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Identification of Porcine Coagulation Factor VIII Domains Responsible for High Level Expression via Enhanced Secretion*

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Blood coagulation factor VIII has a domain structure designated A1-A2-B-*ap*-A3-C1-C2. Human factor VIII is present at low concentration in normal plasma and, comparably, is produced at low levels *in vitro* and *in vivo* using transgenic expression techniques. Heterologous expression of B domain-deleted porcine factor VIII in mammalian cell culture is significantly greater than B domain-deleted human or murine factor VIII. Novel hybrid human/porcine factor VIII molecules were constructed to identify porcine factor VIII domains that confer high level expression. Hybrid human/porcine factor VIII constructs containing the porcine factor VIII A1 and *ap*-A3 domains expressed at levels comparable with recombinant porcine factor VIII. A hybrid construct containing only the porcine A1 domain expressed at intermediate levels between human and porcine factor VIII, whereas a hybrid construct containing the porcine *ap*-A3 domain expressed at levels comparable with human factor VIII. Additionally, hybrid murine/porcine factor VIII constructs containing the porcine factor VIII A1 and *ap*-A3 domain sequences expressed at levels significantly higher than recombinant murine factor VIII. Therefore, the porcine A1 and *ap*-A3 domains are necessary and sufficient for the high level expression associated with porcine factor VIII. Metabolic radiolabeling experiments demonstrated that high level expression was attributable to enhanced secretory efficiency.

Factor VIII (fVIII)[1] is a plasma protein that functions in proteolytically activated form as a cofactor within the intrinsic pathway of blood coagulation to increase the rate of proteolytic activation of factor X by activated factor IX. fVIII contains a domain structure designated A1-A2-B-*ap*-A3-C1-C2 that is defined based on internal sequence homology (1, 2). The fVIII A domains share homology with the copper-binding protein ceruloplasmin (3, 4), which has an A1-A2-A3 domain structure in which the three A domains are arranged along a pseudo-3-fold axis of symmetry (5). Before cell secretion, fVIII is cleaved at the B/*ap*-A3 domain junction into A1-A2-B (heavy chain) and *ap*-A3-C1-C2 (light chain) subunits. fVIII circulates in the plasma as an inactive heavy chain/light chain heterodimeric procofactor that is non-covalently bound to von Willebrand factor. Proteolytic activation of fVIII by thrombin results from cleavages at Arg-372 between the A1 and A2 domains, Arg-740 between the A2 and B domains, and Arg-1689 between the *ap* and A3 domains. During this process, the covalent linkage between the A1 and A2 domains is lost, and the B domain and 41-residue *ap* are released, producing a heterotrimeric, A1/A2/A3-C1-C2 subunit structure (6).

Genetic mutations leading to insufficient or defective fVIII are responsible for the sex-linked bleeding disorder hemophilia A, which affects 1 in 5,000–10,000 males worldwide. Hemophilia A treatment consists of intravenous administration of plasma-derived or recombinant fVIII protein, the cost of which can exceed $100,000 per patient-year (7). The expense of recombinant fVIII products is partly due to manufacturing costs associated with inefficient production of fVIII in mammalian cell culture (8).

Extensive research has been conducted on the genetic and biochemical regulation of fVIII biosynthesis (for a review see Kaufman *et al.* (9)). Low level expression of endogenous fVIII has complicated the study of the regulation of fVIII expression *in vivo*. Consequently, most studies of fVIII biosynthesis have been performed in heterologous mammalian cell culture systems. Several factors have been proposed to limit fVIII expression, including transcriptional inhibition (10, 11), reduced mRNA accumulation (12), interaction with protein chaperones (9, 13–16), and fVIII instability in the absence of von Willebrand factor (17, 18). Recently, we found that B domain-deleted recombinant porcine fVIII was expressed at 10–14-fold greater levels than B domain-deleted recombinant human fVIII (19). In the current study we used homolog-scanning mutagenesis (20), employing a series of hybrid human/porcine (HP) and hybrid murine/porcine (MP) cDNAs to identify the porcine fVIII domains that confer high level expression and to describe the regulatory mechanism involved.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum, penicillin, streptomycin, HEPES, glutamax (L-alanyl-L-glutamine), sodium pyruvate, Geneticin, Lipofectin, LipofectAMINE, Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, methionine-free DMEM, AIM V medium, and oligonucleotides were purchased from Invitrogen. [^{35}S]Methionine was purchased from Amersham Biosciences. The RL-cytomegalovirus vector and dual-luciferase assay kit were purchased from Promega (Madison, WI). Citrated human fVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KA). Activated partial thromboplastin reagent was purchased from Organon Teknika (Durham, NC). ESH-8, an anti-fVIII C2 domain-specific murine monoclonal antibody, was purchased from American Diagnostica (Greenwich, CT). An anti-fVIII A2 domain-specific murine monoclonal antibody, designated 4C7–1B, was produced by a hybridoma cell line derived from the fusion of a NS-1 myeloma cell line (21) with spleens from mice immunized intravenously with human B domain-deleted fVIII as described previously (22). TriReagent and Triton X-100 were purchased from Sigma. M-PER mammalian protein extraction reagent,

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[1] The abbreviations used are: fVIII, factor VIII; HP, hybrid human/porcine; MP, hybrid murine/porcine; DPBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BHK-M, baby hamster kidney-derived; RT, reverse transcription; rh, recombinant human; rp, recombinant porcine; rm, recombinant murine.

Halt protease inhibitor mixture, ImmunoPure immobilized protein G, ImmunoPure lane marker sample buffer, Tween 80, and Gel Code Blue were purchased from Pierce. Ready-Cast SDS-PAGE gels were purchased from Bio-Rad. Chromatography paper was purchased from Fisher.

Generation of Hybrid fVIII cDNAs—The synthesis of B domain-deleted human, porcine, and murine cDNA constructs, designated rh-fVIII, rp-fVIII, and rm-fVIII, in the mammalian expression vector Re-Neo has been described previously (19, 23). These constructs contain a nucleotide sequence between the A2 and *ap*-A3 domains encoding a linker region of human, porcine, or murine origin (designated SQ, OL, or SQ, respectively), which includes the Arg-His-Gln-Arg recognition sequence for PACE/furin processing (24, 25). HP and MP fVIII constructs were generated by splicing-by-overlap extension mutagenesis (26) using previously published procedures (27–29) and rh-fVIII, rp-fVIII, and rm-fVIII as starting materials (Fig. 1). SQ or OL linker sequences were added to previously described HP fVIII cDNA constructs (28–30) to produce constructs designated HP1, HP18, HP22, and HP30 (Fig. 1). HP1 and HP22 contain the SQ sequence, whereas HP18 and HP30 contain the OL sequence.

fVIII Expression—Transient expression of the fVIII constructs from COS-7 cells and stable expression from baby hamster kidney-derived (BHK-M) cells was performed as described previously (19). Briefly, COS-7 cells were grown to 70–80% confluence in 2-cm^2 wells containing 1 ml of DMEM/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using LipofectAMINE and a 2000:1 mass ratio of fVIII construct:luciferase plasmid DNA. Twenty-four hours after transfection the cells were rinsed twice with DPBS, and 0.5 ml of AIM V serum-free medium was added to each well. Transfection of BHK-M cells was performed using Lipofectin according to the manufacturer's instructions. Transfected cells were cultured for 10 days in the presence of DMEM/F-12 containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml Geneticin. Geneticin-resistant clones were screened for fVIII production, seeded onto 2-cm^2 wells, and grown to 80–90% confluence before being switched to 1 ml of AIM V serum-free medium. COS-7 or BHK-M cells were cultured in serum-free medium for 24 h before measurement of secreted fVIII coagulant activity as described below. Expression of fVIII from COS-7 cells was normalized to expression of luciferase activity, which was measured using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Factor VIII Assays—Measurements of fVIII activity were made by one-stage and two-stage coagulation assays in a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France) using human fVIII-deficient plasma as substrate as described previously (23). The activation quotient is defined as the activity in the two-stage assay divided by the activity in the one-stage assay.

fVIII mRNA Analysis—Measurement of steady-state fVIII RNA levels was performed by real-time reverse transcription (RT)-PCR analysis using the ABI 7000 Sequence Detection System and the ABI SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) as described previously (19). Briefly, reaction mixtures containing 1× SYBR Green PCR master mix, 300 μM forward and reverse oligonucleotide primers, 6.25 units of Multi-Scribe reverse transcriptase, and 5 ng of sample total RNA were incubated at 48 °C for 30 min followed by incubation at 95 °C for 10 min and 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. Post-reaction dissociation curve analysis was performed to confirm correct target amplification. Absolute quantitation was achieved by linear regression and comparison to a standard curve generated using known quantities of *in vitro* transcribed human fVIII RNA. The number of fVIII transcripts per cell was calculated using a value of 35 μg of total RNA per 10^6 BHK-M cells.

Metabolic Radiolabeling and fVIII Immunoprecipitation—BHK-M cells stably expressing rh-fVIII or HP47 were seeded onto 35-mm dishes and grown to 80–90% confluence at which point there were ~8.5×10^5 cells/dish. Before radiolabeling the cells were incubated in methionine-free DMEM for 15 min. For radiolabeling, cells were incubated in methionine-free DMEM supplemented with 100 μCi/ml [^{35}S]methionine (>1000 Ci/mmol), 25 mM HEPES, 110 μg/ml sodium pyruvate, and 2 mM glutamax for 20 min. Where indicated the cells were rinsed twice and incubated for 4 h in 1 ml of DMEM containing 15 μg/ml (100-fold excess) unlabeled methionine (chase period). Cell lysates were prepared by rinsing the cells twice in DPBS followed by the addition of 1 ml of M-PER protein extraction reagent containing Halt protease inhibitor mixture at the concentration specified by the manufacturer. Whole cell lysates were subjected to centrifugation at 12,000 × *g*. The resulting supernatants were used immediately in immunoprecipitation reactions or stored at −70 °C until use. After the 4-h chase period, conditioned medium was collected and subjected to centrifugation at 1000 × *g* for 15 min for removal of cell debris. Halt protease inhibitor mixture was added immediately to supernatants at the concentration specified by the manufacturer. Before immunoprecipitation, cell lysate and conditioned medium samples were pre-cleared by the addition of 25 μl of protein G-agarose and incubation at 4 °C for 1 h with gentle rocking. The protein G-agarose was removed by centrifugation at 12,000 × *g* for 3 min. Next, 5 μg of ESH-8 or 4C7–1B murine monoclonal anti-human fVIII IgG was added to the supernatants followed by incubation at 4 °C for 1 h with gentle rocking. fVIII-containing immune complexes were precipitated by incubation with 50 μg of protein G-agarose at 4 °C overnight with gentle rocking. Protein G-agarose-immune complexes were collected by centrifugation at 12,000 × *g* for 3 min followed by three consecutive washes with 1 ml of 1, 0.1, and 0.05% Triton X-100 in DPBS. Where indicated the final protein G-agarose pellets were re-suspended in 20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80, pH 7.4, before the addition of 10 units/ml porcine thrombin and incubation at 37 °C for 1 h. Bound proteins were eluted from protein G-agarose by the addition of 25 μl of SDS sample buffer and incubation at 95 °C for 10 min. Immunoprecipitated proteins were reduced and separated by SDS-PAGE, vacuum-dried to chromatography paper, and visualized by autoradiography using Kodak BioMax MR film. Quantitation of autoradiograph band intensities was performed using Un-Scan It software (Silk Scientific, Orem, UT).

Purification and Characterization of HP47—HP47 was purified using a two-step ion-exchange chromatography procedure as described previously for the purification of rh-fVIII and rp-fVIII (19) with the following modifications. Conditioned medium containing HP47 was loaded onto a 2.5 × 19.5-cm SP-Sepharose Fast Flow column equilibrated at 25 °C in 0.18 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.01% Tween 80, pH 7.4. HP47 was eluted using a linear 0.18–0.7 M NaCl gradient in the same buffer. fVIII-containing fractions were identified as described above by one-stage and two-stage activation quotient coagulation assays and SDS-PAGE. The specific activity of HP47 was calculated using a molar extinction coefficient determined from the absorbance at 280 nm and the predicted tyrosine, tryptophan, and cysteine content (31). The specific activity of the final material was defined as the weighted number average of the specific activities of the fVIII peak fractions excluding any fractions demonstrating an absorbance at 280 nm less than 0.08 or an activation quotient less than 20.

RESULTS

Expression of HP fVIII Constructs—We previously found that a B domain-deleted porcine fVIII construct designated rp-fVIII was expressed at significantly higher levels than a B domain-deleted human fVIII designated rh-fVIII in COS-7 cells and BHK-M cells (19). To identify the porcine fVIII domains that confer high level expression, we generated a panel of HP fVIII constructs (Fig. 1) and compared their heterologous expression capabilities in transiently transfected COS-7 cells and stably transfected BHK-M cells (Fig. 2, *A* and *B*, respectively). In COS-7 cells expression of rp-fVIII was 4.6 ± 1.2-fold higher than rh-fVIII (mean ± 1 S.D.). Expression of HP44, a construct containing the porcine A1, A2, and *ap*-A3 domain sequences, was 6.9 ± 1.2-fold higher than rh-fVIII and was slightly higher than expression of rp-fVIII ($p = 0.03$, Student's t test). In contrast, HP57, which contains the porcine C1 and C2 domain sequences, expressed similarly to rh-fVIII. Thus, the porcine A domains, but not the C domains, are responsible for the high level expression associated with porcine fVIII. Expression of HP47, which contains the porcine A1 and *ap*-A3 domain sequences, was 5.3 ± 0.75-fold greater than rh-fVIII and was not significantly different from rp-fVIII. HP46, a construct containing the porcine A1 domain sequence, expressed at a level intermediate between rh-fVIII and rp-fVIII. In contrast, HP1 and HP30, which contain the porcine A2 and *ap*-A3 domain sequences, respectively, expressed similarly to rh-fVIII. HP45, which contains the porcine A2 and *ap*-A3 domain sequences, expressed at a level intermediate between rh-fVIII and rp-fVIII.

Results in BHK-M cells were largely consistent with the results in COS-7 cells. Average expression levels of rp-fVIII, HP44, and HP47 individual clonal cell lines were similar and

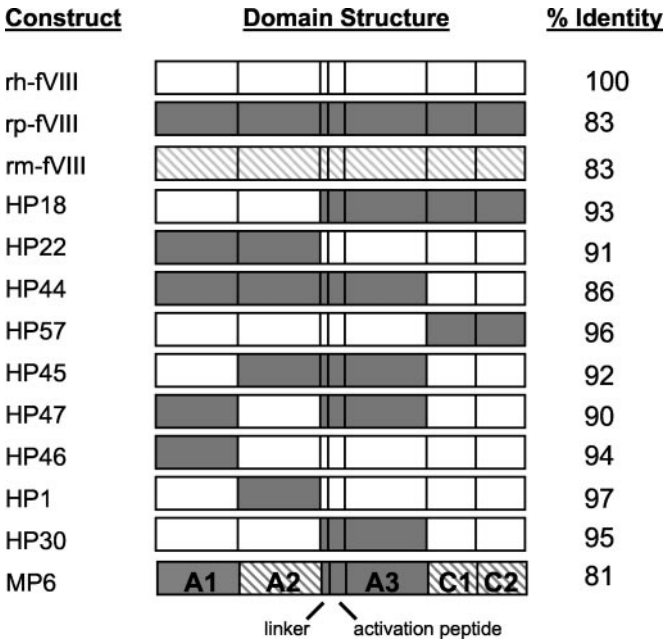

FIG. 1. **Schematic diagram of fVIII expression constructs.** B domain-deleted fVIII expression constructs containing combinations of human (*white*), porcine (*gray*), and murine (*hatched*) domain sequences were generated. The percent amino acid identity to B domain-deleted recombinant human fVIII (rh-fVIII) is given for each construct. fVIII domain boundaries were defined using the human fVIII amino acid sequence numbering (41) as follows; residues 1–372 (A1), 373–740 (A2), 1649–1689 (*ap*), 1690–2019 (A3), 2020–2172 (C1) and 2173–2332 (C2).

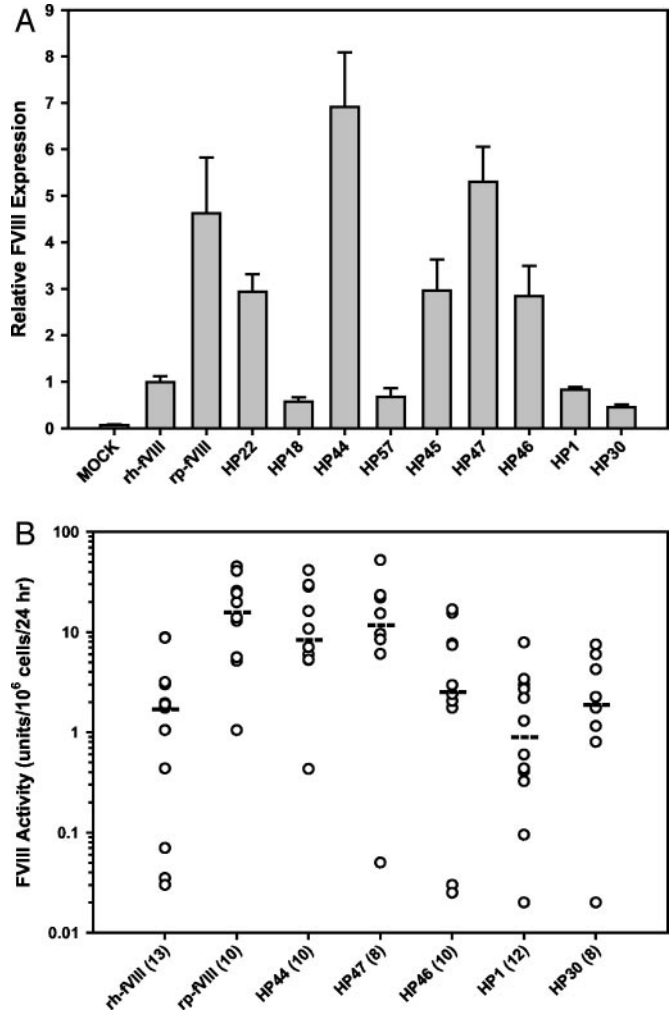

FIG. 2. **Transient and stable expression of HP fVIII constructs.** *A*, fVIII expression constructs and a luciferase control plasmid were transiently transfected into COS-7 cells. fVIII activity in the conditioned medium was determined by one-stage coagulation assay and normalized to luciferase activity measured in the corresponding cell lysate. The data are expressed relative to rh-fVIII as mean ± 1 S.D. from 4 separate transfections and are representative of 3 independent experiments. *B*, BHK-M cells were transfected with fVIII expression constructs and subsequently selected for transgene integration. Individual clones expressing each construct were assayed for fVIII production by one-stage coagulation assay. The *horizontal bars* represent the median value for each set of clones. The number of clones analyzed is given in *parentheses*.

were significantly higher than rh-fVIII ($p < 0.001$, $p = 0.003$ and $p = 0.006$, respectively, Mann-Whitney *U* test). In contrast, expression of HP46 was not significantly different from rh-fVIII expression ($p = 0.34$), although expression from the top-producing clone was ~2-fold greater than the top-producing rh-fVIII clone (17 units/10^6 cells/24 h *versus* 9 units/10^6 cells/24 h). HP1 and HP30 expressed similarly to rh-fVIII. Taken together the results in COS-7 and BHK-M cells indicate that the porcine A1 and *ap*-A3 domains in HP fVIII cDNAs are necessary and sufficient to produce expression levels equivalent to rp-fVIII.

Expression of MP fVIII Constructs—We previously showed that a recombinant B domain-deleted murine fVIII designated rm-fVIII expresses at lower levels than rh-fVIII in BHK-M cells (23). Because the porcine A1 and *ap*-A3 domains confer high level expression in human/porcine fVIII hybrids, we wanted to address whether they increase expression when substituted into murine fVIII. A MP fVIII cDNA containing the porcine A1 and *ap*-A3 domains, MP6, was constructed, and its expression was compared with rm-fVIII in stably transfected BHK-M cells (Fig. 3). Expression of fVIII from the MP6 clones was significantly greater than from the corresponding rm-fVIII clones ($p = 0.001$, Mann-Whitney *U* test). Peak expression for the top-producing MP6 clone was 23 units/10^6 cells/24 h, compared with 0.5 units/10^6 cells/24 h for the top-producing rm-fVIII clone.

Relative Expression Efficiency of Human, Porcine, and HP fVIII—Increased expression of porcine and HP fVIII constructs could be occurring through a differential at the mRNA, translational, or secretory level. Steady-state fVIII mRNA levels in the top expressing clonal cell lines were measured using quantitative real-time RT-PCR (Fig. 4). No correlation between fVIII activity and fVIII transcripts was observed. Further studies designed to uncover the mechanism driving the expression differential were performed using the stably transfected BHK-M/rh-fVIII clone C7 and the BHK-M/HP47 clone F5. fVIII activity measurements and real-time RT-PCR analysis of the steady-state fVIII mRNA levels demonstrated that HP47 clone F5 expressed at a level 9-fold greater than rh-fVIII clone C7 despite the fact that HP47 clone F5 contained 0.7-fold the level of fVIII transcripts (Fig. 5*A*). Relative translational and secretory efficiencies of rh-fVIII and HP47 were determined by pulse-labeling cells for 20 min in medium containing [^{35}S]methionine followed by a 4-h chase incubation in medium containing excess unlabeled methionine and immunoprecipitation using either anti-human fVIII heavy chain (4C7–1B)- or light chain (ESH-8)-specific murine monoclonal antibodies (Fig. 5, *B* and *C*). A band consistent with unprocessed, single chain fVIII was recovered from cell lysates after the pulse-labeling period. This band was not detected in the non-transfected, parental BHK-M cell line (data not shown). Quantitation of the single chain bands revealed rh-fVIII/HP47 ratios of 0.83 and 1.15 when either 4C7–1B or ESH-8, respectively, was used in the immunoprecipitation reaction (Fig. 5*D*). In both cases the differential was not statistically significant. After the chase pe-

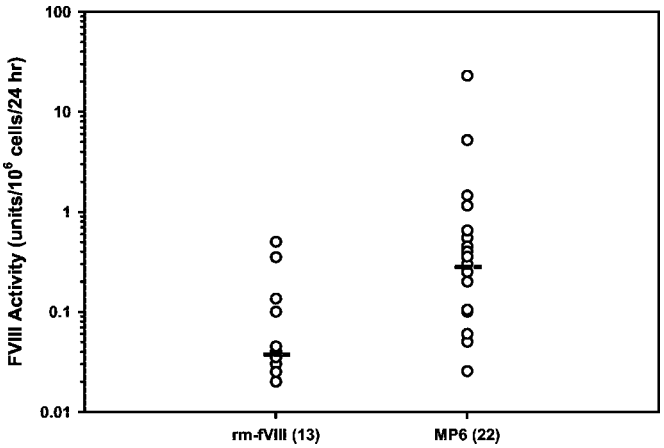

FIG. 3. **High level expression of a MP fVIII construct.** Murine A1 and *ap*-A3 domain sequences were replaced with the corresponding porcine sequences to create a MP fVIII construct designated MP6. Rm-fVIII and MP6 were transfected into BHK-M cells, and expression of fVIII from individual clones was performed as described under "Experimental Procedures." The *horizontal bar* represents the median value, and the value in *parentheses* indicates the number of clones included in the analysis.

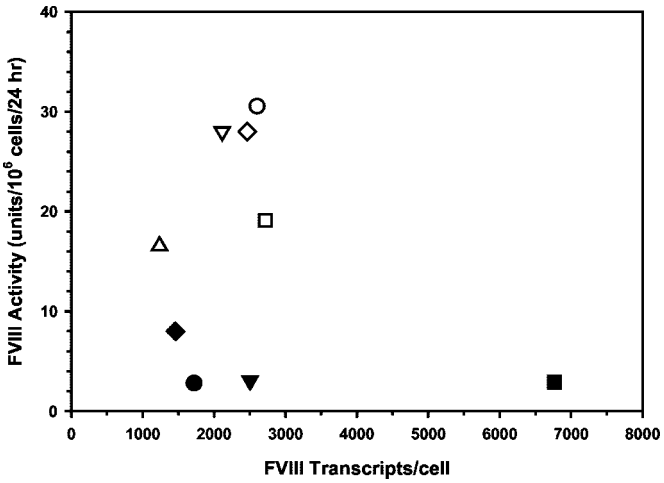

FIG. 4. **fVIII expression levels versus steady-state RNA levels.** Top fVIII-producing BHK-M clones screened from 24–48 stable clones each were seeded into a 75-cm^2 flask, grown to ~90% confluence, and cultured for 24 h in 25 ml of serum-free medium. Conditioned medium was assayed for fVIII activity by one-stage coagulation assay, and the cells were harvested for total RNA purification. Steady-state fVIII RNA levels were determined using quantitative real-time RT-PCR. The symbol designations for the constructs are rh-fVIII (*closed circle*), rp-fVIII (*open circle*), HP22 (*closed diamond*), HP44 (*inverted open triangle*), HP45 (*open square*), HP47 (*open diamond*), HP46 (*open triangle*), HP1 (*inverted closed triangle*), and HP30 (closed square).

riod significantly less single chain HP47 material remained in the cell lysates ($p = 0.005$ and 0.04, for the 4C7–1B and ESH-8 immunoprecipitations, respectively). In contrast, the differentials between pulse and chase rh-fVIII levels were not significant ($p = 0.55$ and 0.1, for the 4C7–1B and ESH-8 immunoprecipitations, respectively). In addition to single chain fVIII, bands consistent with heavy and light chain fVIII were observed in the chase cell lysates (Fig. 5*B*). The identity of these bands was confirmed by subsequent thrombin proteolysis (data not shown). Interestingly, rh-fVIII light chain, but not HP47 light chain, was recovered from the 4C7–1B-immunoprecipitated samples despite the presence of 2.7-fold more HP47 heavy chain material. Additionally, rh-fVIII heavy chain, but not HP47 heavy chain, was recovered from the ESH-8-immunoprecipitated samples. In contrast to the 4C7–1B results, excess free light chain was not observed in the HP47 samples compared with the rh-fVIII samples. Consistent with the observed differential in intracellular single chain fVIII material recovered after the pulse and chase periods, immunoprecipitation of the conditioned medium with 4C7–1B and ESH-8 revealed the presence of 3.8- and 3.9-fold more HP47 than rh-fVIII, respectively (Fig. 5, *C* and *D*). Rh-fVIII- and HP47-conditioned medium samples immunoprecipitated with 4C7–1B contained similar amounts of heavy and light chain, whereas identical samples immunoprecipitated with ESH-8 contained significantly more light chain than heavy chain material ($p = 0.004$ and 0.004, respectively).

It is possible that the mechanism governing the expression differential between BHK-M/rh-fVIII clone C6 and BHK-M/HP47 clone F5 results from genetic alterations that accumulated during the generation of these highly selected clonal cell lines. This possibility was addressed by repeating the radiolabeling experiments described above using mixed populations of 50–100 clonal cell lines stably transfected with the rh-fVIII or HP47 constructs and performing immunoprecipitations with 4C7–1B (Fig. 6). Rh-fVIII and HP47 were expressed from these cell populations at rates of 0.48 ± 0.12 and 2.37 ± 0.25 units/10^6 cells/24 h, respectively. Results of the metabolic labeling analysis were consistent with those obtained for the individual clones. We observed a significant difference in the amount of single chain HP47, but not single chain rh-fVIII, present in the pulse *versus* chase cells lysates ($p = 0.005$ and 0.22, respectively, Student's t test), and significantly more HP47 than rh-fVIII was secreted into the conditioned medium ($p = 0.001$).

Isolation and Characterization of HP47—HP47 was isolated using a two-step ion-exchange chromatography procedure as described under "Experimental Procedures." Approximately 1.3 mg was obtained from 3 liters of conditioned cell culture medium. The purity of HP47 was assessed by SDS-PAGE and compared with rh-fVIII and rp-fVIII (Fig. 7). A small amount of single chain material that was sensitive to cleavage by thrombin was present. No other major contaminants were observed. The specific activity of HP47 was calculated to be 2,530 units/nmol (15,300 units/mg) using a molar extinction coefficient at 280 nm of 254,830 M^{-1} cm^{-1} based on the predicted tyrosine, tryptophan, and cysteine content (31). This value is slightly higher than the previously reported average specific activity of four preparations of rh-fVIII, 1630 ± 530 units/nmol (19).

DISCUSSION

There are significant interspecies differences in the expression, coagulant activity, and antigenicity of fVIII. In previous studies we used homolog-scanning mutagenesis to identify antigenic determinants within human fVIII that are recognized by clinically significant inhibitory antibodies (27–30, 32, 33). In the current study we employed this approach to examine variation in the expression of human and porcine fVIII. We found that the porcine fVIII A1 and *ap*-A3 domains are necessary for high level expression of a HP fVIII construct and sufficient to confer greater expression to a MP fVIII construct. The effect of the porcine A1 and *ap*-A3 domains on expression is synergistic because the summed expression of HP constructs containing only the porcine A1 domain (HP46) or only the *ap*-A3 domains (HP30) is considerably lower than HP47, which contains both the porcine A1 and *ap*-A3 domains (Fig. 2).

We did not observe a correlation between fVIII production and steady-state fVIII mRNA levels (Figs. 4 and 5*A*). This is consistent with our previous observation that there was no difference in the distribution of steady-state mRNA levels between human and porcine fVIII-expressing BHK-M clones despite up to a 14-fold greater production of porcine fVIII activity (19). Therefore, porcine fVIII is expressed with greater efficiency on a per mRNA basis than human fVIII due to an

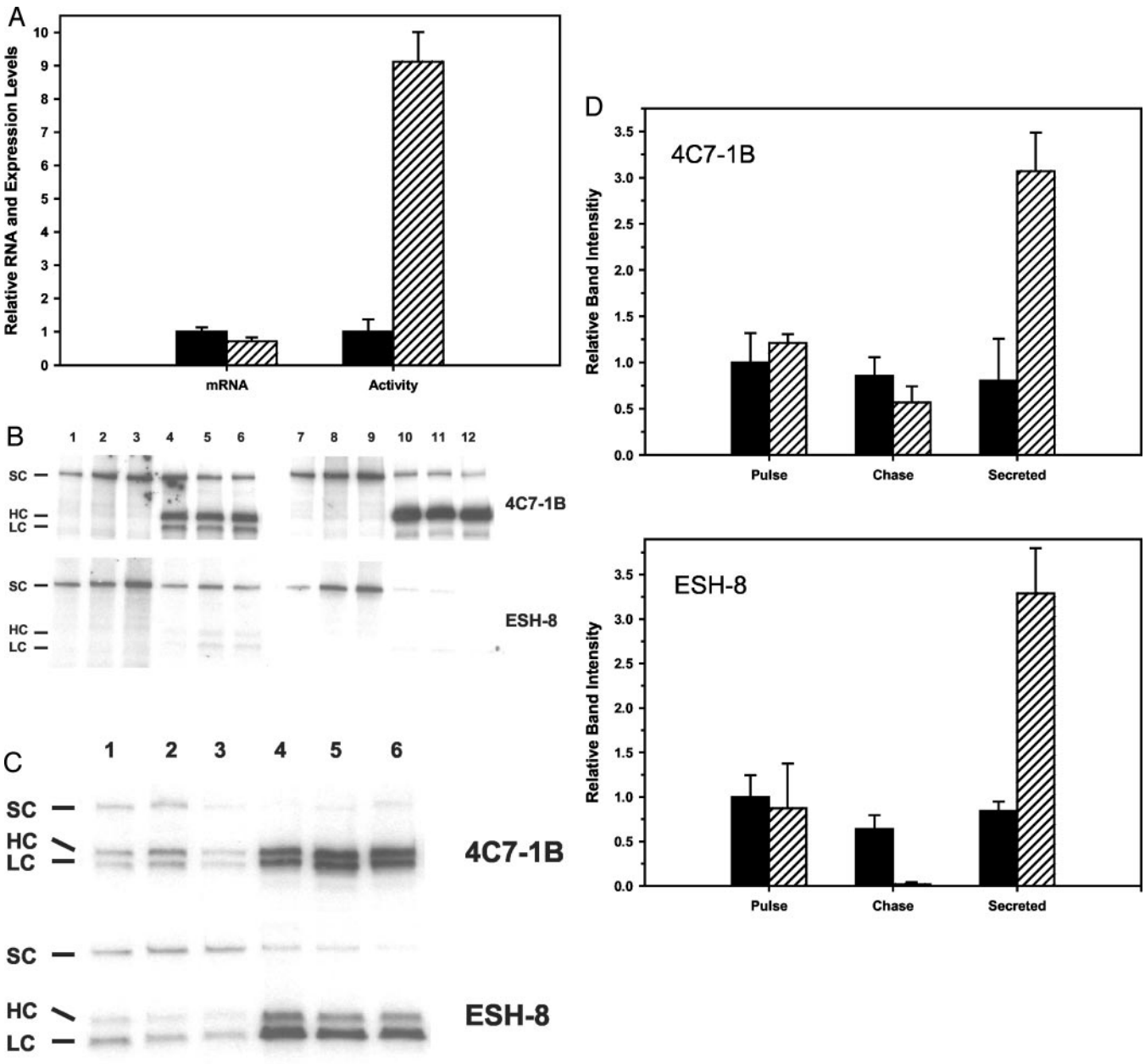

FIG. 5. **Characterization of fVIII biosynthesis in rh-fVIII- and HP47-expressing BHK-M cell lines.** *A*, total RNA and conditioned medium from quadruplicate 35-mm culture dishes containing BHK-M/rh-fVIII clone C7 (*solid bars*) or BHK-M/HP47 clone F5 (*hatched bars*) were harvested and analyzed for fVIII mRNA and fVIII activity levels, respectively, as described under "Experimental Procedures." Data are expressed relative to BHK-M/rh-fVIII clone C7. *B*, BHK-M/rh-fVIII clone C7 (*lanes 1–6*) and BHK-M/HP47 clone F5 (*lanes 7–12*) cell lines were metabolically labeled with [^{35}S]methionine for 20 min. Post-radiolabeling, cells in triplicate 35-mm culture dishes were harvested immediately (*lanes 1–3* and *7–9*) or switched to medium containing excess unlabeled methionine and incubated for 4 h (*lanes 4–6* and *10–12*). fVIII was immunoprecipitated from cell lysates using an anti-human fVIII heavy chain (*top panel*, 4C7–1B)- or light chain (*bottom panel*, ESH-8)-specific antibody. Proteins were resolved by reducing 7.5% SDS-PAGE as described under "Experimental Procedures," Single chain (*SC*), heavy chain (*HC*), and light chain (*LC*) polypeptides are labeled. *C*, Rh-fVIII (*lanes 1–3*) and HP47 (*lanes 4–6*) were immunoprecipitated from conditioned medium after the 4-h chase incubation and resolved as described above. *D*, cell lysate pulse and chase single chain or secreted summed single/heavy/light chain autoradiograph band intensities for rh-fVIII (*solid bars*) and HP47 (*hatched bars*) are presented as the mean ± 1 S.D. relative to the mean value obtained for pulse-labeled rh-fVIII.

inequality in translational and/or posttranslational processing resulting from sequence disparity within the A1 and *ap*-A3 domains. Results of metabolic labeling experiments support the latter as the major determinant of high level expression (Figs. 5, *B* and *C*, and 6). Similar amounts of single chain primary translation product were recovered from rh-fVIII- and HP47-expressing BHK-M cells despite HP47 clone F5 having a slightly lower steady-state fVIII mRNA level (Fig. 5, *A* and *D*). However, since the amount of primary translation product generated during the pulse-labeling period was not significantly different, a slight difference in translational efficiency cannot account for the 3.9-fold difference in fVIII protein recovered from the conditioned medium after the chase period (Fig. 5*D*). Likewise, the 1.6-fold greater specific activity observed for HP47 cannot account for the 9-fold difference in fVIII activity measured in the conditioned medium. The data presented indicate that the major differential in expression between BHK-M/rh-fVIII clone C7 and BHK-M/HP47 clone F5 occurs post-translationally during passage through the endoplasmic reticulum and Golgi apparatus. Similar results were

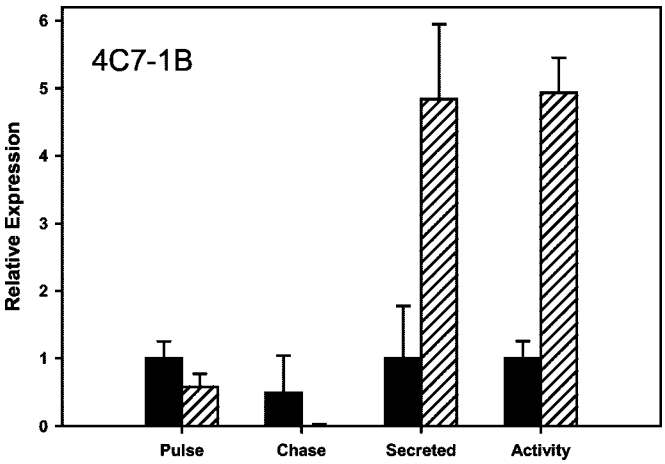

FIG. 6. **Metabolic radiolabeling of mixed populations of rh-fVIII- or HP47-expressing cell lines.** Mixed populations of 50–100 individual BHK-M clones stably transfected with the rh-fVIII and HP47 expression vectors were radiolabeled with [^{35}S]methionine, immunoprecipitated with the fVIII heavy chain-specific antibody 4C7–1B, and analyzed as described in Fig. 5, *A–D*. Cell lysate pulse and chase single chain or secreted summed single/heavy/light chain autoradiograph band intensities for rh-fVIII (*solid bars*) and HP47 (*hatched bars*) are presented as the mean ± 1 S.D. relative to the mean value obtained for pulse-labeled rh-fVII (for the *Pulse* and *Chase* samples) or relative to the amount of rh-fVIII in the conditioned medium (for the *Secreted* and *Activity* samples).

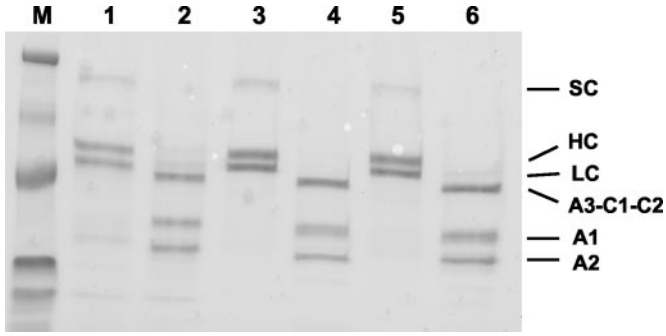

FIG. 7. **SDS-PAGE analysis of HP47.** Two micrograms of rh-fVIII (*lanes 1* and *2*), rp-fVIII (*lanes 3* and *4*) and HP47 (*lanes 5* and *6*) were subjected to 4–15% gradient SDS-PAGE under reducing conditions and visualized by Coomassie Blue staining. *Lanes 2*, *4*, and *6* represent samples that were treated with 100 nM porcine thrombin for 5 min before analysis. Single chain (*SC*), heavy chain (*HC*), light chain (*LC*), A3-C1-C2, A1, and A2 polypeptides are labeled. *Lane M* contains molecular mass markers of 207, 129, 75, 39.7, 32.1 and 17.5 kDa.

obtained when mixed populations of stably transfected BHK-M/rh-fVIII and BHK-M/HP47 cell lines were tested (Fig. 6). Presumably, the transgene copy number and genome integration sites vary between the individual clonal cell lines, and the results obtained represent the average expression capability of the entire population. Therefore, these results should not have been significantly influenced by unrelated genetic alterations occurring in specific clones that could lead to false interpretations of individual cell line data.

It has been demonstrated previously that intracellular proteolytic processing of single chain fVIII occurs after exit from the endoplasmic reticulum and that fVIII heavy and light chain polypeptides are not completely associated within the Golgi apparatus (17). Therefore, the recovery of heavy and/or light chain from the cell lysates after the 4-h chase period is consistent with progression of fVIII from the endoplasmic reticulum into the Golgi apparatus and eventually to secretory vesicles. Consistent with a previous study of recombinant full-length human fVIII (17), disproportionately more light chain, but not heavy chain fVIII, was recovered from the conditioned medium samples after immunoprecipitation with a light chain- or heavy chain-specific antibody, respectively (Fig. 5*C*). The absence of free heavy chain suggests that either 1) free heavy chain is unstable in the conditioned medium and is rapidly degraded or 2) free heavy chain does not escape from the cell until the metal ion-dependent association with the light chain occurs. There are predicted type-1 copper ion binding sites at His-265–Cys-310–His-315–Met-320 in the A1 domain and His-1954–Cys-2000–His-2005–Met-2010 in the A3 domain of human fVIII that have been shown to play a role in the coordination of a single Cu(I) atom (34). Therefore, in light of the observation that the porcine A1 and *ap*-A3 domains are necessary for high level expression, an attractive hypothesis is that a differential in heavy/light chain association is responsible for high level expression. However, this hypothesis is not consistent with several observations. First, more free HP47 heavy chain material than free rh-fVIII heavy chain material was recovered from the chase cell lysates after immunoprecipitation with the heavy chain-specific antibody. Second, co-immunoprecipitation of the heavy and light chains was observed only in the rh-fVIII samples and not in the HP47 samples. Third, the residues currently thought to be involved in Cu(I) atom binding are conserved between human and porcine fVIII.

Within the A1 and *ap*-A3 domains, human and porcine fVIII share 76 and 82% amino acid identity, respectively (35). Several residues within the A1 and A3 domains of fVIII have been identified that are important for fVIII structure and/or expression. Residues within the amino acid sequence 226–336 in the A1 domain are important for the interaction of fVIII with the endoplasmic reticulum lumenal protein BiP (GRP78), which has been proposed to limit expression of fVIII (36, 37). Within this region there are 13 amino acid differences between human and porcine fVIII. The only residue that has been demonstrated to be functionally important in this interaction, Phe-309, is conserved. In contrast, there are interspecies differences in potential *N*-linked glycosylation sites that could be important for post-translational biosynthesis of fVIII. Human fVIII contains two potential glycosylation sites that are not present in porcine fVIII, at Asn-42 near the amino terminus of the A1 domain and at Asn-1685 within the *ap* domain. Conversely, porcine fVIII contains a potential glycosylation site at Asn-213 in the A1 domain that is not conserved in human fVIII. It is possible that glycosylation differences at these sites affect the passage of fVIII through the endoplasmic reticulum/Golgi apparatus through differential interaction with resident lectins responsible for posttranslational processing and quality control. For many secreted proteins (38), including fVIII (13), transit from the endoplasmic reticulum to the intermediate compartment of the Golgi apparatus is a rate-limiting step. Selective transport to the Golgi apparatus does not occur until a protein is correctly folded. Human fVIII is known to interact with several endoplasmic reticulum resident proteins with established roles in protein folding (9, 13, 14). These interactions are not detectable for the homologous protein, blood coagulation factor V, presumably due to more efficient folding of factor V (15, 39). Therefore, it is tempting to speculate that porcine fVIII simply folds more efficiently than human fVIII, resulting in more efficient transport through the endoplasmic reticulum.

As a therapeutic agent in the treatment of hemophilia A, human fVIII possesses several undesirable properties, including low level expression, a short circulatory half-life, and significant immunogenicity. Increasing the expression of fVIII could lead to more economical production of fVIII for intravenous use or to facilitate gene therapy, in which fVIII expression has been a limiting factor (40). In this study we have demonstrated that it is possible to create a fully functional hybrid

fVIII molecule that maintains this interspecies differential characteristic. Through continued humanization of HP47 it may be possible to identify individual amino acids that provide high level expression. From that point a rational design approach could be adopted to further enhance expression properties using non-natural sequence modifications.

REFERENCES

1. 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., and Orr, E. C. (1984) *Nature* **312,** 342–347
2. 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., and. (1984) *Nature* **312,** 337–342
3. Kane, W. H., and Davie, E. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6800–6804
4. Church, W. R., Jernigan, R. L., Toole, J., Hewick, R. M., Knopf, J., Knutson, G. J., Nesheim, M. E., Mann, K. G., and Fass, D. N. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6934–6937
5. Zaitsev, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) *J. Biol. Inorg. Chem.* **1,** 15–23
6. Lollar, P., and Parker, C. G. (1989) *Biochemistry* **28,** 666–674
7. Bohn, R. L., Avorn, J., Glynn, R. J., Choodnovskiy, I., Haschemeyer, R., and Aledort, L. M. (1998) *Thromb. Haemostasis* **79,** 932–937
8. Garber, K. (2000) *Nat. Biotechnol.* **18,** 1133
9. Kaufman, R. J., Pipe, S. W., Tagliavacca, L., Swaroop, M., and Moussalli, M. (1997) *Blood Coagul. Fibrinolysis* **8,** Suppl. 2, 3- 14
10. Koeberl, D. D., Halbert, C. L., Krumm, A., and Miller, A. D. (1995) *Hum. Gene Ther.* **6,** 469–479
11. Hoeben, R. C., Fallaux, F. J., Cramer, S. J., van den Wollenberg, D. J., van Ormondt, H., Briet, E., and van der Eb, A. J. (1995) *Blood* **85,** 2447–2454
12. Lynch, C. M., Israel, D. I., Kaufman, R. J., and Miller, A. D. (1993) *Hum. Gene Ther.* **4,** 259–272
13. Dorner, A. J., Bole, D. G., and Kaufman, R. J. (1987) *J. Cell Biol.* **105,** 2665–2674
14. Fang, X., Chen, C., Wang, Q., Gu, J., and Chi, C. (2001) *Thromb. Res.* **102,** 177–185
15. Pipe, S. W., Morris, J. A., Shah, J., and Kaufman, R. J. (1998) *J. Biol. Chem.* **273,** 8537–8544
16. Tagliavacca, L., Wang, Q., and Kaufman, R. J. (2000) *Biochemistry* **39,** 1973–1981
17. Kaufman, R. J., Wasley, L. C., and Dorner, A. J. (1988) *J. Biol. Chem.* **263,** 6352–6362
18. Kaufman, R. J., Wasley, L. C., Davies, M. V., Wise, R. J., Israel, D. I., and Dorner, A. J. (1989) *Mol. Cell. Biol.* **9,** 1233–1242
19. Doering, C. B., Healey, J. F., Parker, E. T., Barrow, R. T., and Lollar, P. (2002) *J. Biol. Chem.* **277,** 38345–38349
20. Cunningham, B. C., Jhurani, P., Ng, P., and Wells, J. A. (1989) *Science* **243,** 1330–1336
21. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 139–244, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Qian, J., Borovok, M., Bi, L., Kazazian, H. H., Jr., and Hoyer, L. W. (1999) *Thromb. Haemostasis* **81,** 240–244
23. Doering, C. B., Parker, E. T., Healey, J. F., Craddock, H. N., Barrow, R. T., and Lollar, P. (2002) *Thromb. Haemostasis* **88,** 450–458
24. Seidah, N. G., and Chretien, M. (1997) *Curr. Opin. Biotechnol.* **8,** 602–607
25. Lind, P., Larsson, K., Spira, J., Sydow-Backman, M., Almstedt, A., Gray, E., and Sandberg, H. (1995) *Eur. J. Biochem.* **232,** 19–27
26. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990) *Biotechniques* **8,** 528–535
27. Healey, J. F., Lubin, I. M., Nakai, H., Saenko, E. L., Hoyer, L. W., Scandella, D., and Lollar, P. (1995) *J. Biol. Chem.* **270,** 14505–14509
28. Healey, J. F., Barrow, R. T., Tamim, H. M., Lubin, I. M., Shima, M., Scandella, D., and Lollar, P. (1998) *Blood* **92,** 3701–3709
29. Barrow, R. T., Healey, J. F., Gailani, D., Scandella, D., and Lollar, P. (2000) *Blood* **95,** 564–568
30. Lubin, I. M., Healey, J. F., Scandella, D., Runge, M. S., and Lollar, P. (1994) *J. Biol. Chem.* **269,** 8639–8641
31. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci.* **4,** 2411–2423
32. Lubin, I. M., Healey, J. F., Barrow, R. T., Scandella, D., and Lollar, P. (1997) *J. Biol. Chem.* **272,** 30191–30195
33. Barrow, R. T., Healey, J. F., Jacquemin, M. G., Saint-Remy, J. M., and Lollar, P. (2001) *Blood* **97,** 169–174
34. Tagliavacca, L., Moon, N., Dunham, W. R., and Kaufman, R. J. (1997) *J. Biol. Chem.* **272,** 27428–27434
35. Healey, J. F., Lubin, I. M., and Lollar, P. (1996) *Blood* **88,** 4209–4214
36. Marquette, K. A., Pittman, D. D., and Kaufman, R. J. (1995) *J. Biol. Chem.* **270,** 10297–10303
37. Swaroop, M., Moussalli, M., Pipe, S. W., and Kaufman, R. J. (1997) *J. Biol. Chem.* **272,** 24121–24124
38. Lodish, H. F., Kong, N., Snider, M., and Strous, G. J. (1983) *Nature* **304,** 80–83
39. Pittman, D. D., Tomkinson, K. N., and Kaufman, R. J. (1994) *J. Biol. Chem.* **269,** 17329–17337
40. Roth, D. A., Tawa, N. E., Jr., O'Brien, J. M., Treco, D. A., and Selden, R. F. (2001) *N. Engl. J. Med.* **344,** 1735–1742
41. Gitschier, J., Wood, W. I., Goralka, T. M., Wion, K. L., Chen, E. Y., Eaton, D. H., Vehar, G. A., Capon, D. J., and Lawn, R. M. (1984) *Nature* **312,** 326–330

Identification of Porcine Coagulation Factor VIII Domains Responsible for High Level Expression via Enhanced Secretion

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