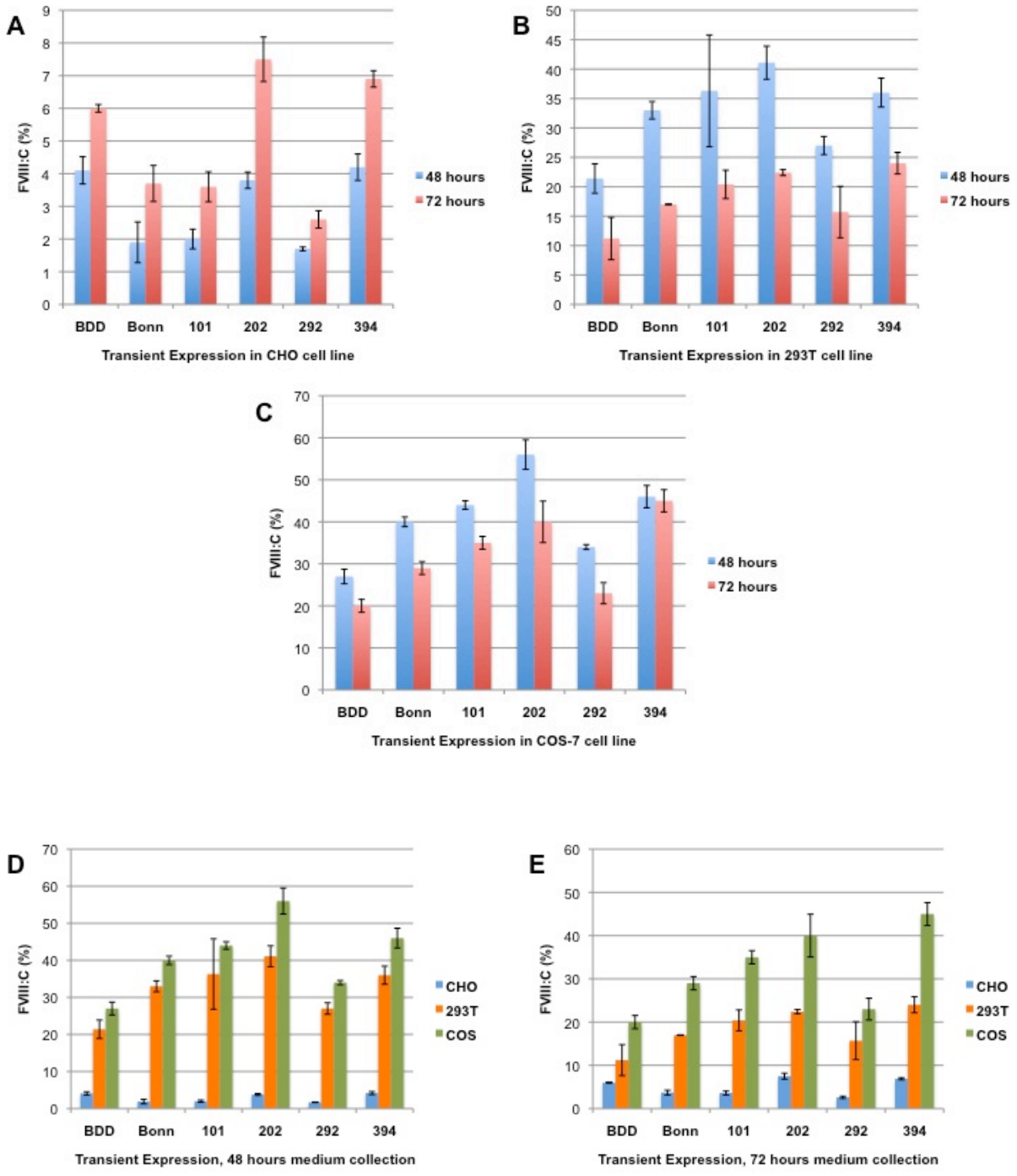

Figure 20: Transient expression of the different constructs in HEK 293T, COS-7 and CHO-DHFR[-] cell lines. A, B and C: FVIII: C in two medium collections for different constructs in CHO, 293T and COS-7 cells respectively. D and E: Activity levels for different constructs in different cell lines were compared for each medium collection. All data are means ± SD.

3.1.3 Functional studies on different B-domain deleted plasmids transiently expressed in COS-7 cells

The COS-7 cell line that showed the highest factor VIII expression (section 3.1.2) was used for further functional assessment of the different B-domain deleted plasmids. For this, COS-7 cells were transfected with the different constructs at 90% confluence. Conditioned medium and cellular lysates collected 48 hours after transfection were used for activity and antigen assessment. All activity and antigen values mentioned in this section are per one million cells per milliliter.

FVIII activity and antigen. Collected media were clarified by centrifugation and assayed for factor VIII activity using the clotting and the chromogenic assays (section 2.4.2). For factor VIII antigen determination, an ELISA assay was used (section 2.4.3). After medium collection, cells were washed directly with PBS, trypsinized, counted and then lysed. Lysates were only used for antigen quantification.

FVIII Activity levels of the two different assay methods are shown in figure 21 A. The clotting assay showed a slightly higher value than the chromogenic assay for all the constructs. However, for the BDD construct, the FVIII: C of chromogenic assay was higher than FVIII: C of clotting assay. As already seen before (3.1.2), the data show that the activity levels are higher in the constructs containing added lengths of the B-domain in comparison to the BDD construct. With the exception of the 292 construct, the different constructs showed an in increase in the FVIII: C with the increase in the B-domain length in both assays methods.

Antigen levels in both the conditioned medium and cellular lysates are shown in figure 21 B. For all expressed plasmids, the FVIII: Ag levels in the medium are much lower than in the lysates. With the exception of the 292 construct, the FVIII: Ag levels are higher in the expression of the constructs containing added lengths of the B-domain in comparison to the BDD construct in medium and lysates. The different constructs showed an increase in the FVIII: Ag with the increase in the B-domain length in the medium except for the 292 construct.

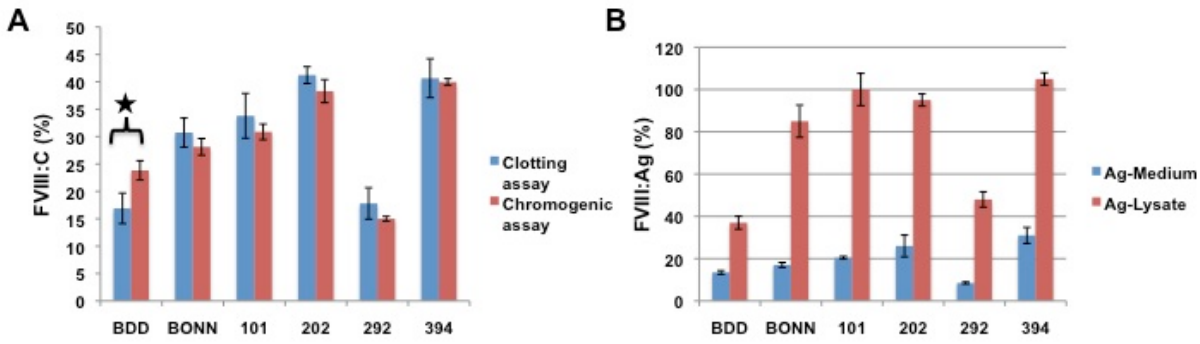

Figure 21: Activity and antigen levels of the different constructs expressed in COS-7 cell line for 48 hours. (A) FVIII: C levels in conditioned medium using the clotting and the chromogenic assays. (B) FVIII: Ag levels in medium and in cellular lysates. All data are means ± SD; *: p value < 0.05.

Specific activity. The functionality of the different constructs was evaluated by their specific activity. The specific activity was calculated by dividing the activity of each construct (measured by the clotting assay) by its corresponding FVIII: Ag value (measured by ELISA) in medium. Considering that normal plasma has an activity of one unit per milliliter and assuming that the one unit FVIII contains 0.15 µg FVIII protein per milliliter (FVIII concentration varies in different publications with a range of 100-300 ng / ml), then the optimal specific activity of FVIII is 6.67 U/µg.

Figure 22 A shows the calculated specific activities of FVIII expressed from different constructs. The data shows that all the constructs are functional with specific activities above the calculated optimal specific activity. The different constructs containing added lengths of the B-domain show higher specific activity in comparison to the BDD construct. Interestingly, the 292 construct that has the lowest FVIII: C and FVIII: Ag compared to the other constructs showed the highest specific activity.

Total expressed FVIII. The total expressed FVIII protein of the different constructs is measured as the sum of the quantity of protein secreted into the medium and that retained in cellular lysates (Fig. 22 B). Data indicate that the different constructs containing added lengths of the B-domain show the higher total protein quantity. With the exception of the 292 construct, the total expressed FVIII increased with the increase in the B-domain length.

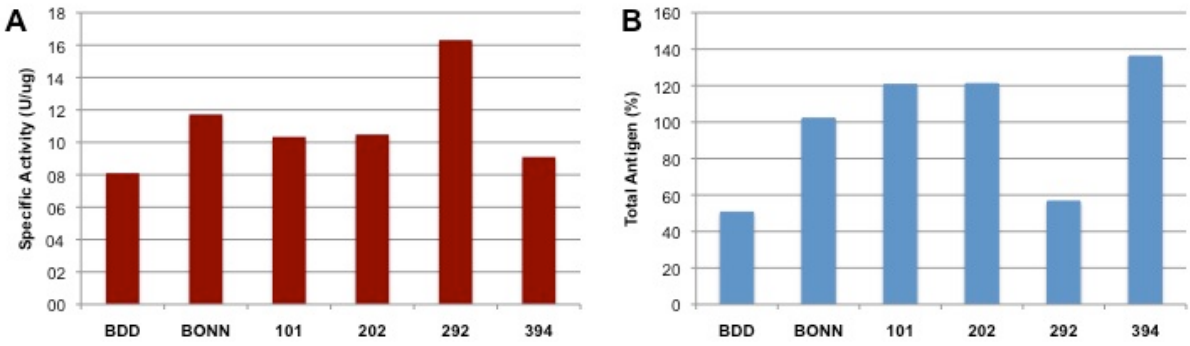

Figure 22: Specific activity of the different expressed FVIII proteins and the total expressed FVIII in COS-7 cells. (A) The specific activity of different constructs as unit per µg FVIII in conditioned medium. (B) The total expressed FVIII (in medium and cellular lysate) of the different constructs.

3.1.4 Generation of stable cell lines in CHO-DHFR[-] cells

Linearized, ethanol precipitated expression plasmids representing the different B-domain deleted constructs were transfected by lipofection into CHO-DHFR[-] cells (see section 2.3.3.1). Two days later, the cells were splitted.

Selection. Transfected cells were selected for plasmid uptake by culturing them in nucleoside deficient complete medium and in different concentrations of MTX. The medium was changed every second day until well-defined new colonies appeared 2-3 weeks after selection (see Fig. 23). New colonies were then pooled and assayed for FVIII: C upon confluency. As a result, only the CHO-BDD, CHO-202, CHO-394 and CHO-full-length cultures showed FVIII productivity at a certain MTX concentration upon selection (see table 9, selection part). Productive cultures were expanded into many dishes for storage, amplification and cloning.

Limited dilution cloning. Productive cultures from the four stable cultures were cloned by limited dilution cloning (see section 2.3.3.2). For this, minimums of three serially diluted 96-well plates were made for each stable culture. Approximately, 20 to 40 wells per construct were marked as having a single cell. Single cells were followed for two weeks until they reached full colonies. A maximum of 24 colonies arose from single cells were propagated into 24-well plates, each in a well until a confluent culture is reached. Conditioned medium was then collected and assayed for FVIII productivity.

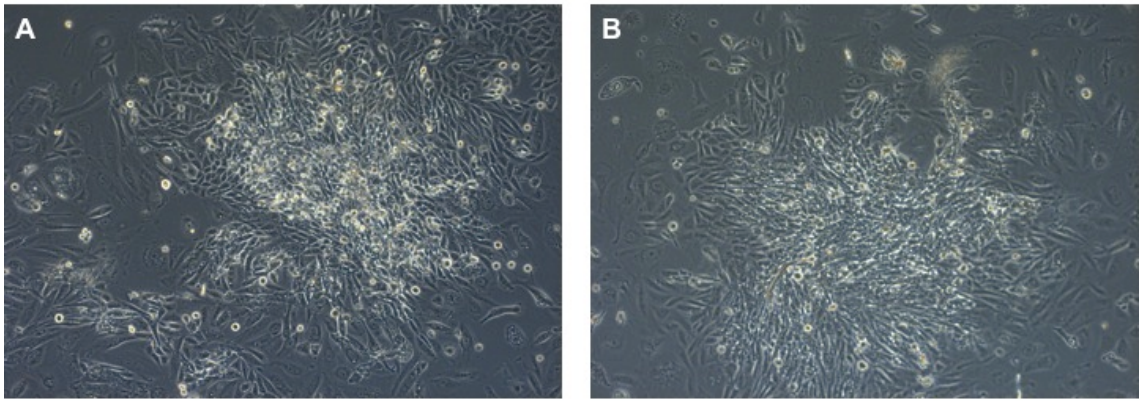

Figure 23: Examples of well-defined new colonies appeared 2-3 weeks after selection procedure. (A) A new colony appeared upon selection of pMT2-F8-BDD transfected cells. (B) A new colony appeared upon selection of pMT2-F8-394 transfected cells.

With this approach, a few productive clones were identified with increased FVIII: C for each stable culture. The highest identified productive clones (CHO-BDD clone F2, CHO-202 clone G3, CHO-394 clone F4 and CHO-Full clone C6) are listed in table 9 (cloning part) with their respective MTX concentration and FVIII: C. Productive clones were expanded for storage, amplification and sub-cloning.

Amplification. Clones that still show low FVIII: C after the cloning procedure (CHO-202 clone G3 and CHO-394 clone F4) was amplified (section 2.3.3.1). One amplification step (MTX concentration from 200 nM to 800 nM) for CHO-394 clone F4 was enough to increase the productivity more than four times (16% to 77%) and several amplification steps (MTX concentration from 400 nM to 6.0 μM) were required for CHO-202 clone G3 to increase the productivity more than 5 times (5% to 28%) (Table 9, Amplification). The new more productive cultures were also expanded, stored and further sub-cloned.

Sub-cloning. Sub-cloning was performed for moderately productive cell lines (CHO-BDD clone F2, CHO-202 clone G3 and CHO-394 clone F4) using the same limited dilution approach (see limited dilution cloning section) to guarantee the clonality of the new cell lines and to obtain a higher productive cell lines. The MTX concentration corresponds to the latest concentration used (in amplification or in cloning steps). Sub-cloning resulted in the following high productive cell lines: CHO-BDD clone F2H1, CHO-202 clone G3E6 and CHO-394 clone F4F3 (Table 9, Sub-cloning).

	Selection		Cloning			Amplification			Sub-cloning		
	MTX	Activity*	Clone	MTX	Activity*	Clone	MTX	Activity*	Clone	MTX	Activity*
CHO-BDD	200 nM	6 %	F2	200 nM	55 %	-	-	-	F2H1	200 nM	90 %
CHO-202	400 nM	2 %	G3	400 nM	5 %	G3	6.0 uM	28 %	G3E6	6.0 µM	50 %
CHO-394	200 nM	9 %	F4	200 nM	16 %	F4	800 nM	77 %	F4F3	800 nM	>150 %
CHO-Full	600 nM	264%	C6	600 nM	>250 %	-	-	-	-	-	-

Table 9: FVIII productivity at different stages of stable cell lines generation.

***** FVIII activity is per 1*106 cells per 24 hours.

3.1.5 Purification and thrombin activation of different recombinant FVIII proteins

The four high productive stable cell lines, CHO-BDD (clone F2H1), CHO-202 (clone G3E6), CHO-394 (clone F4F3) and CHO-Full (clone C6), were expanded in complete medium containing the corresponding MTX concentration. For each cell line, 300 ml medium was collected and ten folds concentrated. FVIII: C were measured after concentration using the clotting assay (section 2.4.2.1) and was > 300 % for all of them.

FVIII purification. FVIII protein in the concentrated medium (30-35 ml) was captured by FVIII-Select beads (section 2.4.1). Beads were then washed and FVIII was then eluted as batches by buffer B. The FVIII: C of the 8 collected batches for each cell line was tested and found to be above 300 % in all the collected batches from the four different cell lines.

Productive batches were then pooled, buffer exchanged and concentrated (section 2.4.1). Concentrated material (~ 250 µl) containing purified FVIII protein were tested for FVIII: C and FVIII: Ag (see tables 10 and 11).

Cell line	FVIII-BDD	FVIII-202	FVIII-394	FVIII-Full
FVIII: C	> 300 %	134 %	> 300 %	223 %

Table 10: FVIII activity (Clotting assay) of the purified products diluted 1:100 in FVIII deficient plasma.

Cell line	FVIII-BDD	FVIII-202	FVIII-394	FVIII-Full
FVIII: Ag	145 %	90 %	> 145 %	145 %

Table 11: FVIII antigen concentration of the purified products diluted 1:100 in buffer A.

FVIII activation by thrombin. Purified FVIII proteins were activated by thrombin (Factor IIa) and the A2 domain cleavage (see fig. 4) was assessed according to section 2.4.4. For this, a master mixture for ten reactions (1 U FVIII, 0.5 nM FIIa and 1x FIIa buffer per reaction) was prepared at 37 °C. Aliqoutes were collected every 15 seconds and reactions were stopped by mixing them with sample loading buffer directly. Samples were then heat denatured and run over PAGE, western blotted against PVDF membrane and detected by an antibody against the A2 domain. Figure 24 show the western blots of the A2 generation for the purified FVIII- BDD and FVIII-394 proteins. The rate of A2 generation was calculated as a percentage by dividing the generated A2 domain band density (integrated density value) by the total A2 density in the reaction (A2 domain band, A1+A2 domains band and any undigested FVIII material). Data in figure 25 show that the BDD construct is slightly faster activated as compared to the 394 construct, since it showed higher A2 generation at point zero and reached a 50% A2 generation in a shorter time.

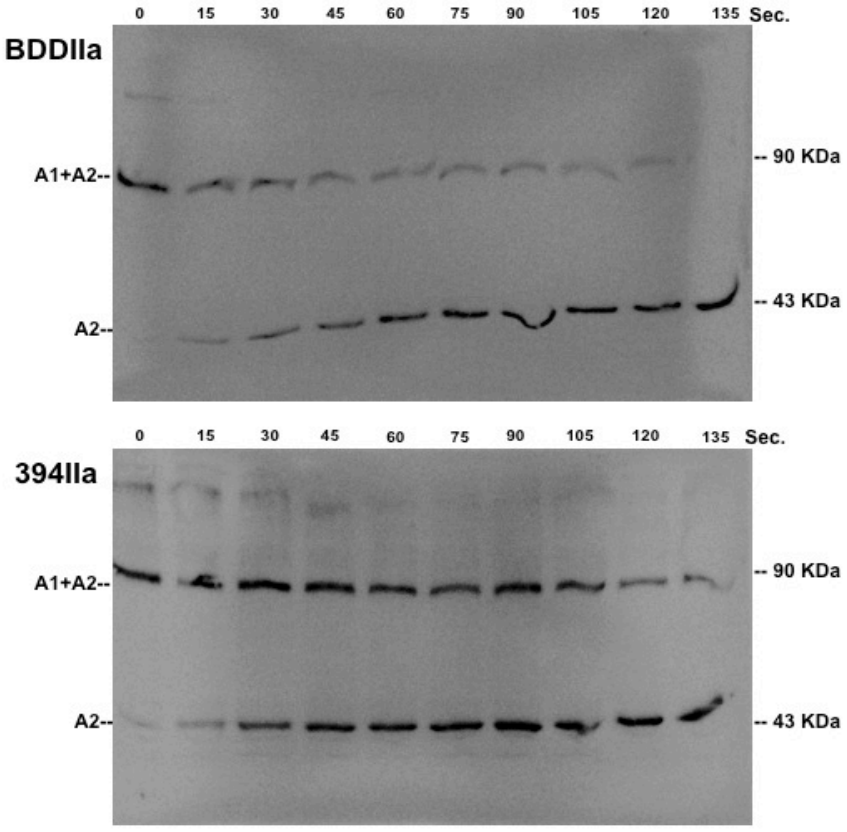

Figure 24: Western blots demonstrating A2 generation of purified FVIII-BDD and FVIII-394 proteins at different time points after thrombin digestion.

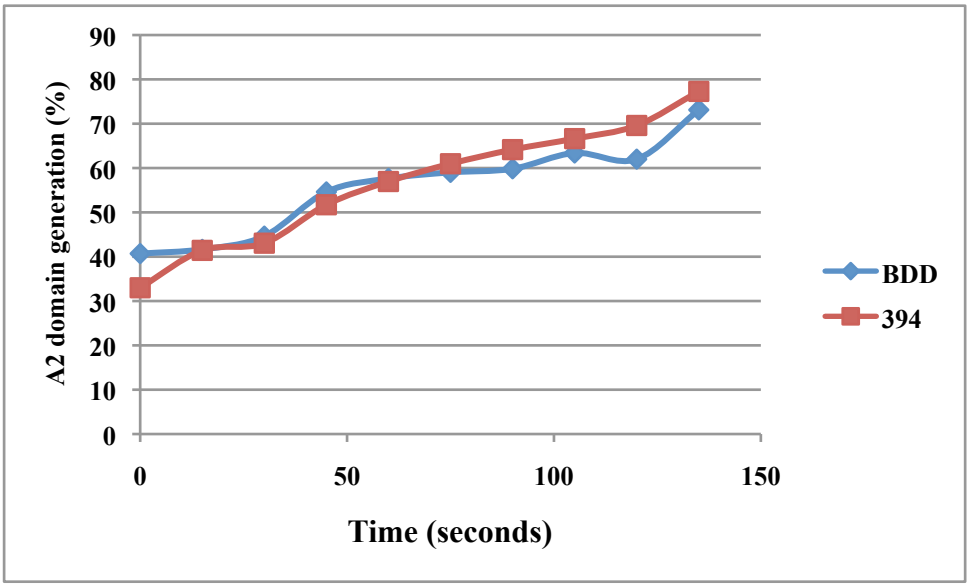

Figure 25: Rate of A2 domain generation measured in 15-second intervals. The BDD construct shows a higher A2 generation at point zero and over time a faster A2 generation than the 394 construct.

3.2 Construction of a new helper vector for the production of helper-dependent adenovectors for the use in hemophilia gene therapy

3.2.1 Construction of the plasmid pBac-AAV2-Ad5

The shuttle plasmid pBac-AAV2-Ad5LR (~8777 bp) was generated by introducing an AAV2- 5'ITR / Ad5LR / AAV2-3'ITR construct into the basic plasmid pFastBac-1 (see section 2.2.10). The shuttle plasmid was then linearized with PmeI (Fig. 26 A), purified, dephosphorylated and then mixed with pAdEasy-1 containing the adenoviral genome from 3.5 to 36 kb (Fig. 26 B) in BJ8153 electrocompetent cells. The cells were then electroporated. Electroporation resulted in the introduction of the Ad5-genome into the shuttle plasmid by homologous recombination and the generation of pBac-AAV2-Ad5 (~35824 bp). Figure 26 C shows a mini prepared DNA of several picked pBac-AAV2-Ad5 clones from the electroporation plates. Possibly correct clones are those that show a super coiled and a relaxed band above the marker level (above the 12.2 kb band), which reflect a size of ~35 kb. Those clones were further characterized by restriction enzyme analysis. Clones that showed smaller size fragments (lower than 12.2 kb band) were neglected (those clones are marked by an x in fig. 26 C). Possibly correct clones were stabilized by transforming them into XL10-Gold bacteria; DNA was then mini prepared (Fig. 27 A).

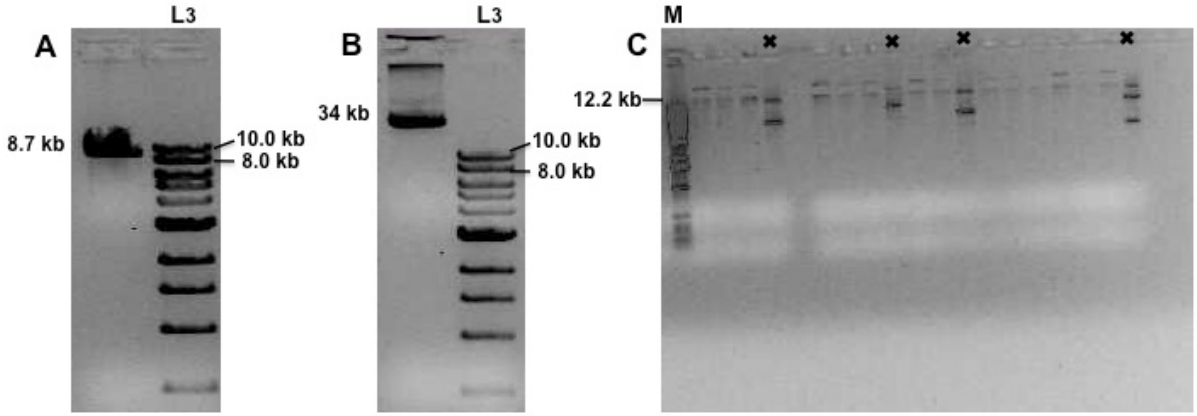

Figure 26: Cloning of pBac-AAV2-Ad5. (A) Linearization of the shuttle plasmid pBac-AAV2-Ad5LR (~8.7 kb). (B) pAdEasy-1 (~34 kb). (C) Mini prepared clones picked from the electroporation plates. Lanes labeled with **x** stands for wrong clones (don't have the expected size). L3: 1kb DNA ladder (0.5 – 10 kb; New England Biolabs); M: DNA molecular weight marker X (0.07 – 12.2 kb; Roche).

Clones with correct size (clones 1,2,4,5and 6 in Fig. 27 A) were further characterized by restriction analysis and DNA sequencing of the adenoviral construct borders. Digestion with SmaI (which should give the following fragments: 6447, 11, 195 [5'-ITR], 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230, 652, 2278, 2262, 550 [3'-ITR] and 11 bp) confirmed the presence of the Ad5 genome insert, however showed a deletion in the 3'ITR region. Normally, SmaI should cut twice in each ITR region, however the loss of its cut sites in the 3'ITR (positions 34153 and 34164 in the pBac-AAV2-Ad5 genome) resulted in joining the fragments 6447,550 and 11 bp in one band: 7008 bp (Fig. 27 B).

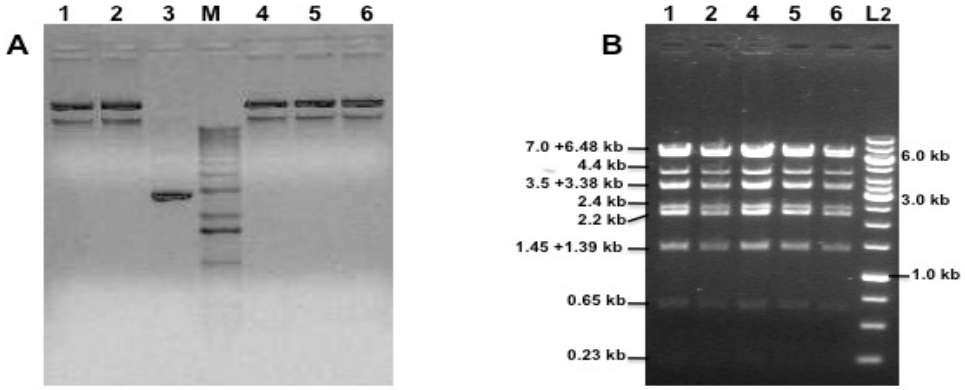

Figure 27: Characterization of pBac-AAV2-Ad5. (A) DNA mini preparation of selected clones after transforming the possible correct pBac-AAV2-Ad5 clones into XL10-Gold bacteria. (B) SmaI map of pBac- AAV2-Ad5 clones that shows the absence of 6447, 550 and 11 bp fragments and the presence of a 7 kb band instead. M: DNA molecular weight marker X (0.07 – 12.2 kb; Roche). L2: Gene Ruler 1kb DNA ladder (0.25 – 10 kb; Fermentas).

DNA sequencing across the 5'ITR region (using the primer Ad5-4231a, see section 2.2.10) revealed an intact 5'ITR. DNA sequencing across the 3'ITR region using the primer Ad5-35687s confirmed a 3'ITR deletion from nt 34120 until 34249 (Fig. 28).

pBac-EGFP and pBac-AAV2-Rep were constructed according to the cloning strategy outlined in section 2.2.10. Expression of the AAV2-Rep gene was tested in both Sf9 and 293T cells. SF9 cells were transfected with pBac-AAV2-Rep for 48 hours and total RNA was then extracted (see section 2.2.2 and 2.2.10). Total RNA was subject for RT PCR using Superscript III reverse transcriptase. Taq-PCR was performed using the primer pairs AAV2-2310a / AAV2-1380s and AAV2-2310a / AAV2-321s. Both gave the expected PCR product of 1.0 kb and 2.0 kb, respectively (Fig. 29 lanes 2 &3 and 5 & 6). 293T cells were transfected with pBac-AAV2-Rep and pDG (as a positive control for Rep gene expression) plasmids for 48 hours and total RNA was then extracted (see section 2.2.2 and 2.2.10). Again, total RNA was subjected for RT PCR using the primer pairs AAV2-2310a / AAV2-1380s and AAV2-2310a / AAV2-321s. Both gave the expected product of 1.0 kb and 2.0 kb, respectively (Fig. 29 lanes 8 & 9 for pDG and 10 & 11 for pBac-AAV2-Rep using the first primer pair and lanes 13 & 14 for pDG and 15 & 16 for pBac-AAV2-Rep using the second primer pair).

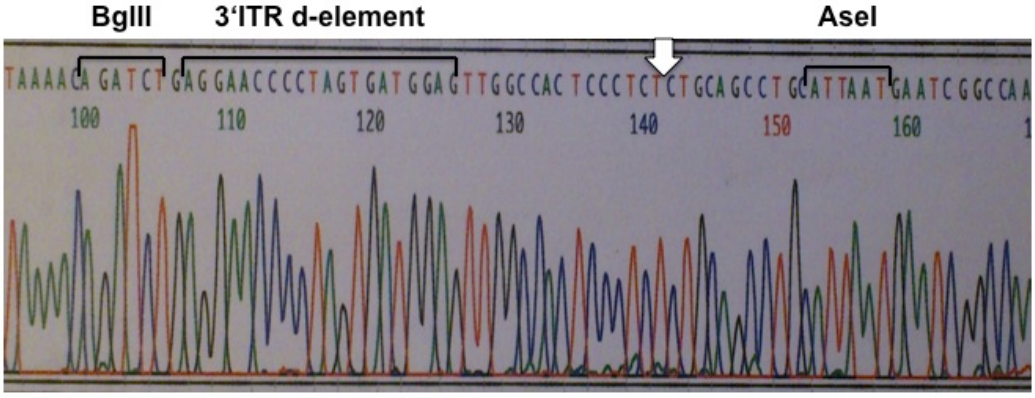

Figure 28: DNA sequence of pBac-AAV2-Ad5 3'ITR region. The white arrow indicates the deletion place where the pBac-AAV2-Ad5 sequence stopped at position 34121 and continued at position 34250.

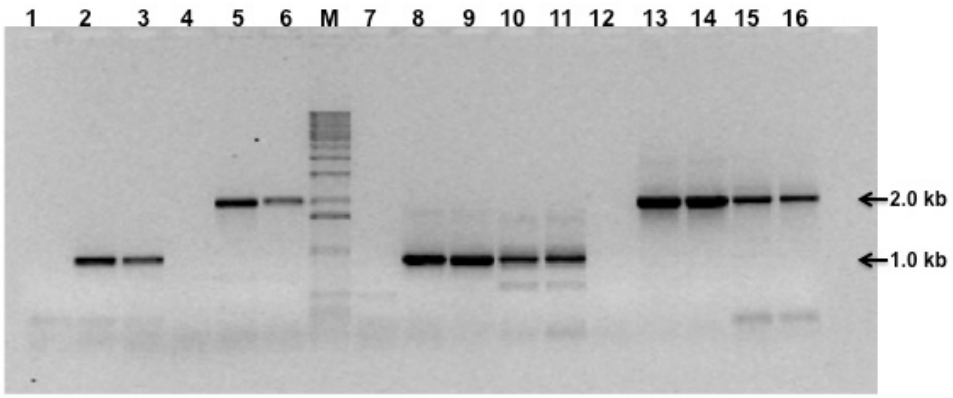

Figure 29: Testing the expression of the AAV2-Rep gene in SF9 and 293T cells. RT-PCR for Rep expression in SF9 cells (lanes 1 to 6) and in 293T cells (lanes 7 to 16). Lanes 1,4,7 and 12 are negative controls. M: DNA molecular weight marker X (0.07 – 12.2 kb; Roche).

3.2.2 Generation of the bacmid bBac-AAV2-Ad5

Recombinant bacmids were generated by site-specific transposition in DH10Bac cells that contain the baculovirus shuttle vector (bacmid). The **p**lasmids **p**Bac-AAV2-Ad5, **p**Bac-EGFP and **p**Bac-MMTV-bGH-AAVRep served as shuttle vectors to generate the recombinant **b**acmids **b**Bac-AAV2-Ad5, **b**Bac-EGFP and **b**Bac-AAV2-Rep respectively in DH10Bac cells according to the protocol detailed in section 2.2.10.

Recombinant bacmid DNA was isolated according to Bac-to-Bac Baculovirus Expression System manual (section 2.2.1). Figure 30 A shows six selected bBac-AAV2-Ad5 clones. Those clones were characterized by Taq-PCR analysis. When we amplified them with M13 forward and reverse primers (which cover all the insert region), no product could be seen (Fig. 30 B) which reflects the presence of our expected very large insert within the bacmid DNA. After amplifying them with M13 forward / Ad5-4231a primers we could see a ~3.1 kb product (Fig. 30 D left) which confirms the presence of the 5'ITR end. The amplification of the 3'ITR-end (primers Ad5-35687s / M13 reverse) resulted in a ~1.55 kb product (Fig. 30 D right) which confirms the presence of the 3'ITR end. Figure 32 C shows an example of empty bacmids where amplification by M13 forward and reverse primers resulted in a 273 bp product. bBac-EGFP was used as a positive control for M13 forward and reverse amplifications (marked P; Fig. 30 B and C) and resulted in a product of ~ 3.6 kb.

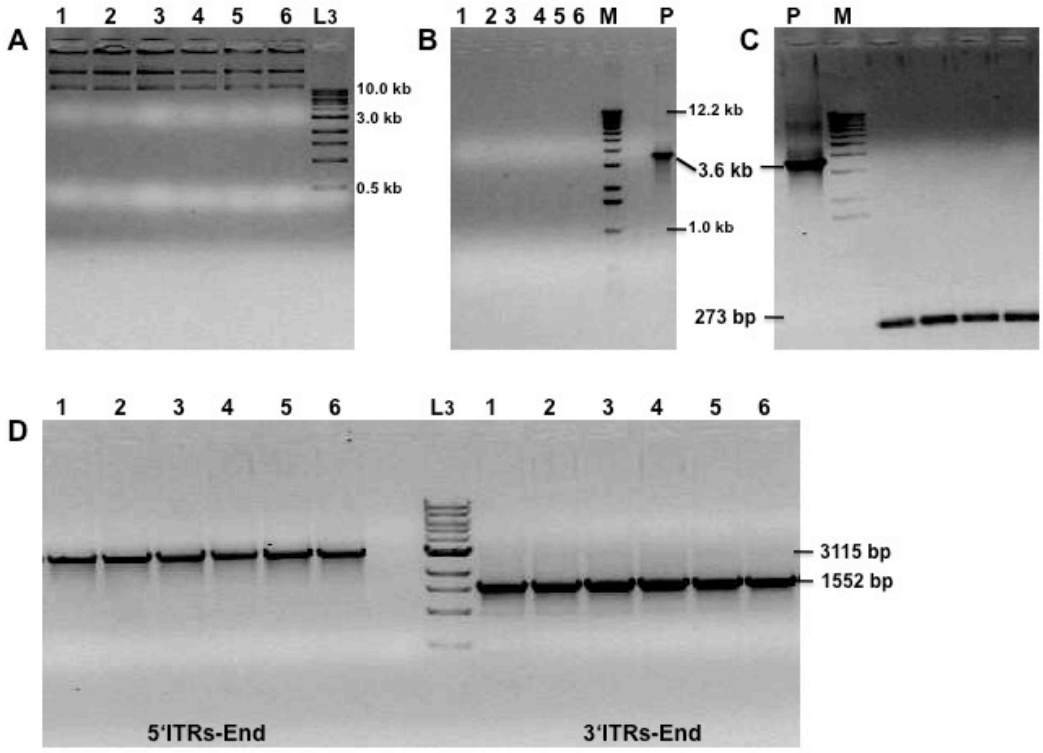

Figure 30: Characterization of bBac-AAV2-Ad5 by Taq-PCR. (A) Isolated bacmid DNA from a selected bBac-AAV2-Ad5 clones. Those clones were characterized by Taq-PCR using M13 forward and reverse primers (Image B), M13 forward / Ad5-4231a primers (Image D left) and Ad5-35687s / M13 reverse primers (Image D right). (C) Is an example of empty bacmids. P: bBac-EGFP as a positive control for M13 forward and reverse amplifications. L3: 1kb DNA ladder (0.5 – 10 kb; New England Biolabs); M: DNA molecular weight marker X (0.07 – 12.2 kb; Roche).

bBac-AAV2-Rep was also characterized by Taq-PCR. Amplifying 10 selected clones by M13 forward and reverse primers resulted in a 5.3 kb PCR product in clones 1,2,3,5,6,7,8 and 9 and a 273 bp PCR product in clones 4 and 10 (Fig. 31 A). While the 5.3 kb product confirms the existence of an MMTV(promoter)-AAVRep insert in the bacmid DNA, the 273 bp product indicates empty clones. To further confirm the presence and the integrity of the MMTV(promoter)-AAVRep insert, the 5'-end of the insert were amplified with M13 forward / MMTV-1347a primers and the 3'-end were amplified with AAV2-321s / M13 reverse primers. Both resulted in the expected 2.3 kb and 2.8 kb PCR products in the same positive clones respectively (Fig. 31 B & C).

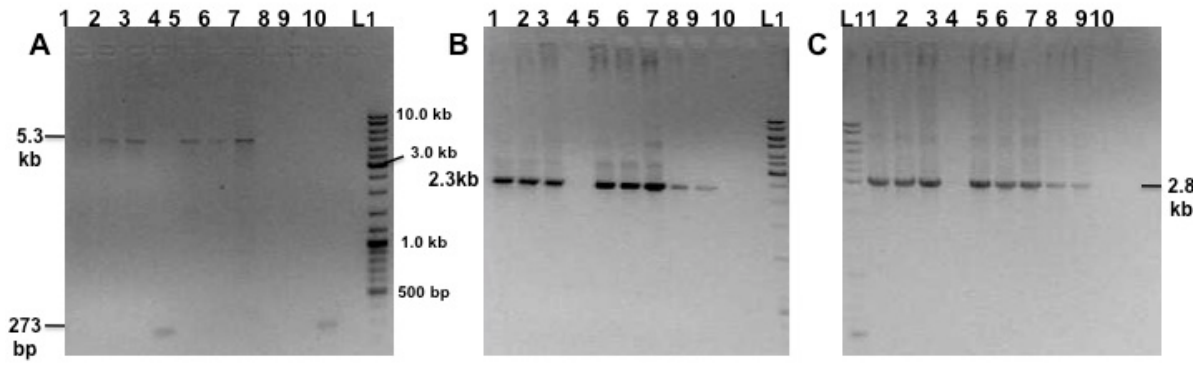

Figure 31: Characterization of bBac-AAV2-Rep by Taq-PCR. Ten isolated bBac-AAV2-Rep clones were characterized by Taq-PCR using M13 forward and reverse primers (Image A), M13 forward / MMTV-1347a primers (Image B) and AAV2-321s / M13 reverse primers (Image C). L1: Gene Ruler DNA ladder mix (0.1 – 10 kb; Fermantas).

3.2.3 Generation of helper recombinant baculovirus in insect cells

3.2.3.1 Transfection of SF9 cells with bacmid DNA and collection of P1 viral stock

SF9 cells (Fig. 32 A) were seeded at 9 * 10^5 cells per well in 6 well plates. After attachment, they were transfected with 1.0 µg purified bacmid DNA (bBac-AAV2-Ad5 and/or bBac-AAV2-Rep) according to section II.3.4.1. Two days after transfection, SF9 cells showed an increase in cellular diameter. Three days after transfection, SF9 cells showed a cessation of growth, cellular swelling and granular appearance which indicates viral budding (Fig. 32 B, C). One day later, cells show massive detachment and lysis.

Medium were collected on the fourth day after transfection in sterile tubes, centrifuged and the clarified supernatant containing the virus was then transferred into a fresh tube and labeled as P1 **v**iral stock for **v**Bac-AAV2-Ad5 as well as for **v**Bac-AAV2-Rep.

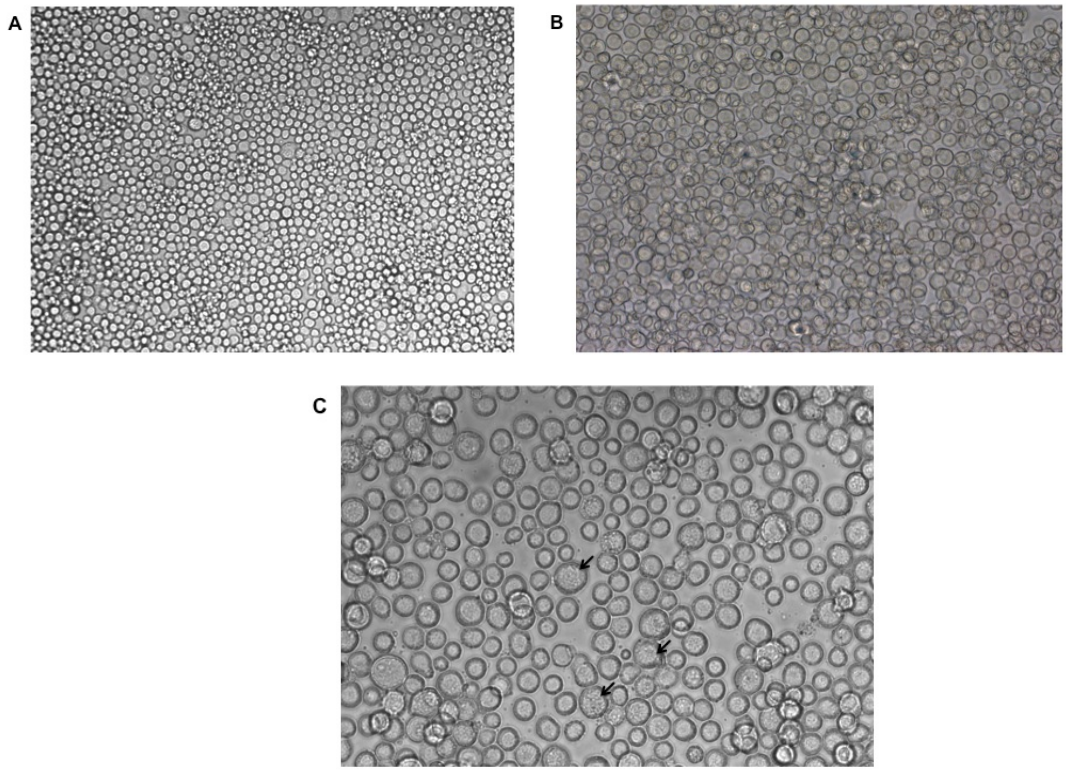

Figure 32: Transfection of SF9 cells: (A) confluent wild type SF9 culture (10x magnification). (B) SF9 cells three days after transfection with bBac-AAV2-Ad5 (10x magnification). The cells show cellular swelling and granular appearance. (C) A 20x magnification of a part of foto (B) where arrows indicate cells with granulation due to viral budding.

3.2.3.2 Amplification of P1 viral stock

To obtain a higher titer ranging from $1 * 10^7$ to $1 * 10^8$ pfu/ml, P1 viral stock was amplified in SF9 cells. 0.04 ml P1 viral inoculum were used to infect SF9 cells seeded at $2 * 10^6$ cells per well in 6-well plates. Two days after infection, SF9 cells showed all the signs of infection (Increased cell diameter, cessation of growth, granular appearance, detachment and cell lysis). Medium containing the virus was collected, pelleted and the clarified supernatant was transferred into a fresh tube labeled P2 viral stock.

3.2.3.3 Viral purification: Plaque assay

Clarified baculoviral stock P2 (from vBac-AAV2-Ad5 and vBac-AAV2-Rep) was diluted to an 8-log serial dilution and 1.0 ml inoculum was used to infect wild type SF9 cells seeded at $1 * 10^6$ cells per well in 6-well plates for one hour (see section 2.3.4.3). The medium was then replaced by a plaquing medium and plates were incubated until plaques formation.

Ten days later, plaques were visible at all viral dilutions wells (Fig. 33) and countable only at the highest dilution (10^{-8}). Infection with vBac-AAV2-Ad5 P2 viral stock resulted in the appearance of 50 plaques at 10^{-8} dilution, which reflects a viral titer of $5 * 10^9$ pfu/ml. Infection with vBac-AAV2-Rep P2 viral stock resulted in the appearance of 40 plaques at 10^{-8} dilution, which reflects a viral titer of $4 * 10^9$ pfu/ml. The titer of the viral stock was then calculated according to the following formula:

$$\text{Titer (pfu/ml)} = \text{number of plaques} * \text{dilution factor} * \frac{1}{\text{ml of inoculum / well}}$$

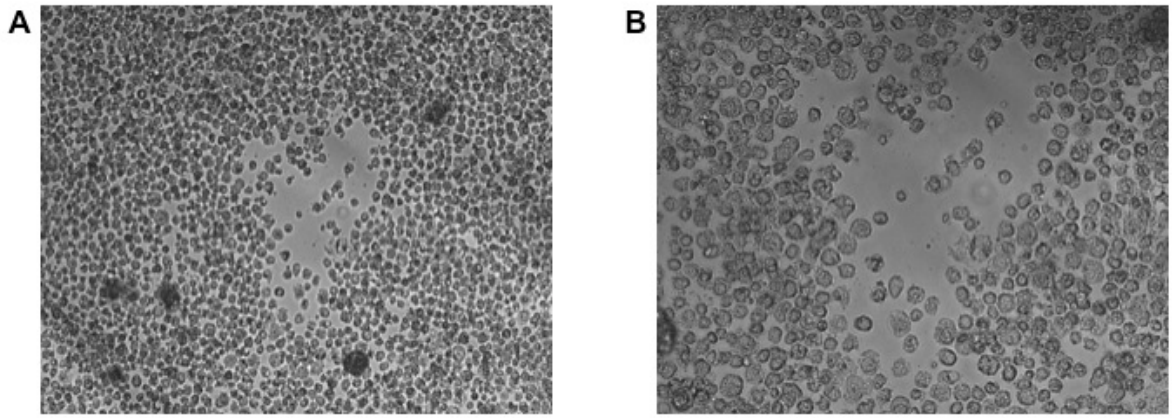

Figure 33: Palques of vBac-AAV2-Ad5 in SF9 cells. (A) 10 days after infection with vBac-AAV2-Ad5 (10x magnification). (B) Is a 20x magnification of the plaque in photo A.

A viral stock from single viral clones was generated by plaque purification. Clear well separated plaques were picked (20 plaques for vBac-AAV2-Ad5 and 10 for vBac-AAV2-Rep) and each agarose plug was transferred separately into 500 µl Grace's medium, mixed and used to infect one well of SF9 cells seeded at $1 * 10^6$ cells per well in 6-well plates. Three days later, the plaque purified viral stock was collected and then amplified according to section 2.3.4.2.

3.2.3.4 PCR analysis of the purified virus

Purified viral DNA was extracted from the purified amplified viral stock using the extra chromosomal DNA isolation protocol (see section 2.2.1). Two viral DNA clones (originating from two different plaques) from vBac-AAV2-Ad5 as well as from vBac-AAV2-Rep were verified by Taq-PCR.

Amplification of vBac-AAV2-Ad5 clones (plaques 9 and 16 in Fig. 34 A) with M13 forward and reverse primers that cover the whole insert region resulted in no product (Fig. 34 A, a), which reflects the presence of our very large insert (Ad5 genome) within the baculoviral genome. Amplifying them with M13 forward / Ad5-4231a primers resulted in a ~3.1 kb product (Fig. 34 A, b) which confirms the presence of the 5'ITR region (the 5' end of the insert). Amplifying them with Ad5-35687s / M13 reverse primers resulted in a ~1.55 kb product (Fig. 34 A, c) which confirms the presence of the 3'ITR region (the 3' end of the insert). The two clones were also amplified with Ad5-4000xs / Ad5-7000a primers (cover part of the Ad5 sequence) which gave a 3.0 kb band (Fig. 34 A, d) and further confirmed the presence of the Ad5 insert.

vBac-AAV2-Rep clones (plaques 1 and 5 in Fig. 34 B) were also characterized by Taq-PCR. Amplification with M13 forward and reverse primers that cover the whole insert region and resulted in a 5.3 kb PCR product (Fig. 34 B, a) which confirms the existence of our MMTV-AAVRep insert in the viral DNA. PCR amplification with M13 forward / MMTV-1347a primers resulted in a 2.3 kb PCR product which double confirms the 5'-end of the insert (Fig. 34 B, b). Amplification with AAV2-321s / M13 reverse primers that cover the 3' end of the insert further confirmed the existence of an intact MMTV-AAVRep insert and resulted in a 2.8 kb PCR product (Fig. 34 B, c).

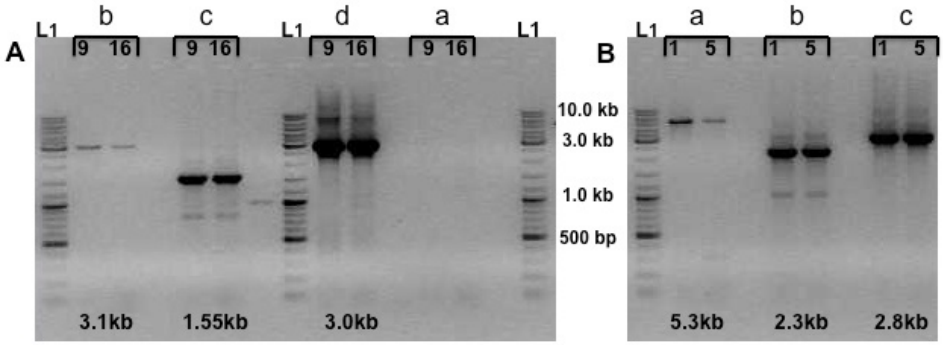

Figure 34: Characterization of vBac-AAV2-Ad5 and vBac-AAV2-Rep by Taq-PCR. (A) Two clones of vBac-AAV2-Ad5 were amplified by Taq-PCR using M13 forward and reverse primers which cover the whole insert region (a), M13 forward / Ad5-4231a primers which cover the 5' end of the insert (b), Ad5-35687s / M13 reverse primers which cover the 3' end of the insert (c) and Ad5-4000xs / Ad5-7000a primers which cover part of the Ad5 sequence (d). (B) Two vBac-AAV2-Rep clones were characterized by Taq-PCR using M13 forward and reverse primers which cover the whole MMTV-AAVRep insert (a), M13 forward / MMTV-1347a primers which cover the 5' end of the insert (b) and AAV2-321s / M13 reverse primers which cover the 3' end of the insert (c). L1: Gene Ruler DNA ladder mix (0.1 – 10 kb; Fermantas).

3.2.4 Rescuing the helper dependent adenovirus in HEK 293T cells

The ability of the baculovirus to infect HEK 293 was tested. Confluent HEK 293 cells were infected with 200 µl vBac-EGFP P2 viral stock once for one hour at 37 °C and once for four hours at 28 °C (see infection protocol, section 2.3.5). One day after infection, cultures were observed under UV light and found to be efficiently infected in both conditions as can be seen by GFP fluorescence (Fig. 35).

HEK 293 cells were transfected at 90% confluence with a linearized mini-Ad5 helper dependent vector (pAd5-AAT-hf8), which contains the human factor VIII cDNA, by lipofection in 6-well plates. Cells were then infected with vBac-AV2-Ad5 clones at 2000 and 500 MOI in combination with vBac-AAV2-Rep particles at 200 MOI as detailed in table 8. One well was infected only with vBac-AAV2-Rep as a negative control for infection. Infection mixtures were incubated with cells for 5 hours and medium was changed then.

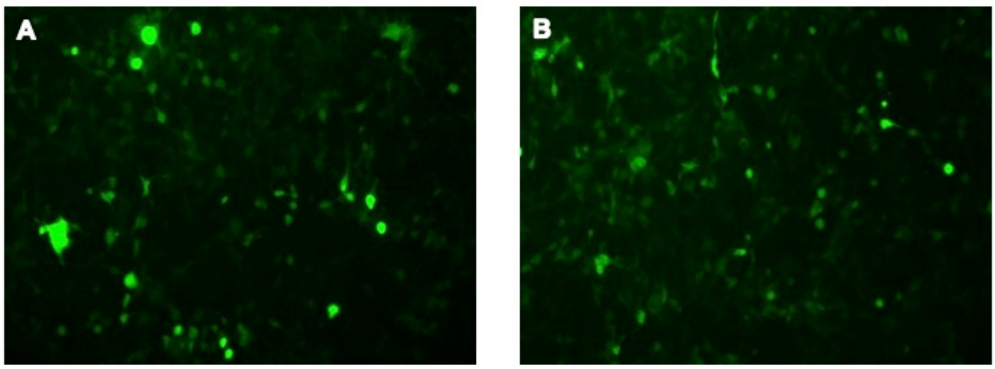

Figure 35: The ability of the baculovirus to infect HEK 293 cells. A: HEK 293 cells infected with vBac-EGFP for one hour at 37 °C. B: HEK 293 cells infected with vBac-EGFP for four hours at 28 °C.

Four days after infection (five days after transfection), medium was collected and cells were washed with PBS and lysed by four rouds of freeze / thaw cycles. Medium was concentrated 10 times, assayed for FVIII: Ag and showed no FVIII antigen in all wells. Extra chromosomal DNA was isolated from cellular lysates according to section 2.2.1. Gel elctrophoresis of 2 µl of the extracted extra chromosomal DNA showed a fragment representing the size of a linearized mini-Ad5 helper dependent vector in all the wells (Fig. 36, lanes marked a).

To decide whether the extracted extra chromosomal DNA resulted from the original introduced plasmid or has been produced inside HEK 293 cells under the support of our helper hybrid virus, the extra chromosomal DNA was digested with DpnI. Gel electrophoresis of 10 µl DpnI digested DNA showed the disappearance of the extra chromosomal DNA fragment (Fig. 36, lanes marked b). Cellular lysates were used for a second round of infection (according to section 2.3.5). No signs of viral production were seen over four days. Cells were then lysed and lysates were used for extra chromosomal DNA isolation. DpnI digestion showed the same result. Lysates (from the second round of infection) were used for a third round of infection that showed the same result.

The disappearance (digestion) of the extra chromosomal DNA after DpnI digestion indicates that it was all from bacterial origin and our designed helper hybrid virus (vBac-AAV2-Ad5 in combination with vBac-AAV2-Rep) could not support an in vivo production of the helper dependent vector.

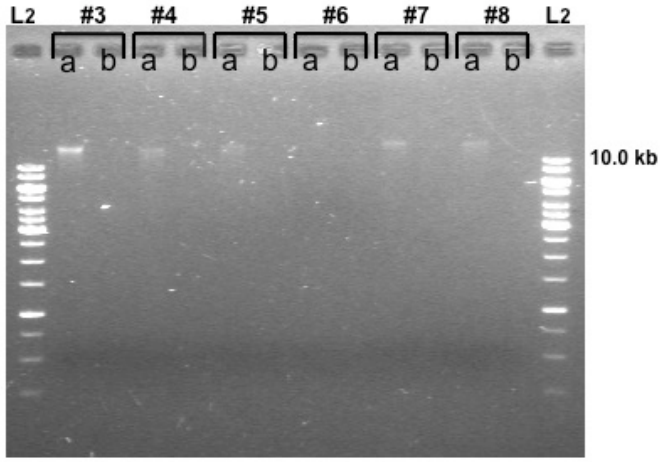

Figure 36: DpnI digestion of extra chromosomal DNA. Extra chromosomal DNA was isolated from cellular lysates (lanes marked a) representing different infection wells (wells 3à8 according to table 8). DpnI digestion showed no product indicating totally degraded material (lanes b). L2: Gene Ruler 1kb DNA ladder (0.25 – 10 kb; Fermantas).

4. Discussion:

4.1 Functional studies on factor VIII protein variants containing different lengths of the B-domain

Haemophilia A is an X-linked hereditary deficiency of functional coagulation factor VIII (FVIII). In the absence of treatment, patients with severe haemophilia A (plasma levels of FVIII below 1% of normal) will suffer repeated bleeds, often in muscles and joints. To prevent this development, patients with severe haemophilia A are treated with recombinant or plasma-derived FVIII concentrates (Christiansen *et al.,* 2010). Recombinant FVIII has become the preferred form of factor replacement for hemophilia A patients as it eliminates the risk of transmission of human blood-borne infections (Rogoff *et al.,* 2002; Key and Negrier, 2007). Current developments in rFVIII technology have enabled the production of rFVIII devoid of any human protein exposure during the cell culture fermenting process and in final formulation (Mitterer *et al.,* 2003).

Two forms of recombinant FVIII are currently manufactured: full-length recombinant FVIII (FL-rFVIII) and B-domain-deleted recombinant FVIII (BDD-rFVIII). While BDD-rFVIII is effectively used in the treatment of haemophilia A patients, recent experimental and clinical studies revealed a number of issues related to its equivalency to pd-FVIII (Gruppo *et al.,* 2003; Lollar 2003; Mikaelsson *et al.,* 2001). In particular, it was shown that the activity of BDD-rFVIII determined by one-stage clotting assay is up to 50% lower than that determined by chromogenic assay, thus complicating determination of BDD-rFVIII activity in haemophilia A patient's blood (Barrowcliffe *et al.,* 2002). The meta-analysis of the data accumulated during long-term clinical usage of BDD-rFVIII revealed its lower efficiency in prophylaxis, giving a higher incidence of bleeding in comparison with full-length FVIII (Gruppo *et al.,* 2003 and 2004).

In this study, we evaluated the importance and the effect of the B domain region on FVIII expression and function by constructing factor VIII expression plasmids containing different lengths of the B domain. Those plasmids were expressed in mammalian cells and expression results were compared to the expression of a B-domain deleted factor VIII plasmid. For transient expression, three mammalian cell lines were transfected with the different constructs. For stable expression, we used CHO-DHFR[-] cell line. FVIII activity was

assessed in culture medium and FVIII antigen was assessed in culture medium and cellular lysates. FVIII proteins were purified from the produced cell lines that stably express FVIII.

As the B domain comprises approximately 40% of the FVIII molecule and is located within the FVIII gene (exon 14), a complex cloning strategy was adopted in constructing those plasmids. The pMT2-FVIII vector containing the full length FVIII cDNA was used as a starting material. This vector contains a strong mammalian expression cassette and a gene for selection and amplification (DHFR). To construct the various expression plasmids, pMT2-FVIII was digested with RE to remove a large fragment within the FVIII cDNA representing part of the heavy chain, all the B domain and part of the light chain (from nt 1695 to nt 7302 in the vector which corresponds to aa 195 to 2064 in the protein). The end of the B domain (aa 1634 to 1648) along with light chain part was then returned by a mutagenesis PCR to create the intermediate vector pMT2-FVIII-SNA. Fragments starting from position 1695 (aa 195) and stopping at different serine positions in the B domain (aa 743, 844, 945, 1035 and 1137) were then amplified by a mutagenesis PCR using the pMT2-FVIII as template and cloned into the intermediate vector pMT2-FVIII-SNA. With this cloning strategy we have successfully constructed the expression plasmids: pMT2-F8-BDD, pMT2-F8-101, pMT2-F8-202, pMT2-F8-292 and pMT2-F8-394 (see appendix). The correct cloning was then confirmed by restriction enzyme analysis and DNA sequencing (Fig. 17 and 18).

To evaluate our tranfection method (Lipofection) and to select an optimal cell line to use for the functional studies, we have transiently expressed the different plasmids in three mammalian cell lines (HEK 293, COS-7 and CHO) and tested the level of FVIII expression in two medium collections. Our data show that lipofection is an efficient method of transfection as judged by the GFP expression of the pEGFP-C1 positive control plasmid that was transfected along with the different B-domain deleted expression plasmids in the three cell lines (Fig. 19). The CHO cell line, which has the shortest doubling time (24 hours) among the tested cell lines (DSMZ), showed the lowest FVIII expression level (as controlled by clotting assay FVIII: C). In both medium collections the average FVIII activity for the different constructs ranged from 1.67 % to 4.27 % in the first medium collection and from 2.5 % to 7.27 % in the second medium collection. In contrast to the CHO cell line, HEK 293 and COS-7 cell lines showed a higher expression level in the first medium collection compared to the second collection, which meet the time required for their doubling (COS-7 doubles every 35-48 hours and HEK 293 doubles every 24-30 hours; DSMZ). In the first medium collection,

the average activity of the different constructs ranged from 8.4 % to 46.73 % in HEK 293 cells and from 33.7 % to 56.33 % in COS-7 cells. In the second medium collection, the average activity of the different constructs ranged from 4.47 % to 24.4 % in HEK 293 cells and from 22.67 % to 44 % in COS-7 cells. Despite the fact that HEK 293 cells are of a human origin, our data showed a lower FVIII expression level in this cell line compared to COS-7 cell line in both medium collections, which indicate that the FVIII B-domain variants expressed more efficiently in COS-7 cells.

The COS-7 cell line, which showed the highest expression in terms of FVIII activity levels among the three cell lines, was then used for the functional studies on those constructs. The functionality was assessed by measuring the FVIII activity in culture medium using: (i) the clotting and the chromogenic assays, (ii) measuring the antigen level in culture medium and cellular lysates, (iii) calculating the specific activity and the total expressed protein of the different constructs, and (iv) comparing generally the expression of the constructs containing variable lengths of the B domain to the BDD construct. For this, COS-7 cells were transiently transfected with the different B-domain deleted constructs and the culture medium and cellular lysates was collected 48 hours after transfection.

FVIII: C was determined in standard FVIII one-stage clotting and two-stage chromogenic assays, using a WHO FVIII standard as calibrator. The different constructs containing various lengths of the B domain showed close values between the two assay methods (Fig. 21) with the clotting assay showing slightly higher values, but there was no significant difference between the two assays values for each construct. This can be seen from figure 37, presenting the ratio between the FVIII: C values obtained in the one-stage clotting assay and the chromogenic assay. In each case it was 1.0 approximately. In contrast to this, the BDD construct showed higher FVIII activity value in the chromogenic assay in comparison to the clotting assay (fig. 21 and fig. 37). The difference between both results was significant as calculated by the student's t-test ($p < 0.05$). This result confirms what was reported earlier in literature regarding the BDD-rFVIII (Barrowcliffe *et al.,* 2002; Mikaelsson *et al.,* 2001). From this data, we conclude that addition of even short parts of the B domain has a direct effect on FVIII physiology as such additions eliminate the assay discrepancy. This knowledge is important for the production of further recombinant FVIII concentrates, because until now, the difference of one-stage and chromogenic assays in determining the FVIII: C within patient's plasma prevents a good, standardized clinical monitoring of hemophilia A patients

after substitution with BDD-FVIII.

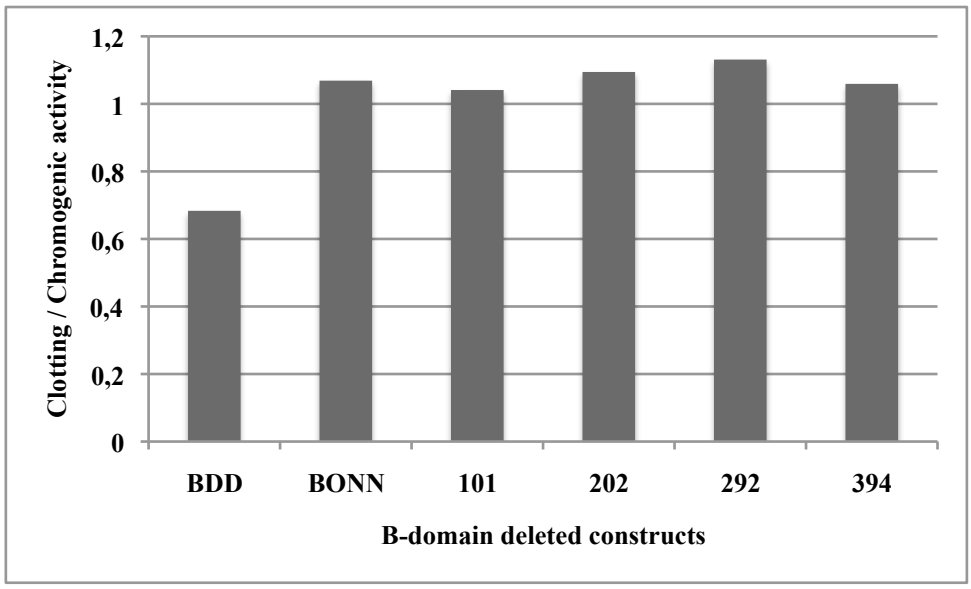

Figure 37: Ratio of the clotting assay activity value and chromogenic assay activity value for each of the different constructs containing various lengths of the B domain.

The different constructs containing various lengths of the B domain showed higher clotting FVIII activity levels than the BDD construct (see fig. 21), ranging from slightly more than one fold to 2.5 folds as Figure 38 shows. The different constructs also showed a higher total expressed protein as measured by FVIII: Ag ELISA (see fig. 22) than the BDD construct, ranging from more than one fold to more than 2.5 folds (Fig. 39). Evaluating the functionality of the different constructs in terms of FVIII specific activity, our data show that the different constructs containing various lengths of the B domain show a higher specific activity than the BDD construct (Fig. 22). This high specific activity could also be seen within the medium of cells transfected with the 292 construct that has the lowest expression. This data indicate that the added lengths of the B domain improve the overall activity, protein synthesis and functionality of FVIII in comparison to the BDD-rFVIII.

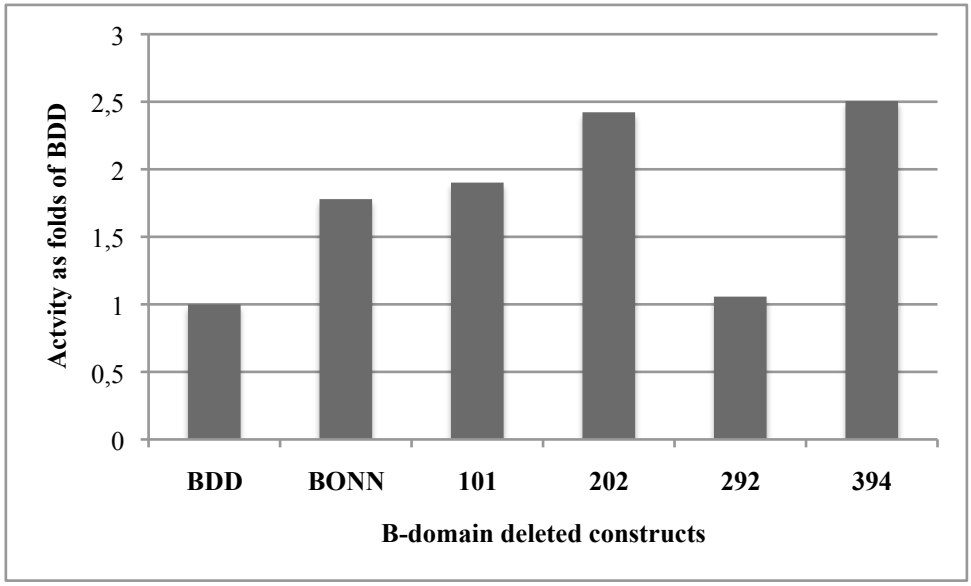

Figure 38: The clotting FVIII activity in culture medium of the different constructs containing various lengths of the B domain given as multiple of the BDD construct activity.

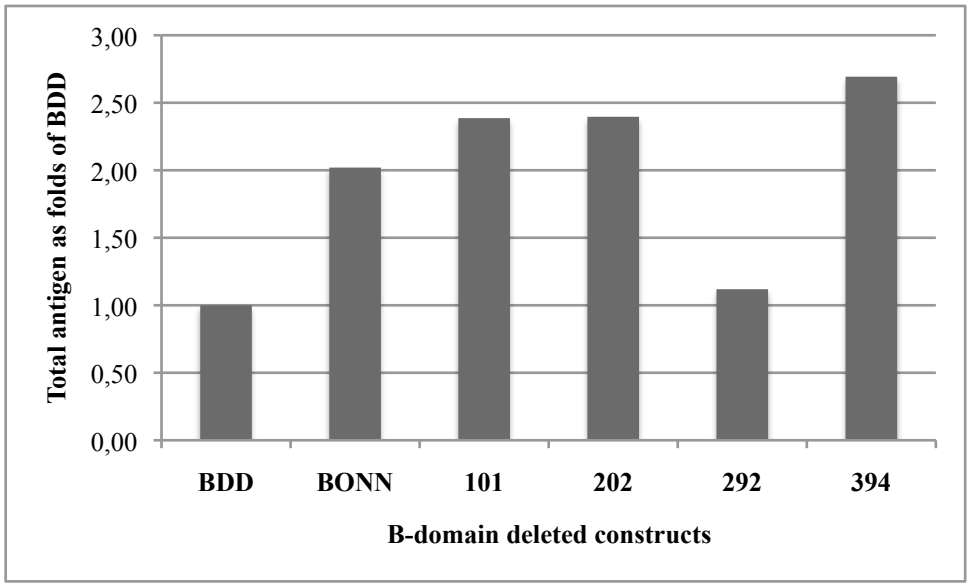

Figure 39: The total amount of expressed FVIII antigen (in culture medium and cellular lysates) of the different constructs containg various lengths of the B domain given as a multiple of the expressed BDD-FVIII construct.

The increase in the secretion efficiency is attributed to not only the added length of the B-domain, but also to the added glycosylation sites (Fig. 16), which improves the protein trafficking through the secretory pathway. This data agrees with previous literature studied FVIII secretion (Miao *et al.,* 2004; Pittman *et al.,* 1993).

The very low antigen secreted into the medium of the 292 construct (Fig. 21 B) but its high specific activity (Fig. 22 A) indicates that the FVIII product of this construct is functional but

poorly processed through the secretory pathway. This could be of a misfolded protein structure that will lead to degradation in the endoplasmic reticulum, but it has no influence on the functional activity of the small amount of secreted protein.

The approximately similar activity and antigen values of the Bonn (in which the B-domain region is deleted between aa 852 and 1525, so this construct has ~113 aa of the amino terminal of the B domain and ~124 aa of the carboxy terminal of the B domain) and the 101 construct (Fig. 21) indicates that the ~124 included amino acids from the B-domain end in the Bonn construct has no added effect on FVIII expression. This was expected as this part is naturally removed when FVIII undergoes intracellular proteolysis (Kaufman *et al*., 1988, van de Ven WJM *et al*., 1990, Hutton 1990, Barr 1991). FVIII is cleaved after residues 1313 and 1648 within the B domain to generate a 200 kD heavy chain and an 80 kDa light chain (see fig. 4), giving rise to the heterodimeric molecule that circulates in plasma (Kaufman 1992).

To have enough material for FVIII purification, the different recombinant FVIII constructs were stably expressed in CHO-DHFR[-]. In the CHO/DHFR expression system, the integrated gene of interest is expected to be coamplified with the DHFR gene by exposing rCHO cells to MTX. However, despite the stepwise selection, the foreign protein expression level of rCHO clones is not always increased with increasing concentrations of MTX (Kim *et al.*, 2001). Clonal variation in regard to foreign protein expression naturally occurs probably because of different integration sites of the foreign gene as a result of unpredictable chromosomal rearrangement during gene amplification (Davies and Reff 2001; Flintoff *et al.,* 1984). CHO-DHFR[-] were transfected with the different constructs and cloning by limiting dilution method was performed twice, once after a round of selection of parental cell clones and once after obtaining high producer clones. We have successfully generated a high FVIII producing rCHO cell lines for the BDD (clone F2H1), the 202 (clone G3E6), the 394 (clone F4F3) and the Full length (clone C6) construct (see table 9). The different rCHO cell lines show different FVIII productivities and different growth rates that are slower than nonproducing clones. This data agrees with previous studies on producing recombinant clones in CHO cell line (Gu *et al.* 1994; Kim *et al.,* 1998).

Culture medium collected from the high producing rCHO cell lines was used for FVIII purification. FVIII was purified by a small-scale affinity purification method using FVIII-Select beads. Purified materials were shown to be functional by the clotting FVIII: C assay

after diluting them in FVIII-deficient plasma (Table 10). After quantifying the purified material by FVIII ELISA (Table 11), equal antigen concentrations were used for thrombin cleavage. Comparing A2 cleavage patterns of BDD-rFVIII and 394-rFVIII, we could see that the BDD- rFVIII is more susceptible for thrombin activation showing a faster A2 generation pattern (Fig. 25). This result agrees with those of Eaton *et al.,* 1986 where a B-domain deleted molecule was shown to exhibit increases sensitivity to thrombin activation compared with wild type and those of Meulin *et al.,* 1988 where a B-domain deletion molecule was activated to a greater extent than wild type FVIII. However, our result disagrees with those of Pittman *et al.,* 1993 in which no difference was detected in the rate of heavy chain cleavage of the BDD molecule and wild type FVIII by thrombin. In view of our result, we suppose that the B domain region has an important physiological role in FVIII activation.

In conclusion, our data show that the addition of short lengths (~100-400 amino acids) of the B domain improved the overall expression of FVIII, eliminates the assay discrepancy and stabilizes the molecule upon activation.

3.2 Construction of a new helper vector for the production of helper-dependent adenovectors for the use in hemophilia gene therapy

Current treatment of hemophilia A consists of protein substitution therapies based on plasma-derived or recombinant FVIII. Although FVIII substitution therapy has significantly improved the quality of life of patients suffering from hemophilia A, it is not curative and a life-long dependency on clotting factor treatment is required (Ragni, 2004). Furthermore, the patients are still at risk of spontaneous bleeding episodes, which might cause significant morbidity and sometimes even mortality. Hence, it would be ideal if a cure for hemophilia A could be developed such that sustained therapeutic FVIII levels could be achieved. Hemophilia A is therefore considered to be a particularly attractive target disease for gene therapy. The molecular biology and pathology of this disease is relatively well understood and only a relatively slight increase in FVIII levels (above 1% of physiologic levels) suffices to convert a severe to a moderate hemophilic phenotype, which constitutes a major clinical improvement (Chuah *et al.,* 2004).

During the last few decades, a variety of different gene delivery technologies have been developed, each with their own advantages and limitations. Vectors derived from human

adenoviruses exhibit a number of features that have made them extremely useful gene transfer reagents. Most notably, these vectors can transduce a broad range of both dividing and post-mitotic cells of different origin. The ultimate form of adenovirus vector modification is the creation of fully deleted 'helper-dependent' adenovirus vectors (HD-AdVs). These are adenovirus-based vectors that contain only the cis elements necessary for replication and packaging, but lack all adenovirus genes. As they do not express any viral genes, they have a significantly improved safety profile compared with early-generation adenoviral vectors. HD-AdVs could give rise to prolonged transgene expression and were associated with significantly reduced acute and chronic hepatotoxicity and reduced inflammatory responses compared with first-generation adenoviral vectors, even in large animal models such as primates or dogs, which are more susceptible to the toxic side effects of adenoviral vectors than mice (Chuah *et al.,* 2004). HD-AdVs created so far share a major disadvantage which is the use of helper viruses or plasmid cotransfection to provide the necessary virus proteins in trans. The use of helper viruses almost always results in some contamination of the vector by the helper virus.

Recombinant baculoviruses are increasingly used as gene delivery vectors for mammalian cells (Kost *et al.,* 2005). This relatively new gene delivery approach offers a number of advantages including the inability of the virus to replicate in mammalian cells, the absence or virtual absence of cytotoxicity, technical simplicity and superior biosafety profile as compared to mammalian cell-derived viral vectors (Kost *et al.,* 2000). The demonstration that a recombinant baculovirus containing a 38 kb insert can be generated and propagated in a genetically stable manner in insect cells provides important direct evidence of the large cloning capacity of the virus (Cheshenko *et al.,* 2001).

In the present study, we have examined the potential utility of a novel helper system for the production of a HD-AdV carrying a B-domain-deleted factor VIII-cDNA without any risk of contamination. In this system, a packaging deficient, replication-competent adenovirus Helper construct was delivered by a baculovirus. This baculovirus / adenovirus hybrid genome was then delivered into HEK 293T cells along with the HD-AdV, where the adenoviral sequences integrated into the hybrid genome should provide (in trans) all of the functions necessary for the propagation of HD-AdVs.

In designing our novel helper (the baculovirus / adenovirus hybrid) genome, we included only the Ad5 genes (nts 4000 to 35832) necessary for replication and packaging of HD-AdV. The adenoviral 5'ITR (nts 1-190), packaging signal (nts 191-358) and 3'ITR (nts 35833-35935) as well as E1/E3 region were deleted in this helper construct, thus rendering it replication incompetent and packaging incompetent. The replication capacity of the helper construct was then regained by fusing the Adeno-associated virus 2 (AAV2)-ITRs (5'ITR; 3'ITR) into it. AAV2-ITRs serves as origins of replication for the helper construct provided that AAV2-rep78/68 genes are provided in trans. (See also section 2.2.9 and 2.3.5) The helper construct (AAV2[5'ITR] /Ad5/ AAV2[3'ITR]) was then integrated into the Baculovirus genome generating the helper vector Bac-AAV2-Ad5.

The cloning of such a big helper construct required a tedious lab effort. The big insert size (~ 32 kb) with respect to the vector size (the Bac-shuttle plasmid of around 6 kb in size) required the screening of around 100 recombinant clones by size and restriction enzyme analysis. Doing this, we were able to isolate a clone, however with a small deletion within the 3'ITR region. This deletion was confirmed by Sma I restriction and DNA sequencing (Fig. 27 and 28). After consultation with Prof. W Poller research group (our gene therapy collaborating group; Freie Universitaet Berlin), we continued work as this group assured that one ITR is enough to serve as an origin of replication.

To guarantee the genetic stability of the helper construct in insect and mammalian cells and to prevent its self-circularization, we have provided the Rep genes in trans. in a separate baculoviral construct. The expression of the AAV2-Rep genes has been shown in SF9 and HEK 293 cells by RT-PCR (Fig. 29). The EGFP gene was cloned in a separate baculoviral construct and served as a positive control for HEK 293 cells infection (Fig. 35).

Recombinant bacmids (bBac-AAV2-Ad5, bBac-AAV2-Rep and bBac-EGFP) were generated by site specific transposition with their corresponding shuttle plasmids and confirmed by Taq-PCR using M13 forward and reverse primers and insert specific primers (Fig. 30 and 31).

For virus production, recombinant bacmids were used to rescue the recombinant Bac virus (vBac-AAV2-Ad5, vBac-AAV2-Rep and vBac-EGFP) in Sf9 insect cells. Viral titers of 5 * 10_9 pfu/ml and 4 * 10_9 pfu/ml were achieved for vBac-AAV2-Ad5 and vBac-AAV2-Rep respectively and selected plaques were amplified. Viral DNA was extracted from purified

amplified viral stocks and tested by Taq-PCR to have the Ad5-chimera and the Rep genes. We have successfully isolated plaques (9 and 16) of vBac-AAV2-Ad5 and plaques (1 and 5) of vBac-AAV2-Rep that showed a genetic stability over passaging and found to have the correct inserts by Taq-PCR (Fig. 34). As the structure of the Ad5 insert in vBac-AAV2-Ad5 did not change during passaging, this indicates that the presence of adenoviral sequences in the recombinant hybrid genome did not interfere with baculovirus growth.

For the main experiment, the purified amplified Bac-AAV2-Ad5 helper virus (at MOI of 500 and 2000) was used in combination with Bac-AAV2-Rep (at MOI of 200) to rescue the HD-AdV in HEK293 cells. As a positive control of transfection, one well was infected with pBac-EGFP. As a negative control of rescuing the HD-AdV, one well was infected with Bac-AAV2-Rep (at MOI of 500) only (no helper adenoviral construct). Assuming that inside the HEK 293 cells, the AAV2-ITR will function as an origin of replication and as a termination signal supported by the expression of the Rep78/68 protein and that the Ad5 helper construct in the hybrid genome will provide all Ad5 proteins necessary for replication and packaging of HD-AdV, the HD-AdV should be produced. This viral production should lead to lysis of HEK 293 as well as BDD-rFVIII should be expressed.

Our results were not as expected as we have not seen any cytopathic effects after four days infection period. Nonetheless, to see whether the results were positive or no, medium was collected and HEK 293 cells were lysed. Lysates were partially used for extrachromsomal DNA extraction to control wether DNA of HD-AdV has been amplified in HEK 293 cells. Digestion with DpnI indicated that our isolated extrachromosomal DNA was not produced *in vivo* inside HEK 293 cells, but was of bacterial origin belonging to the transfected HD-AdV (Fig. 36). Culture medium was used for FVIII: Ag testing which was not detectable. A second portion of the lysates was used for a second round of infection (to amplify the helper dependent viral particles in case they were produced in a very low quantity) using fresh confluent HEK 293 cells in the presence of the same concentrations of the helper virus. Four days later, cells were lysed and lysates were used for a third round of HEK 293 infection. No signs of infection could be seen in the three rounds of infection. These results indicate that our helper system could not support the production of the helper dependent adenovector.

Cheshenko *et al.,* 2001 developed an alternative system for the propagation of HD-AdVs, in which the adenoviral genes (flanked by adenoviral ITRs) essential for replication and

packaging of the helper vector are delivered into producer cells by a similar baculovirus-adenovirus hybrid. This hybrid was constructed to carry a Cre recombinase-excisable copy of a packaging-deficient adenovirus genome. Their experiments resulted in a vector that was completely free of helper virus and was able to transduce cultured 293 cells. However, scaling-up of HD-AdV production was prevented by the eventual emergence of replication-competent adenovirus (RCA), apparently the product of homologous recombination between adenovirus sequences contained in 293 cells and the adenoviral helper genome (Kost and Condreay, 2002). The major difference between this system and the helper-dependent system is that, instead of deleting the packaging signal from the helper genome during the vector propagation, we have deleted it before it gets into cells. This modification precludes the packaging of this genome into adenovirus virions. Flanking the adenoviral genome by AAV2-ITRs instead of Ad5-ITRs will also prevent the appearance of RCA.

In the design of this system we took advantage of a unique feature of the baculovirus AcMNPV to infect human cells efficiently while remaining virtually nontoxic and transcriptonally and replicatively inactive. The genome of AcMNPV consists of 131 kb double-stranded circular DNA molecule (Ayres *et al.*, 1994). Although it was generally reported that the extra packaging capacity of baculovirus is high (Cheshenko *et al.,* 2001), we could prove that the recombinant baculovirus Bac-AAV2-Ad5 created in this study was able to accomodate an insert of ~35824 bp, which apparently did not affect the virus viability. Our data suggest that, despite the presence of such a large insert, the genetic structure of Bac-AAV2-Ad5 remained stable, allowing passaging and expansion of the virus. In addition, we we were able to propagate those recombinant baculoviruses to relatively high titers (5 * 109 pfu/ml) and further concentrate them by centrifugation, which agrees with Hofmann *et al.,* 1995.

Since baculovirus genes are not expressed in mammalian cells, it is unlikely that the Bac/Ad5 hybrid genome will replicate in mammalian cells. However, replication of adenovirus sequences in the helper hybrid genome is essential to generate the large number of templates for the synthesis of the large amounts of viral proteins that are necessary to obtain high yields of HD-AdV. Thus, in order to support the replication and packaging of the HD-AdV in an efficient fashion, the adenovirus helper genome should be able to replicate. To permit replication directed from the AAV2-ITRs flanking the Ad5 sequences, the AAV2-Rep genes were provided in trans in a separate baculovirus that was produced into high titers and

coinfected with Bac-AAV2-Ad5 helper hybrid.

In conclusion, as the baculovirus was able to infect HEK 293 cells as shown by vBac-EGFP infection and the Rep genes are expressed in those cells, we propose that the HD-AdV was not produced because of inefficient or non occurring replication of the Ad5 genes in the Bac/Ad5 hybrid. The presence of only one ITR may be a possible cause that replication did not work (efficiently). Reconstructing such a helper hybrid to have both ITRs would validate our novel system and show its ability to support the HD-AdVs.

5. Summary:

5.1 Functional studies on factor VIII protein variants containing different lengths of the B-domain

To evaluate the role of the factor VIII (FVIII) B-domain on FVIII expression, (activity and activation), we constructed several FVIII expression constructs with various lengths of the B-domain and expressed them transiently as well as stably.

Practically, B-domain deleted recombinant FVIII (BDD-rFVIII) was generated from the full-length FVIII-cDNA by ligating (on the protein level) Ser743 to Arg1634; the corresponding FVIII-cDNA was then cloned into the pMT2 expression vector. Then, cDNA-fragments of different length (on the protein level: 101, 202, 292, and 394 amino acid long fragments of the B-domain starting from its amino terminal) were cloned between Ser743 and Arg1634. Constructs were then transiently expressed in COS-7, HEK 293 and CHO cells and stably expressed in CHO-DHFR(-) cells by methotrexate selection and amplification. FVIII proteins were then assessed for activity in culture medium by the clotting assay and the chromogenic assay and for antigen in cellular lysates by ELISA. FVIII proteins were purified from stable cell lines media.

Our transient expression studies showed that FVIII expression was the highest in COS-7 cells followed by HEK 293 cells. The CHO cells showed the lowest FVIII expression. Transient expression in COS-7 cells (for forty-eight hours) revealed that the constructs containing added lengths of the B-domain showed a higher FVIII: C (~1.1-2.5 folds) and a higher overall expressed protein (total antigen in medium and cellular lysates; ~1.1-3 folds) compared the BDD-rFVIII. The functionality of the expressed proteins in terms of FVIII specific activity was higher in all constructs in comparison to the BDD construct. Comparing the clotting FVIII activity by that of the chromogenic assay, we found that those constructs showed a slightly higher FVIII value within the clotting assay than within the chromogenic assay. In contrast, the BDD construct showed the opposite effect and also showed a significant discrepancy between both assay methods. Activation of purified FVIII proteins by thrombin revealed that the BDD-rFVIII showed a faster generation of the A2 domain than the constructs containing added lengths of the B-domain.

In conclusion, our data showed that the inclusion of a 100 amino acids or more of the B-domain sequence eliminates the assay discrepancy and increases the overall expression and functionality of rFVIII protein.

5.2 Construction of a new helper vector for the production of helper-dependent adenovectors for the use in hemophilia gene therapy

Fully deleted Helper-Dependent Adenovectors (HD-AdV) are so far the ultimate form of Adenovirus vector modifications which lack all the adenovirus genes except the replication and packaging elements. They can accommodate an insert of up to 36 kb. HD-AdVs are produced in HEK 293 cells in the presence of a helper adenovirus that supplies the viral genes in trans. Using this system very high titers of HD-AdV can be prepared now, with small but still measurable helper adenovirus contamination.

Within this study we have tried to design a novel hybrid helper virus that can support the production of a HD-AdV, carrying a B-domain-deleted factor VIII-cDNA, in high efficiency without any contamination. In order to avoid contamination of HD-AdV production, the adenoviral sequences in the novel hybrid helper virus was lacking the adenoviral ITRs and the packaging signal, but was flanked by adeno-associated virus (AAV) ITRs. This adenoviral helper sequences was then cloned into the baculovirus expression system.

The lack of the Adenovirus packaging signal renders the hybrid helper virus packaging incompetent and thus eliminates the risk of contaminating the HD-AdV preparations. Since the helper virus also lacks the adenoviral ITRs, it will not compete with the HD-AdV for the Adenovirus 5 replication proteins. The lack of the capsid genes within the AAV-sequences should also prevent the generation of AAV particles.

To drive the replication of the Ad5-genome in the hybrid helper virus theAAV2-Rep genes were cloned in a separate baculoviral construct. A GFP baculoviral construct was prepared to be used as positive control for transfecting insect cells and infecting HEK 293 cells.

We successfully constructed the hybrid helper vector pBac-AAV2-Ad5 with a deletion in the 3'ITR region. The recombinant baculovirus particles (vBac-AAV2-Ad5, vBac-AAV2-Rep and vBac-EGFP) was then produced in insect cells, plaque purified and amplified to high

titers. Since the deletion within the 3'-ITR region should have no effect on helper virus production, the produced baculoviruses were used to infect HEK 293 cells, together with the HD-AdV carrying the B-domain-deleted factor VIII-cDNA. Depending on the presence of AAV-ITRs, we expected that the genome of the helper-virus will be replicated, thus improving its efficiency in providing helper function. To verify our strategy in synthesizing the helper virus, we showed that the baculovirus is able to infect HEK 293 cells by eGFP expression and that the Rep gene was expressed in both SF9 cells and HEK 293 cells.

After three rounds of infecting HEK293 cells with the helper hybrid virus, we couldn't see any signs of HD-AdV production. Unexpectedly, the analysis of extracted extra chromosomal DNA showed that the material was the original transfected material and not synthesized within the HEK 293 cells.

In conclusion, as the baculovirus was able to infect HEK 293 cells and the Rep genes were also expressed in those cells, we propose that the HD-AdV was not produced because of inefficient or non occurring replication of the Ad5 genes in the Bac/Ad5 hybrid. The presence of only one ITR may be a possible cause. The reconstruction of an helper hybrid with both ITRs would clarify the function of such an adenoviral helper virus system.

6. References:

ANDERSSON, L. O., FORSMAN, N., HUANG, K., LARSEN, K., LUNDIN, A., PAVLU, B., SANDBERG, H., SEWERIN, K. & SMART, J. 1986. Isolation and characterization of human factor VIII: molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. *Proc Natl Acad Sci U S A,* 83**,** 2979-83.

ANDREWS, J. L., KADAN, M. J., GORZIGLIA, M. I., KALEKO, M. & CONNELLY, S. 2001. Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. *Mol Ther,* 3**,** 329-36.

ANTONARAKIS, S. E., KAZAZIAN, H. H. & TUDDENHAM, E. G. 1995. Molecular etiology of factor VIII deficiency in hemophilia A. *Hum Mutat,* 5**,** 1-22.

ARAD, U. 1998. Modified Hirt procedure for rapid purification of extrachromosomal DNA from mammalian cells. *Biotechniques*. 24(5), 760-2.

ARMENTANO, D., SOOKDEO, C. C., HEHIR, K. M., GREGORY, R. J., ST GEORGE, J. A., PRINCE, G. A., WADSWORTH, S. C. & SMITH, A. E. 1995. Characterization of an adenovirus gene transfer vector containing an E4 deletion. *Hum Gene Ther,* 6**,** 1343-53.

ARRUDA, V. R., FIELDS, P. A., MILNER, R., WAINWRIGHT, L., DE MIGUEL, M. P., DONOVAN, P. J., HERZOG, R. W., NICHOLS, T. C., BIEGEL, J. A., RAZAVI, M., DAKE, M., HUFF, D., FLAKE, A. W., COUTO, L., KAY, M. A. & HIGH, K. A. 2001. Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. *Mol Ther,* 4**,** 586-92.

AYRES, M. D., HOWARD, S. C., KUZIO, J., LOPEZ-FERBER, M., POSSEE, R. D. 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology. 1994, 202(2):586-605.

AZZI, A., DE SANTIS, R., MORFINI, M., ZAKRZEWSKA, K., MUSSO, R., SANTAGOSTINO, E. & CASTAMAN, G. 2001. TT virus contaminates first-generation recombinant factor VIII concentrates. *Blood,* 98**,** 2571-3.

BAGNALL, R. D., WASEEM, N., GREEN, P. M. & GIANNELLI, F. 2002. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood,* 99**,** 168-74.

BALAGUE, C., ZHOU, J., DAI, Y., ALEMANY, R., JOSEPHS, S. F., ANDREASON, G., HARIHARAN, M., SETHI, E., PROKOPENKO, E., JAN, H. Y., LOU, Y. C., HUBERT-LESLIE, D., RUIZ, L. & ZHANG, W. W. 2000. Sustained high-level expression of full-length human factor VIII and restoration of clotting activity in hemophilic mice using a minimal adenovirus vector. *Blood,* 95**,** 820-8.

BARROWCLIFFE, T. W., RAUT, S., SANDS, D. & HUBBARD, A. R. 2002. Coagulation and chromogenic assays of factor VIII activity: general aspects, standardization, and recommendations. *Semin Thromb Hemost,* 28**,** 247-56.

BERKNER, K. L. 1988. Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques,* 6**,** 616-29.

BI, L., LAWLER, A. M., ANTONARAKIS, S. E., HIGH, K. A., GEARHART, J. D. & KAZAZIAN, H. H., JR. 1995. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet,* 10**,** 119-21.

BOYCE, F. M. & BUCHER, N. L. 1996. Baculovirus-mediated gene transfer into mammalian cells. *Proc Natl Acad Sci U S A,* 93**,** 2348-52.

BRACKMANN, H. H., OLDENBURG, J. & SCHWAAB, R. 1996. Immune tolerance for the treatment of factor VIII inhibitors--twenty years' 'bonn protocol'. *Vox Sang,* 70 Suppl 1**,** 30-5.

BRETTLER, D. B., FORSBERG, A. D., LEVINE, P. H., PETILLO, J., LAMON, K. & SULLIVAN, J. L. 1989. Factor VIII:C concentrate purified from plasma using monoclonal antibodies: human studies. *Blood,* 73**,** 1859-63.

BROZE, G. J., JR. 1992. The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Semin Hematol,* 29**,** 159-69.

CHAO, H., MAO, L., BRUCE, A. T. & WALSH, C. E. 2000. Sustained expression of human factor VIII in mice using a parvovirus-based vector. *Blood,* 95**,** 1594-9.

CHESHENKO, N., KROUGLIAK, N., EISENSMITH, R. C. & KROUGLIAK, V. A. 2001. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. *Gene Ther,* 8**,** 846-54.

CHRISTIANSEN, M. L., BALLING, K. W., PERSSON, E., HILDEN, I., BAGGER-SORENSEN, A., SORENSEN, B. B., VIUFF, D., SEGEL, S., KLAUSEN, N. K., EZBAN, M., LETHAGEN, S., STEENSTRUP, T. D. & KJALKE, M. Functional characteristics of N8, a new recombinant FVIII. *Haemophilia,* 16**,** 878-87.

CHRISTIANSEN, M. L., BALLING, K. W., PERSSON, E., HILDEN, I., BAGGER-SORENSEN, A., SORENSEN, B. B., VIUFF, D., SEGEL, S., KLAUSEN, N. K., EZBAN, M., LETHAGEN, S., STEENSTRUP, T. D. & KJALKE, M. 2010. Functional characteristics of N8, a new recombinant FVIII. *Haemophilia,* 16**,** 878-87.

CHUAH, M. K., COLLEN, D. & VANDENDRIESSCHE, T. 2001. Gene therapy for hemophilia. *J Gene Med,* 3**,** 3-20.

CHUAH, M. K., COLLEN, D. & VANDENDRIESSCHE, T. 2004. Clinical gene transfer studies for hemophilia A. *Semin Thromb Hemost,* 30**,** 249-56.

CHUAH, M. K., SCHIEDNER, G., THORREZ, L., BROWN, B., JOHNSTON, M., GILLIJNS, V., HERTEL, S., VAN ROOIJEN, N., LILLICRAP, D., COLLEN, D., VANDENDRIESSCHE, T. & KOCHANEK, S. 2003. Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. *Blood,* 101**,** 1734-43.

CLEMENS, P. R., KOCHANEK, S., SUNADA, Y., CHAN, S., CHEN, H. H., CAMPBELL, K. P. & CASKEY, C. T. 1996. In vivo muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes. *Gene Ther,* 3**,** 965-72.

COLMAN, R. W., MARDER, V. J., SALZMAN, W. W., HIRSH, J. 1994. Overview of hemostasis. In Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W. (eds): Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 3rd edn. JB Lippencott, Philadelphia, pp. 3-18.

CONNELLY, S., ANDREWS, J. L., GALLO, A. M., KAYDA, D. B., QIAN, J., HOYER, L., KADAN, M. J., GORZIGLIA, M. I., TRAPNELL, B. C., MCCLELLAND, A. & KALEKO, M. 1998. Sustained phenotypic correction of murine hemophilia A by in vivo gene therapy. *Blood,* 91**,** 3273-81.

CONNELLY, S., GARDNER, J. M., LYONS, R. M., MCCLELLAND, A. & KALEKO, M. 1996. Sustained expression of therapeutic levels of human factor VIII in mice. *Blood,* 87**,** 4671-7.

CONNELLY, S., GARDNER, J. M., MCCLELLAND, A. & KALEKO, M. 1996. High-level tissue-specific expression of functional human factor VIII in mice. *Hum Gene Ther,* 7**,** 183-95.

CONNELLY, S., MOUNT, J., MAUSER, A., GARDNER, J. M., KALEKO, M., MCCLELLAND, A. & LOTHROP, C. D., JR. 1996. Complete short-term correction of canine hemophilia A by in vivo gene therapy. *Blood,* 88**,** 3846-53.

CUNNINGHAM, M. A., PIPE, S.W., HAURI, H. P., KAUFMAN, R.J. 2001. The interaction of coagulation factor VIII with ERGIC-53 requires specific N-linked oligosaccharide structures within the B domain. *Blood,* 98, 705a (Abst.).

DAVIE, E. W., FUJIKAWA, K. & KISIEL, W. 1991. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry,* 30**,** 10363-70.

DAVIES, J. & REFF, M. 2001. Chromosome localization and gene-copy-number quantification of three random integrations in Chinese-hamster ovary cells and their amplified cell lines using fluorescence in situ hybridization. *Biotechnol Appl Biochem,* 33**,** 99-105.

DORNER, A. J., BOLE, D. G. & KAUFMAN, R. J. 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J Cell Biol,* 105**,** 2665-74.

DORNER, A. J., WASLEY, L. C. & KAUFMAN, R. J. 1989. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J Biol Chem,* 264**,** 20602-7. EATON, D. L., HASS, P. E., RIDDLE, L., MATHER, J., WIEBE, M., GREGORY, T. & VEHAR, G. A. 1987. Characterization of recombinant human factor VIII. *J Biol Chem,* 262**,** 3285-90.

EATON, D. L., WOOD, W. I., EATON, D., HASS, P. E., HOLLINGSHEAD, P., WION, K., MATHER, J., LAWN, R. M., VEHAR, G. A. & GORMAN, C. 1986. Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. *Biochemistry,* 25**,** 8343-7.

ELDER, B., LAKICH, D. & GITSCHIER, J. 1993. Sequence of the murine factor VIII cDNA. *Genomics,* 16**,** 374-9.

ESMON, C. T. 1989. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem,* 264**,** 4743-6.

EVANS, J. P., BRINKHOUS, K. M., BRAYER, G. D., REISNER, H. M. & HIGH, K. A. 1989. Canine hemophilia B resulting from a point mutation with unusual consequences. *Proc Natl Acad Sci U S A,* 86**,** 10095-9.

FAKHARZADEH, S. S., ZHANG, Y., SARKAR, R. & KAZAZIAN, H. H., JR. 2000. Correction of the coagulation defect in hemophilia A mice through factor VIII expression in skin. *Blood,* 95**,** 2799-805.

FAY, P. J. 2004. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev,* 18**,** 1-15.

FAY, P. J. 2006. Factor VIII structure and function. *Int J Hematol,* 83**,** 103-8.

FAY, P. J., COUMANS, J. V. & WALKER, F. J. 1991. von Willebrand factor mediates protection of factor VIII from activated protein C-catalyzed inactivation. *J Biol Chem,* 266**,** 2172-7.

FAY, P. J., HAIDARIS, P. J. & HUGGINS, C. F. 1993. Role of the COOH-terminal acidic region of A1 subunit in A2 subunit retention in human factor VIIIa. *J Biol Chem,* 268**,** 17861-6.

FAY, P. J. & JENKINS, P. V. 2005. Mutating factor VIII: lessons from structure to function. *Blood Rev,* 19**,** 15-27.

FAY, P. J., MASTRI, M., KOSZELAK, M. E. & WAKABAYASHI, H. 2001. Cleavage of factor VIII heavy chain is required for the functional interaction of a2 subunit with factor IXA. *J Biol Chem,* 276**,** 12434-9.

FAY, P. J., SMUDZIN, T. M. & WALKER, F. J. 1991. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. *J Biol Chem,* 266**,** 20139-45.

FISHER, K. D., STALLWOOD, Y., GREEN, N. K., ULBRICH, K., MAUTNER, V. & SEYMOUR, L. W. 2001. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther,* 8**,** 341-8.

FLINTOFF, W. F., LIVINGSTON, E., DUFF, C. & WORTON, R. G. 1984. Moderate-level gene amplification in methotrexate-resistant Chinese hamster ovary cells is accompanied by chromosomal translocations at or near the site of the amplified DHFR gene. *Mol Cell Biol,* 4**,** 69-76.

FOSTER, P. A., FULCHER, C. A., HOUGHTEN, R. A. & ZIMMERMAN, T. S. 1988. An immunogenic region within residues Val1670-Glu1684 of the factor VIII light chain induces antibodies which inhibit binding of factor VIII to von Willebrand factor. *J Biol Chem,* 263**,** 5230-4.

FRANCHINI, M. & LIPPI, G. Von Willebrand factor-containing factor VIII concentrates and inhibitors in haemophilia A. A critical literature review. *Thromb Haemost,* 104**,** 931-40.

FRANCHINI, M. & LIPPI, G. Recombinant factor VIII concentrates. *Semin Thromb Hemost,* 36**,** 493-7.

FREIJE, D. & SCHLESSINGER, D. 1992. A 1.6-Mb contig of yeast artificial chromosomes around the human factor VIII gene reveals three regions homologous to probes for the DXS115 locus and two for the DXYS64 locus. *Am J Hum Genet,* 51**,** 66-80.

FRESHNEY, R. I. 2000. Culture of Animal Cells: A Manual of Basic Techniques, 4th edn.

FURIE, B. & FURIE, B. C. 2005. Thrombus formation in vivo. *J Clin. Invest.,* 115 (12), 3355-62.

FURIE, B. & FURIE, B. C. 1992. Molecular and cellular biology of blood coagulation. *N Engl J Med,* 326**,** 800-6.

FURIE, B., LIMENTANI, S. A. & ROSENFIELD, C. G. 1994. A practical guide to the evaluation and treatment of hemophilia. *Blood,* 84**,** 3-9.

GALLO-PENN, A. M., SHIRLEY, P. S., ANDREWS, J. L., TINLIN, S., WEBSTER, S., CAMERON, C., HOUGH, C., NOTLEY , C., LILLICRAP , D., KALEKO,M. & CONNELLY, S. 2001. Systemic delivery of an adenoviral vector encoding canine factor VIII results in short-term phenotypic correction, inhibitor development, and biphasic liver toxicity in hemophilia A dogs. *Blood,* 97**,** 107-13.

GAO, G. P., YANG, Y. & WILSON, J. M. 1996. Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J Virol,* 70**,** 8934-43.

GIRMA, J-P., FRESSINAUD, E., HOULLIER, A., LAURIAN, Y., AMIRAL, J. & MEYER, D. 1998. Assay of factor VIII antigen (VIII:CAg) in 294 haemophilia A patients by a new commercial ELISA using monoclonal antibodies. *Haemophilia*, **4**, 98–103.

GNATENKO, D. V., SAENKO, E. L., JESTY, J., CAO, L. X., HEARING, P. & BAHOU, W. F. 1999. Human factor VIII can be packaged and functionally expressed in an adeno-associated virus background: applicability to haemophilia A gene therapy. *Br J Haematol,* 104**,** 27-36.

GORZIGLIA, M. I., KADAN, M. J., YEI, S., LIM, J., LEE, G. M., LUTHRA, R. & TRAPNELL, B. C. 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol,* 70**,** 4173-8.

GRANADOS, R. R. *et al.* 1994. A new cell line from *Trichoplusia ni (BTI-Tn-5B1-4)* susceptible to *Trichoplusia ni* single enveloped nuclear polyhedrosis virus. *J. Invertebr. Pathol.* 64, 260-266.

GRUPPO, R. A., BROWN, D., WILKES, M. M. & NAVICKIS, R. J. 2003. Comparative effectiveness of full-length and B-domain deleted factor VIII for prophylaxis--a meta-analysis. *Haemophilia,* 9**,** 251-60.

GRUPPO, R. A., BROWN, D., WILKES, M. M. & NAVICKIS, R. J. 2004. Meta-analytic evidence of increased breakthrough bleeding during prophylaxis with B-domain deleted factor VIII. *Haemophilia,* 10**,** 747-50.

GRUPPO, R. A., BROWN, D., WILKES, M. M. & NAVICKIS, R. J. 2004. Increased breakthrough bleeding during prophylaxis with B-domain deleted factor VIII--a robust meta-analytic finding. *Haemophilia,* 10**,** 449-51.

GU, M. B., TODD, P. & KOMPALA, D. S. 1994. Analysis of foreign protein over production in recombinant CHO cells. Effect of growth kinetics and cell cycle traverse. *Ann N Y Acad Sci,* 721**,** 194-207.

HE, T. C., ZHOU, S., DA COSTA, L. T., YU, J., KINZLER, K. W., VOGELSTEIN, B. 1998. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci, USA, 95(5), 2509-14.

HIGH, K. A. 2003. Gene transfer as an approach to treating hemophilia. *Semin Thromb Hemost,* 29**,** 107-20.

HILL-EUBANKS, D. C., PARKER, C. G. & LOLLAR, P. 1989. Differential proteolytic activation of factor VIII-von Willebrand factor complex by thrombin. *Proc Natl Acad Sci U S A,* 86**,** 6508-12.

HOFMANN, C., SANDIG, V., JENNINGS, G., RUDOLPH, M., SCHLAG, P. & STRAUSS, M. 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc Natl Acad Sci U S A,* 92**,** 10099-103.

HORTELANO, G., AL-HENDY, A., OFOSU, F. A. & CHANG, P. L. 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood,* 87**,** 5095-103.

HOUGH, C., KAMISUE, S., CAMERON, C., NOTLEY, C., TINLIN, S., GILES, A. & LILLICRAP, D. 2002. Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia. *Thromb Haemost,* 87**,** 659-65.

HOYER, L. W. 1994. Hemophilia A. *N Engl J Med,* 330**,** 38-47.

HUTTON, D. A., PEARSON, J. P., ALLEN, A. & FOSTER, S. N. 1990. Mucolysis of the colonic mucus barrier by faecal proteinases: inhibition by interacting polyacrylate. *Clin Sci (Lond),* 78**,** 265-71.

INGRAM, G. I. 1976. The history of haemophilia. *J Clin Pathol,* 29**,** 469-79.

JONES, P. K. & RATNOFF, O. D. 1991. The changing prognosis of classic hemophilia (factor VIII "deficiency"). *Ann Intern Med,* 114**,** 641-8.

KANE, W. H. & DAVIE, E. W. 1988. Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. *Blood,* 71**,** 539-55.

KAUFMAN, R. J. 1992. Expression and structure-function properties of recombinant factor VIII. *Transfus Med Rev,* 6**,** 235-46.

KAUFMAN, R. J. 1992. Biological regulation of factor VIII activity. *Annu Rev Med,* 43**,** 325-39.

KAUFMAN, R. J. 1999. Advances toward gene therapy for hemophilia at the millennium. *Hum Gene Ther,* 10**,** 2091-107.

KAUFMAN, R. J., WASLEY, L. C., DAVIES, M. V., WISE, R. J., ISRAEL, D. I. & DORNER, A. J. 1989. Effect of von Willebrand factor coexpression on the synthesis and secretion of factor VIII in Chinese hamster ovary cells. *Mol Cell Biol,* 9**,** 1233-42.

KAUFMAN, R. J., WASLEY, L. C. & DORNER, A. J. 1988. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem,* 263**,** 6352-62.

KAY, M. A., MANNO, C. S., RAGNI, M. V., LARSON, P. J., COUTO, L. B., MCCLELLAND, A., GLADER, B., CHEW, A. J., TAI, S. J., HERZOG, R. W., ARRUDA, V., JOHNSON, F., SCALLAN, C., SKARSGARD, E., FLAKE, A. W. & HIGH, K. A. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet,* 24**,** 257-61.

KEMBALL-COOK, G., TUDDENHAM, E. G. & WACEY, A. I. 1998. The factor VIII Structure and Mutation Resource Site: HAMSTeRS version 4. *Nucleic Acids Res,* 26**,** 216-9.

KEY, N. S. & NEGRIER, C. 2007. Coagulation factor concentrates: past, present, and future. *Lancet,* 370**,** 439-48.

KIM, N. S., BYUN, T. H. & LEE, G. M. 2001. Key determinants in the occurrence of clonal variation in humanized antibody expression of cho cells during dihydrofolate reductase mediated gene amplification. *Biotechnol Prog,* 17**,** 69-75.

KIM, S. J., KIM, N. S., RYU, C. J., HONG, H. J. & LEE, G. M. 1998. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. *Biotechnol Bioeng,* 58**,** 73-84.

KHRENOV, A. V., ANANYEVA, N. M., SAENKO, E. L. 2006. Role of the B domain in proteolytic inactivation of activated coagulation factor VIII by activated protein C and activated factor X. *Blood Coagul Fibrinolysis,* 17(5): 379-88.

KOCHANEK, S. 1999. Development of high-capacity adenoviral vectors for gene therapy. *Thromb Haemost,* 82**,** 547-51.

KOCHANEK, S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther,* 10**,** 2451-9.

KOCHANEK, S., CLEMENS, P. R., MITANI, K., CHEN, H. H., CHAN, S. & CASKEY, C. T. 1996. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A,* 93**,** 5731-6.

KOEDAM, J. A., HAMER, R. J., BEESER-VISSER, N. H., BOUMA, B. N. & SIXMA, J. J. 1990. The effect of von Willebrand factor on activation of factor VIII by factor Xa. *Eur J Biochem,* 189**,** 229-34.

KOST, T. A. *et al.* 2000. Biosafety and viral gene transfer vectors. In *Biological Safety, Principles and practices* (3rd edn) (Flemming, D. O. and Hunt, D. L., eds), pp. 579-597, ASM Press.

KOST, T. A. & CONDREAY, J. P. 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends Biotechnol,* 20**,** 173-80.

KOST, T. A., CONDREAY, J. P. & JARVIS, D. L. 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol,* 23**,** 567-75.

KOZARSKY, K. F. & WILSON, J. M. 1993. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev,* 3**,** 499-503.

KROUGLIAK, V. & GRAHAM, F. L. 1995. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. *Hum Gene Ther,* 6**,** 1575-86.

LAPAN, K. A. & FAY, P. J. 1997. Localization of a factor X interactive site in the A1 subunit of factor VIIIa. *J Biol Chem,* 272**,** 2082-8.

LENTING, P. J., PEGON, J. N., CHRISTOPHE, O. D. & DENIS, C. V. 2010. Factor VIII and von Willebrand factor--too sweet for their own good. *Haemophilia,* 16 Suppl 5**,** 194-9.

LENTING, P. J., VAN MOURIK, J. A. & MERTENS, K. 1998. The life cycle of coagulation factor VIII in view of its structure and function. *Blood,* 92**,** 3983-96.

LEVINSON, B., KENWRICK, S., GAMEL, P., FISHER, K. & GITSCHIER, J. 1992. Evidence for a third transcript from the human factor VIII gene. *Genomics,* 14**,** 585-9.

LEVINSON, B., KENWRICK, S., LAKICH, D., HAMMONDS, G., JR. & GITSCHIER, J. 1990. A transcribed gene in an intron of the human factor VIII gene. *Genomics,* 7**,** 1-11.

LEYTE, A., VAN SCHIJNDEL, H. B., NIEHRS, C., HUTTNER, W. B., VERBEET, M. P., MERTENS, K. & VAN MOURIK, J. A. 1991. Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem,* 266**,** 740-6.

LI, X. & GABRIEL, D. A. 1997. The physical exchange of factor VIII (FVIII) between von Willebrand factor and activated platelets and the effect of the FVIII B-domain on platelet binding. *Biochemistry,* 36**,** 10760-7.

LIN, P. H., LAIBELMAN, A. M. & SINHA, U. 1997. Reversible acylation of factor Xa as a potential therapy for hemophilia. *Thromb Res,* 88**,** 365-72.

LJUNG, R. C. 1999. Prophylactic infusion regimens in the management of hemophilia. *Thromb Haemost.* 82(2), 525-30.

LOLLAR, P. 2003. The factor VIII assay problem: neither rhyme nor reason. *J Thromb Haemost,* 1**,** 2275-9.

LOLLAR, P., FAY, P. J. & FASS, D. N. 1993. Factor VIII and factor VIIIa. *Methods Enzymol,* 222**,** 128-43.

LOZIER, J. N., METZGER, M. E., DONAHUE, R. E. & MORGAN, R. A. 1999. Adenovirus-mediated expression of human coagulation factor IX in the rhesus macaque is associated with dose-limiting toxicity. *Blood,* 94**,** 3968-75.

MANN, K. G. 1999. Biochemistry and physiology of blood coagulation. *Thromb Haemost,* 82**,** 165-74.

MANNO, C. S., CHEW, A. J., HUTCHISON, S., LARSON, P. J., HERZOG, R. W., ARRUDA, V. R., TAI, S. J., RAGNI, M. V., THOMPSON, A., OZELO, M., COUTO, L. B., LEONARD, D. G., JOHNSON, F. A., MCCLELLAND, A., SCALLAN, C., SKARSGARD, E., FLAKE, A. W., KAY, M. A., HIGH, K. A. & GLADER, B. 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood,* 101**,** 2963-72.

MARQUETTE, K. A., PITTMAN, D. D. & KAUFMAN, R. J. 1995. A 110-amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. *J Biol Chem,* 270**,** 10297-303.

MEULIEN, P., FAURE, T., MISCHLERF, HARRER, H., ULRICH, P., BOUDER- BALA, B., DOTT, K., SAINTEMARIE, M., MAZURIERC, CAZENAVE, J-P., COURTNEY, M., PAVIRANI, A. 1988. A new recombinant procoagulant protein derived from the cDNA encoding human factor VIII. *Protein Eng*, 2:301.

MIAO, H. Z., SIRACHAINAN, N., PALMER, L., KUCAB, P., CUNNINGHAM, M. A., KAUFMAN, R. J. & PIPE, S. W. 2004. Bioengineering of coagulation factor VIII for improved secretion. *Blood,* 103**,** 3412-9.

MIKAELSSON, M., OSWALDSSON, U. & JANKOWSKI, M. A. 2001. Measurement of factor VIII activity of B-domain deleted recombinant factor VIII. *Semin Hematol,* 38**,** 13-23.

MILLER, L. K., 1997. The Baculoviruses. Plenum Press, New York.

MITANI, K., GRAHAM, F. L., CASKEY, C. T. & KOCHANEK, S. 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc Natl Acad Sci U S A,* 92**,** 3854-8.

MORRAL, N., O'NEAL, W., RICE, K., LELAND, M., KAPLAN, J., PIEDRA, P. A., ZHOU, H., PARKS, R. J., VELJI, R., AGUILAR-CORDOVA, E., WADSWORTH, S., GRAHAM, F. L., KOCHANEK, S., CAREY, K. D. & BEAUDET, A. L. 1999. Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc Natl Acad Sci U S A,* 96**,** 12816-21.

MORSY, M. A., GU, M., MOTZEL, S., ZHAO, J., LIN, J., SU, Q., ALLEN, H., FRANLIN, L., PARKS, R. J., GRAHAM, F. L., KOCHANEK, S., BETT, A. J. & CASKEY, C. T. 1998. An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc Natl Acad Sci U S A,* 95**,** 7866-71.

MOUSSALLI, M., PIPE, S. W., HAURI, H. P., NICHOLS, W. C., GINSBURG, D. & KAUFMAN, R. J. 1999. Mannose-dependent endoplasmic reticulum (ER)-Golgi intermediate compartment-53-mediated ER to Golgi trafficking of coagulation factors V and VIII. *J Biol Chem,* 274**,** 32539-42.

MURPHY, S. L. & HIGH, K. A. 2008. Gene therapy for haemophilia. *Br J Haematol,* 140**,** 479-87.

NILSSON, I. M. 1992. The management of hemophilia patients with inhibitors. *Transfus Med Rev,* 6**,** 285-93.

NOGAMI, K., SHIMA, M., HOSOKAWA, K., NAGATA, M., KOIDE, T., SAENKO, E. L., TANAKA, I., SHIBATA, M. & YOSHIOKA, A. 2000. Factor VIII C2 domain contains the thrombin-binding site responsible for thrombin-catalyzed cleavage at Arg1689. *J Biol Chem,* 275**,** 25774-80.

NOGAMI, K., SHIMA, M., HOSOKAWA, K., SUZUKI, T., KOIDE, T., SAENKO, E. L., SCANDELLA, D., SHIBATA, M., KAMISUE, S., TANAKA, I. & YOSHIOKA, A. 1999. Role of factor VIII C2 domain in factor VIII binding to factor Xa. *J Biol Chem,* 274**,** 31000-7.

O'BRIEN, D. P., JOHNSON, D., BYFIELD, P. & TUDDENHAM, E. G. 1992. Inactivation of factor VIII by factor IXa. *Biochemistry,* 31**,** 2805-12.

O'REILLY, D. R., MILLER, L. K. and LUCKOW, V.A. 1992. Baculovirus Expression Vectors: A Laboratory Manual. W. H. Freeman, New York.

O'REILLY, D.R. *et al.*, eds (1994). *Baculovirus Expression Vectors*, Oxford University Press.

PAGE, S. M. & BROWNLEE, G. G. 1997. An ex vivo keratinocyte model for gene therapy of hemophilia B. *J Invest Dermatol,* 109**,** 139-45.

PARKS, R. J. & GRAHAM, F. L. 1997. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol,* 71**,** 3293-8.

PENNOCK, G. D., SHOEMAKER, C. & MILLER, L. K. 1984. Strong and regulated expression of Escherichia coli beta-galactosidase in insect cells with a baculovirus vector. *Mol Cell Biol,* 4**,** 399-406.

PIERONI, L. & LA MONICA, N. 2001. Towards the use of baculovirus as a gene therapy vector. *Curr Opin Mol Ther,* 3**,** 464-7.

PIETERS, J., LINDHOUT, T. & HEMKER, H. C. 1989. In situ-generated thrombin is the only enzyme that effectively activates factor VIII and factor V in thromboplastin-activated plasma. *Blood,* 74**,** 1021-4.

PIPE, S. W. 2009. Functional roles of the factor VIII B domain. *Haemophilia,* 15**,** 1187-96.

PIPE, S. W., MORRIS, J. A., SHAH, J. & KAUFMAN, R. J. 1998. Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *J Biol Chem,* 273**,** 8537-44.

PITTMAN, D. D., ALDERMAN, E. M., TOMKINSON, K. N., WANG, J. H., GILES, A. R. & KAUFMAN, R. J. 1993. Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. *Blood,* 81**,** 2925-35.

PITTMAN, D. D. & KAUFMAN, R. J. 1989. Structure-function relationships of factor VIII elucidated through recombinant DNA technology. *Thromb Haemost,* 61**,** 161-5.

PITTMAN, D. D., MILLENSON, M., MARQUETTE, K., BAUER, K. & KAUFMAN, R. J. 1992. A2 domain of human recombinant-derived factor VIII is required for procoagulant activity but not for thrombin cleavage. *Blood,* 79**,** 389-97.

POOLE, S., FIRTEL, R. A., LAMAR, E. & ROWEKAMP, W. 1981. Sequence and expression of the discoidin I gene family in Dictyostelium discoideum. *J Mol Biol,* 153**,** 273-89.

POUSTKA, A., DIETRICH, A., LANGENSTEIN, G., TONIOLO, D., WARREN, S. T. & LEHRACH, H. 1991. Physical map of human Xq27-qter: localizing the region of the fragile X mutation. *Proc Natl Acad Sci U S A,* 88**,** 8302-6.

POWELL, J. S., RAGNI, M. V., WHITE, G. C., 2ND, LUSHER, J. M., HILLMAN-WISEMAN, C., MOON, T. E., COLE, V., RAMANATHAN-GIRISH, S., ROEHL, H., SAJJADI, N., JOLLY, D. J. & HURST, D. 2003. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood,* 102**,** 2038-45.

RAGNI, M. V. 2004. Hemophilia gene transfer: comparison with conventional protein replacement therapy. *Semin Thromb Hemost,* 30**,** 239-47.

RATNOFF O. D. (1991) Evolution of knowledge about hemostasis. In RATNOFF O. D., FORBES C. D. (eds): Disorders of hemostasis, 2nd edn. WB Saunders, Philadelphia, pp. 1-17.

RAY, M. J. & HAWSON, G. A. 1989. A comparison of two APTT reagents which use silica activators. *Clin Lab Haematol,* 11**,** 221-32.

REDDY, P. S., SAKHUJA, K., GANESH, S., YANG, L., KAYDA, D., BRANN, T., PATTISON, S., GOLIGHTLY, D., IDAMAKANTI, N., PINKSTAFF, A., KALOSS, M., BARJOT, C., CHAMBERLAIN, J. S., KALEKO, M. & CONNELLY, S. 2002. Sustained human factor VIII expression in hemophilia A mice following systemic delivery of a gutless adenoviral vector. *Mol Ther,* 5**,** 63-73.

REGAN, L. M., O'BRIEN, L. M., BEATTIE, T. L., SUDHAKAR, K., WALKER, F. J. & FAY, P. J. 1996. Activated protein C-catalyzed proteolysis of factor VIIIa alters its interactions within factor Xase. *J Biol Chem,* 271**,** 3982-7.

RICK, M. E., ESMON, N. L. & KRIZEK, D. M. 1990. Factor IXa and von Willebrand factor modify the inactivation of factor VIII by activated protein C. *J Lab Clin Med,* 115**,** 415-21.

ROGOFF, E. G., GUIRGUIS, H. S., LIPTON, R. A., SEREMETIS, S. V., DIMICHELE, D. M., AGNEW, G. M., KARPATKIN, M., BARISH, R. J., JONES, R. L., BIANCO, C., KNOTHE, B. D. & LEE, M. S. 2002. The upward spiral of drug costs: a time series analysis of drugs used in the treatment of hemophilia. *Thromb Haemost,* 88**,** 545-53.

ROSENBERG, J. B., GREENGARD, J. S. & MONTGOMERY, R. R. 2000. Genetic induction of a releasable pool of factor VIII in human endothelial cells. *Arterioscler Thromb Vasc Biol,* 20**,** 2689-95.

ROTH, D. A., TAWA, N. E., JR., O'BRIEN, J. M., TRECO, D. A., SELDEN, R. F. & FACTOR, V. T. T. S. G. 2001. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med,* 344**,** 1735-42.

SAENKO, E. L., SHIMA, M. & SARAFANOV, A. G. 1999. Role of activation of the coagulation factor VIII in interaction with vWf, phospholipid, and functioning within the factor Xase complex. *Trends Cardiovasc Med,* 9**,** 185-92.

SAMBROOK, J. and RUSSELL, D. W. 2001. *Molecular Cloning: A Laboratory Manual* (3rd ed.). Cold Spring Harbor Laboratory Press.

SANCAR, G. B., SMITH, F. W. & SANCAR, A. 1983. Identification and amplification of the E. coli phr gene product. *Nucleic Acids Res,* 11**,** 6667-78.

SANDBERG, H., ALMSTEDT, A., BRANDT, J., GRAY, E., HOLMQUIST, L., OSWALDSSON, U., SEBRING, S. & MIKAELSSON, M. 2001. Structural and functional characteristics of the B-domain-deleted recombinant factor VIII protein, r-VIII SQ. *Thromb Haemost,* 85**,** 93-100.

SANDIG, V., HOFMANN, C., STEINERT, S., JENNINGS, G., SCHLAG, P. & STRAUSS, M. 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. *Hum Gene Ther,* 7**,** 1937-45.

SARAFANOV, A. G., ANANYEVA, N. M., SHIMA, M. & SAENKO, E. L. 2001. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J Biol Chem,* 276**,** 11970-9.

SCHAFER, A. I. 1994 Coagulation Cascade: an Overview. In LOSCALZO J., SSCHAFER A. I. (eds): Thrombosis and Hemorrhage. Blackwell Scientific, Boston, pp. 3-12.

SCHAGEN, F. H., OSSEVOORT, M., TOES, R. E. & HOEBEN, R. C. 2004. Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion. *Crit Rev Oncol Hematol,* 50**,** 51-70.

SCHIEDNER, G., MORRAL, N., PARKS, R. J., WU, Y., KOOPMANS, S. C., LANGSTON, C., GRAHAM, F. L., BEAUDET, A. L. & KOCHANEK, S. 1998. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet,* 18**,** 180-3.

SCHNELL, M. A., ZHANG, Y., TAZELAAR, J., GAO, G. P., YU, Q. C., QIAN, R., CHEN, S. J., VARNAVSKI, A. N., LECLAIR, C., RAPER, S. E. & WILSON, J. M. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol Ther,* 3**,** 708-22.

SCHWARZ, H. P., LENTING, P. J., BINDER, B., MIHALY, J., DENIS, C., DORNER, F. & TURECEK, P. L. 2000. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood,* 95**,** 1703-8.

SHOJI, I., AIZAKI, H., TANI, H., ISHII, K., CHIBA, T., SAITO, I., MIYAMURA, T. & MATSUURA, Y. 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. *J Gen Virol,* 78 (Pt 10)**,** 2657-64.

SMITH, G. E., SUMMERS, M. D. & FRASER, M. J. 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol,* 3**,** 2156-65.

SOLLERBRANT, K., ELMEN, J., WAHLESTEDT, C., ACKER, J., LEBLOIS-PREHAUD, H., LATTA-MAHIEU, M., YEH, P. & PERRICAUDET, M. 2001. A novel method using baculovirus-mediated gene transfer for production of recombinant adeno-associated virus vectors. *J Gen Virol,* 82**,** 2051-60.

SOUCIE, J. M., EVATT, B. & JACKSON, D. 1998. Occurrence of hemophilia in the United States. The Hemophilia Surveillance System Project Investigators. *Am J Hematol,* 59**,** 288-94.

SROUR, M. A., GRUPP, J., ABURUBAIHA, Z., ALBERT, T., BRONDKE, H., OLDENBURG, J. & SCHWAAB, R. 2008. Modified expression of coagulation factor VIII by addition of a glycosylation site at the N terminus of the protein. *Ann Hematol,* 87**,** 107-12.

STUBBS, J. D., LEKUTIS, C., SINGER, K. L., BUI, A., YUZUKI, D., SRINIVASAN, U. & PARRY, G. 1990. cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. *Proc Natl Acad Sci U S A,* 87**,** 8417-21.

SWAROOP, M., MOUSSALLI, M., PIPE, S. W. & KAUFMAN, R. J. 1997. Mutagenesis of a potential immunoglobulin-binding protein-binding site enhances secretion of coagulation factor VIII. *J Biol Chem,* 272**,** 24121-4.

THOMPSON, A. R. 2003. Structure and function of the factor VIII gene and protein. *Semin Thromb Hemost,* 29**,** 11-22.

THORREZ, L., VANDENDRIESSCHE, T., COLLEN, D. & CHUAH, M. K. 2004. Preclinical gene therapy studies for hemophilia using adenoviral vectors. *Semin Thromb Hemost,* 30**,** 173-83.

TOOLE, J. J., KNOPF, J. L., WOZNEY, J. M., SULTZMAN, L. A., BUECKER, J. L., PITTMAN, D. D., KAUFMAN, R. J., BROWN, E., SHOEMAKER, C., ORR, E. C. & ET AL. 1984. Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature,* 312**,** 342-7.

TUDDENHAM, E. G. 2003. In search of the eighth factor: a personal reminiscence. *J Thromb Haemost,* 1**,** 403-9.

TUDDENHAM, E. G., SCHWAAB, R., SEEHAFER, J., MILLAR, D. S., GITSCHIER, J., HIGUCHI, M., BIDICHANDANI, S., CONNOR, J. M., HOYER, L. W., YOSHIOKA, A. & ET AL. 1994. Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene, second edition. *Nucleic Acids Res,* 22**,** 3511-33.

TURI, D. C. & PEERSCHKE, E. I. 1986. Sensitivity of three activated partial thromboplastin time reagents to coagulation factor deficiencies. *Am J Clin Pathol,* 85**,** 43-9.

URNOV, F. D., MILLER, J. C., LEE, Y. L., BEAUSEJOUR, C. M., ROCK, J. M., AUGUSTUS, S., JAMIESON, A. C., PORTEUS, M. H., GREGORY, P. D. & HOLMES, M. C. 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature,* 435**,** 646-51.

VAN DE VEN, W. J., VOORBERG, J., FONTIJN, R., PANNEKOEK, H., VAN DEN OUWELAND, A. M., VAN DUIJNHOVEN, H. L., ROEBROEK, A. J. & SIEZEN, R. J. 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol Biol Rep,* 14**,** 265-75.

VAN DEN BESSELAAR, A. M., NEUTEBOOM, J. & BERTINA, R. M. 1993. Effect of synthetic phospholipids on the response of the activated partial thromboplastin time to heparin. *Blood Coagul Fibrinolysis,* 4**,** 895-903.

VAN DIEIJEN, G., TANS, G., ROSING, J. & HEMKER, H. C. 1981. The role of phospholipid and factor VIIIa in the activation of bovine factor X. *J Biol Chem,* 256**,** 3433-42.

VANDENDRIESSCHE, T., VANSLEMBROUCK, V., GOOVAERTS, I., ZWINNEN, H., VANDERHAEGHEN, M. L., COLLEN, D. & CHUAH, M. K. 1999. Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. *Proc Natl Acad Sci U S A,* 96**,** 10379-84.

VAUGHN, J. L., GOODWIN, R. H., TOMPKINS, G. J. & MCCAWLEY, P. 1977. The establishment of two cell lines from the insect Spodoptera frugiperda (Lepidoptera; Noctuidae). *In Vitro,* 13**,** 213-7.

VEHAR, G. A., KEYT, B., EATON, D., RODRIGUEZ, H., O'BRIEN, D. P., ROTBLAT, F., OPPERMANN, H., KECK, R., WOOD, W. I., HARKINS, R. N. & ET AL. 1984. Structure of human factor VIII. *Nature,* 312**,** 337-42.

VLOT, A. J., KOPPELMAN, S. J., VAN DEN BERG, M. H., BOUMA, B. N. & SIXMA, J. J. 1995. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood,* 85**,** 3150-7.

VORBURGER, S. A. & HUNT, K. K. 2002. Adenoviral gene therapy. *Oncologist,* 7**,** 46-59. Williams Hematology, 5th edition, 1995.

WION, K. L., KELLY, D., SUMMERFIELD, J. A., TUDDENHAM, E. G. & LAWN, R. M. 1985. Distribution of factor VIII mRNA and antigen in human liver and other tissues. *Nature,* 317**,** 726-9.

WOOD, W. I., CAPON, D. J., SIMONSEN, C. C., EATON, D. L., GITSCHIER, J., KEYT, B., SEEBURG, P. H., SMITH, D. H., HOLLINGSHEAD, P., WION, K. L. & ET AL. 1984. Expression of active human factor VIII from recombinant DNA clones. *Nature,* 312**,** 330-7.

YAO, S. N., WILSON, J. M., NABEL, E. G., KURACHI, S., HACHIYA, H. L. & KURACHI, K. 1991. Expression of human factor IX in rat capillary endothelial cells: toward somatic gene therapy for hemophilia B. *Proc Natl Acad Sci U S A,* 88**,** 8101-5.

YEH, P., DEDIEU, J. F., ORSINI, C., VIGNE, E., DENEFLE, P. & PERRICAUDET, M. 1996. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. *J Virol,* 70**,** 559-65.

ZOU, L., ZHOU, H., PASTORE, L. & YANG, K. 2000. Prolonged transgene expression mediated by a helper-dependent adenoviral vector (hdAd) in the central nervous system. *Mol Ther,* 2**,** 105-13.

7. Appendix

Schematic representation of the newly constructed B-domain deleted expression vectors (pMT2-F8-BDD, pMT2-F8-101, pMT2-F8-202, pMT2-F8-292, pMT2-F8-394).