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LOCALIZATION WITHIN THE 106 N-TERMINAL AMINO ACIDS OF von WILLEBRAND FACTOR (vWF) OF THE EPITOPE CORRESPONDING TO A MONOCLONAL ANTIBODY WHICH INHIBITS vWF BINDING TO FACTOR VIII

G.Piétu*, A.S. Ribba, P. Meulien*, and D.Meyer

INSERM U. 143, Hôpital de Bicêtre, le Kremlin Bicêtre and Transgène S.A.*, Strasbourg, France

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SUMMARY : We have used an in vitro transcription-translation system to localize the epitope corresponding to a monoclonal antibody (MAb 418) to vWF which specifically inhibits its binding to F.VIII. We have subcloned 1.5 Kb of vWF cDNA encoding for the N-terminal part of the vWF subunit which contains a binding site for F.VIII into the Bluescribe expression vector. After in vitro transcription and translation using the resulting construction (pBS-TG3522), a polypeptide of mol wt 60,000 (AA 1-495) was immunoprecipitated with a polyclonal antibody to vWF and with MAb 418. The MAb 418 epitope was further localized by reducing the size of the cDNA insert. This resulted in the production of two polypeptides of 18,000 (AA 1 to 142) and 13,000 (AA 1 to 106) which both retained reactivity with MAb 418. Thus the epitope corresponding to MAb 418 is localized to the first 106 AA of the mature vWF subunit.

vWF is a multimeric plasma glycoprotein composed of identical subunits (mol wt 270 000), which has two main functions in hemostasis. Firstly, it mediates platelet adhesion to the injured vessel wall. Functional binding domains to platelet membrane glycoprotein Ib (1-3), glycoprotein IIb/IIIa (4), collagen (5-6) and heparin (7) have been defined on the vWF subunit. Secondly, vWF serves as a carrier protein for coagulation F.VIII, stabilizing the activity of the plasmatic (8) or the recombinant (9) protein. In vivo, vWF and F.VIII circulate as a non-covalent complex.

vWF is synthesized in endothelial cells as a precursor called pre-pro-vWF composed of a signal peptide (22 AA), a pro-peptide or vWAgII (741 AA) and the

*Correspondence : Dr. G. Piétu, INSERM U.143, Hôpital de Bicêtre, 94275 le Kremlin Bicêtre Cedex, France.

ABBREVIATIONS

vWF, von Willebrand Factor ; mol wt , molecular weight ; F.VIII, Factor VIII ; AA, amino acid ; MAb, monoclonal antibody ; Ab, antibody ; PMSF, phenylmethane sulfonylfluoride ; SDS, sodium dodecylsulfate ; DEPC, diethylpyrocarbonate ; CIP, calf intestin phosphatase ; RNasin, ribonuclease inhibitor ; pBS-M13, Bluescribe M13 ; BMV, Brome Mosaic Virus.

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mature vWF subunit (2050 AA). The sequence of the vWF subunit, comprising 2050 AA, has been determined by DNA sequencing (10-12) and AA (13) analysis. A number of repetitive structural domains are observed : D1, D2, D', D3, A1, A2, A3, D4, B1, B2, C1, C2 (10-11).

A F.VIII binding domain has been recently identified on the N- terminal portion of the vWF subunit within AA 1 to 298 or 272 by degradation of purified vWF with plasmin or trypsin (P34 fragment) (14-15). This F.VIII binding domain corresponds to the structural domains D' and D3 and contains a very high percentage of cysteine residues which are unevenly distributed along the complete vWF subunit in two major groups at the N- and C-terminal parts of the molecule (13). Potential glycosylation sites have also been located in this region of the subunit (13).

MAbs specific for the different domains of vWF have provided a useful tool for studying the structure-function relationship of the protein. We have recently identified a MAb to vWF (MAb 418) which is directed towards the P34 fragment and specifically inhibits the binding of vWF or of P34 fragment to F.VIII (14).

In this study, we have used an in vitro transcription-translation system and have localized the epitope recognized by MAb 418 on a polypeptide corresponding to the 106 N-terminal residues of the vWF subunit.

MATERIALS AND METHODS

MATERIALS

Triton X100, leupeptine, leucine, PMSF, agarose, EDTA, SDS, spermidine, acrylamide, DEPC were from Sigma Chemical Co, St Louis, MO, USA ; ^{3}H-leucine and Amplify from Amersham International, Amersham, UK; Protein A Sepharose from Pharmacia Fine Chemicals AB, Uppsala, Sweden; restriction enzymes, CIP, RNasin and T7 RNA polymerase from Boehringer Mannheim, Meylan, France ; rabbit reticulocyte lysate translation system from Promega Biotec, Madison, WI, USA ; X-O Mat films and intensifying screens from Kodak, Marne-la-Vallée, France ; pBS-M13 and transcription kit assay from Stratagene Inc, San Diego, CA, USA. ; all other reagents were of analytical grade and obtained from Merck , France.

METHODS

Construction of pBS-TG3522 expression vector

Appropriate cDNA clones (λ–TG3514, λ–TG3501, λ–TG3502) were obtained while cloning the full length vWF cDNA (16). Clone λ–TG3502 (1.5 Kb), located at the junction between vWAg II and vWF cDNA (Fig. 1A), encodes the N-terminal part of the vWF subunit including the F.VIII binding domain but does not contain the 5' translation initiation codon. An ATG codon was added by fusing clone λ–TG3502 with fragments of clones λ–TG3514 and λ–TG3501 located in the 5' region of vWF cDNA (Fig. 1A). Clone λ–TG3502 was digested by the restriction enzymes BamHI and EcoRI (Fig. 1B) and the 3' part of the fragment obtained by this digestion was ligated to the fragment obtained after digestion of clone λ–TG3501 with the enzyme BamHI and PstI (Fig. 1B). This cDNA fragment was then ligated to the 5' part of clone λ–TG3514 digested with the enzymes EcoRI and PstI (Fig. 1B). A deletion on this construct was performed by loop-out mutagenesis using synthetic oligonucleotide TG1316 (5'-GGGCCGACAGGATAGGCTAGTTCCTTCTGCACAAAG-3') so as to introduce the

translational initiation codon ATG at the N-terminus of the mature vWF subunit (Fig. 1B). The final construction spanning 1700 base pairs (bp) is referred to as M13-TG3520.

The pBS-M13 expression vector was cleaved at the single EcoRI site of the polylinker and dephosphorylated with CIP. The 1.7 Kb M13-TG3520 was inserted in this vector, downstream from the T7 phage promoter (Fig. 1C).The correct orientation (5'-3') of the vWF sequence in the construction was confirmed by restriction enzyme digestion. This construction is referred to as pBS-TG3522 (Fig. 1C).

In vitro transcription of vWF cDNA (pBS-TG3522)

In vitro transcription of pBS-TG3522 was performed as described (17) by using a commercial kit. Plasmid pBS-TG3522 (50 μg) linearized by digestion with the appropriate restriction enzyme (HindIII, BamHI or PvuI) was incubated in transcription buffer (40 mM Tris base pH 8,8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl) with 100 units of RNasin, 0.5 M rATP, rGTP, rCTP, rUTP, 90 mM DTT , 120 units of T7 RNA polymerase in a final volume of 100 μl. Transcription was performed for 1 h at 37 °C. Following the addition of 120 units of T7 RNA polymerase, the mixture was incubated at 37°C for 30 min. The mRNA was extracted with a mixture of phenol- chloroform followed by chloroform alone. The mRNA was precipitated in the presence of 0.1 M NaCl with 2 volumes of ethanol at -20°C for 12 h. The precipitate, collected by centrifugation at 15,000 g for 30 min , was washed in 70% (v/v) ethanol. The pellet was dried and dissolved to a concentration of 1μg/μl in sterilized DEPC-treated H_2O. The transcription product was stored at -20°C and was used for translation within 24 h.

Cell-free translation of transcribed mRNA

The mRNA synthesized in vitro from pBS-TG3522 was translated using the rabbit reticulocyte lysate system. Typically, mixtures contained 35 μl of lysate, 1 μl of an AA mixture lacking leucine, 5 μl of ^{3}H-leucine (1mCi /ml) and 10 μg of mRNA derived from transcription of pBS-TG3522, previously denatured for 5 min at 65°C. As a positive control for correct translation, 0.25 μg of RNA of BMV was used.

Translation was performed for 1h at 30°C. At the end of the incubation, the mixture was then diluted four times in 50 mM Tris HCl, 150 mM NaCl pH 7.4 buffer ("radioimmune precipitation buffer") containing 1 mM leucine, 2 mM PMSF, 1% Triton X100, 0.01 mM leupeptine, 1mM ε aminocaproic acid, 1ul sodium deoxycholate, 10 mM EDTA. The translation product was stored at -20°C for no longer than two weeks.

Analysis of protein products

The protein products of cell-free translation of mRNA were analyzed by SDS-PAGE (0.1% SDS, 15 or 18 % polyacrylamide) according to the method of Laemmli (18). Following electrophoresis for 15 h, the gels were fixed for 30 min in 10% methanol / acetic acid, soaked for 30 min in a solution of Amplify for fluorography, dried and analyzed by autoradiography using X-0 Mat films and intensifying screens at -70°C.

Aliquots (15,000 cpm) of ^{3}H-labeled translation products were precipitated with trichloroacetic acid to estimate the amount of incorporated ^{3}H-leucine .

Immunoprecipitation of the protein products

Immunoprecipitation of the products was performed with aliquots of the translation mixture (150,000 cpm). Polyclonal or monoclonal antibody IgG (10 μg) to vWF were incubated with the ^{3}H-labeled translation products for 15 h at 4°C. Protein A-Sepharose (200 μg) was added to the incubation mixture containing the polyclonal antibody. Anti-mouse IgG antibody (200 μg) coupled to protein A-Sepharose was added to the mixture containing the monoclonal antibodies. The mixture was incubated for 3 h at 4°C with gentle agitation.

The immunoadsorbent was sedimented by centrifugation at 5,260 g for 10 min at 4°C and the pellet was washed twice in "radioimmune precipitation buffer", twice in phosphate buffer (10 mM NaH2P04, 140 mM NaCl, pH 7.2) and once in 50 mM Tris HCl, pH 6.8. The final pellet was suspended in 20 µl of 0.4 M Tris-HCl, pH 6.8, containing 30% glycerol, 6% SDS and 0.008% bromophenol blue. The samples were boiled for 5 min and loaded on 0.1% SDS -15 or 18% acrylamide gels (18). After electrophoresis, the gels were treated as described above.

Polyclonal and monoclonal antibodies to vWF

A rabbit antiserum to human vWF (Ab 42-43) was prepared and rendered monospecific by immunoadsorption of contaminants with plasma from a patient with severe von Willebrand disease as previously described (19) .

MAbs to vWF were produced and characterized as already described (20). MAb 418 specifically inhibits the binding of vWF to F.VIII and reacts with the P34 fragment of the vWF subunit (AA 1-272) (14).

Polyclonal and monoclonal antibodies were used as purified IgG (21). Non-immune rabbit IgG and MAb 345 directed against a part of vWF subunit (AA 1366 to 2050) not involved in binding of vWF to F.VIII were used as controls.

RESULTS AND DISCUSSION

In this study, we have used a recombinant DNA approach to further map on the vWF subunit, the epitope corresponding to a monoclonal antibody which inhibits the binding of vWF to F.VIII. We have used an in vitro transcription and translation system to generate polypeptides of different sizes corresponding to the N- terminal portion of vWF.

The construction of pBS-TG3522 is described under Methods and Fig. 1A. The presence of the translation initiation codon (ATG) is an absolute requirement for the efficient production of proteins from cDNA by in vitro transcription and translation. Because this codon was not present in the vWF clone corresponding to the N-terminal part of the vWF subunit and containing the F.VIII binding domain, it was added by fusing this clone with the 5' end of the vWF cDNA containing the translation initiation site. This 1.7 Kb construction (M13-TG3520) is composed of 131 bp upstream of the initiation codon ATG (Fig. 1B) followed by 78 bp encoding the signal peptide and 4 AA of vWAgII and finally 1485 bp encoding AA Ser 1 to Val 495 of the vWF subunit. A stop codon is positioned at 1486.

In vitro transcription and translation of pBS-TG3522 linearized with HindIII

In vitro transcription of sequences downstream of the T7 promoter has been performed with the T7 RNA polymerase. The restriction site for HindIII used to linearize pBS-TG3522 is localized 45 bp downstream of the vWF insert (Fig. 1C), consequently, the mRNA obtained spans 1.7 Kb of TG3520. This mRNA was used for cell-free translation using the rabbit reticulocyte lysate system. The expected mol wt of the

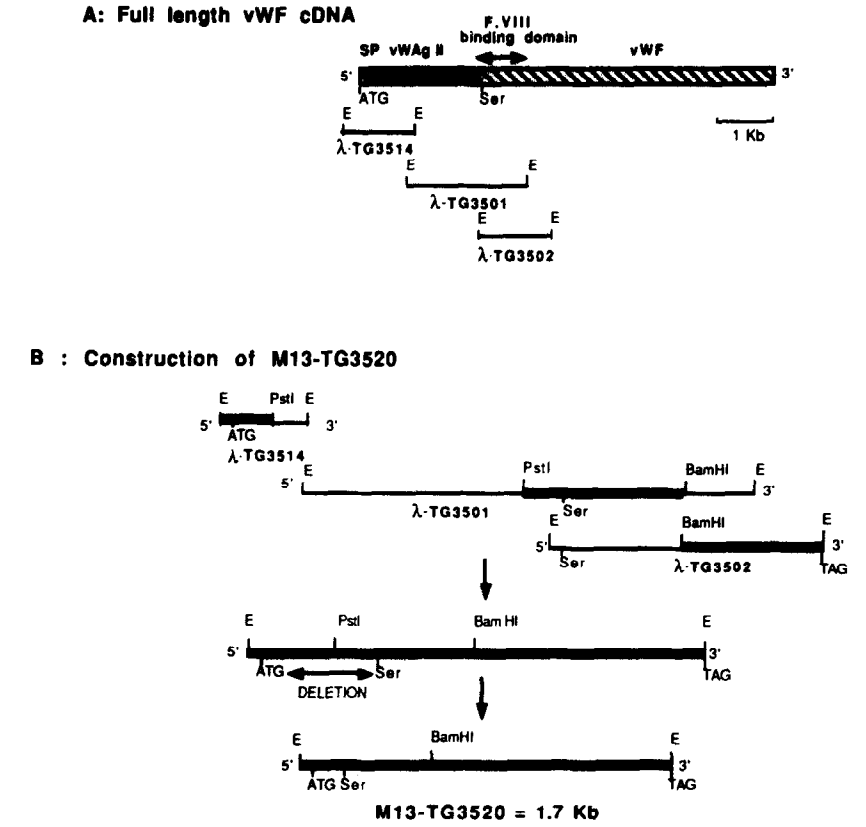

Figure 1 : Construction of pBS-TG3522 expression vector from vWF cDNA clones.

A - **Full length vWF cDNA.**
The vWF cDNA spans 8.8 Kb and codes for the signal peptide SP(22 AA), vWAgII (741 AA) and mature vWF subunit (2050 AA). A F.VIII binding domain is located between AA 1 and 272 of the mature vWF subunit.

The position of cDNA clones λ–TG3514, λ–TG3501 and λ–TG3502 on the full length vWF cDNA is indicated. E = EcoRI restriction site. Ser corresponds to the first AA of the mature vWF subunit.

B - **Construction of vWF clone M13-TG3520.**
vWF clone M13-TG3520 was constructed by engineering cDNA clones λ–TG3514, λ–TG3501 and λ–TG3502. The 3' BamHI-EcoRI fragment of clone λ–TG3502 was ligated to the PstI-BamHI fragment of λ–TG3501. The cDNA fragment was then digested with the enzyme Pst I and ligated to the 5' EcoRI-PstI part of λ–TG3514. A deletion (indicated by the arrow) was made in order to fuse the signal peptide including the ATG codon with the cDNA encoding Ser 1 to Val 495 of the mature vWF subunit. The final construction is referred to as M13-TG3520 and spans 1.7 Kb. E = EcoRI restriction site. TAG = stop codon in the 3' part of clone λ–TG3502.

C - **Construction of pBS-TG3522 expression vector.**
TG3520 cDNA was subcloned in pBS-M13 vector at the EcoRI site downstream of the RNA polymerase T7 phage promoter. This construction is referred to as pBS-TG3522. The orientation of the insert (5'-3') in the vector was determined by digestion of the plasmid with BamHI. Restriction sites for EcoRI (E), PvuI (P),BamHI (B), HindIII (H) are indicated. P_{T7} = T7 phage promoter, P_{T3} = T3 phage promoter. LAC Z = β galactosidase gene, LAC I = β galactosidase gene repressor. AmpR = ampicillin resistance gene.

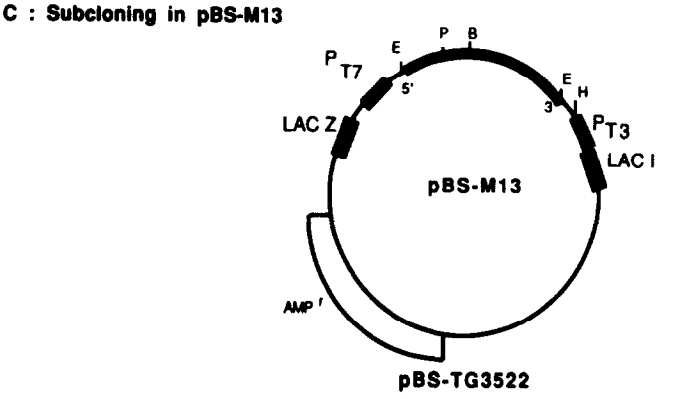

Fig. 1 - Continued

polypeptide was 60,000. Electrophoresis of the polypeptide products revealed a single major protein band with the expected mol wt (Fig. 2A). No band was observed in control experiments in the absence of mRNA. The initiation ATG codon was necessary because an ATG located 18 bp after the beginning of the cDNA insert TG3502 was unable to direct the synthesis of any polypeptide in this system (results not shown).

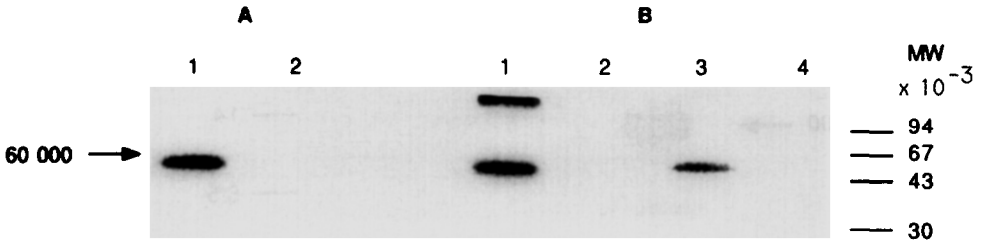

Figure 2 : Electrophoresis (0.1% SDS-15% polyacrylamide) of in vitro translation products of pBS-TG3522 linearized by HindIII.
The restriction site HindIII is localized in the polylinker of the expression vector pBS-M13 (Fig. 1C).

A - **Translation products labeled with ^{3}H-leucine.**
Lane 1 : Translation in the presence of 4 ug of mRNA transcribed in vitro from pBS-TG3522 linearized by HindIII (15 000 cpm).
Lane 2 : Translation in the absence of mRNA (buffer control).
The arrow identifies the position of the synthesized polypeptide (60,000).

B - **Translation products identified by immunoprecipitation.**
10 µg of polyclonal or monoclonal IgG were mixed with translation products (150 000 cpm) and immunoprecipitated in the presence of protein A Sepharose.
Lane 1 : Translation products immunoprecipitated by Ab 42-43 to vWF (IgG).
Lane 2 : Translation products treated with non-immune rabbit IgG.
Lane 3 : Translation products immunoprecipitated by MAb 418 to vWF (IgG) which inhibits binding of vWF to F.VIII.
Lane 4 : Translation products treated with MAb 345 (IgG) to vWF.
MW : Molecular weight markers.

The polypeptide of mol wt 60,000 was immunoprecipitated by polyclonal Ab 42-43 to vWF and MAb 418 (Fig. 2B). No band was observed following immunoprecipitation with non-immune rabbit IgG or with an unrelated MAb to vWF (MAb 345) (Fig. 2B).

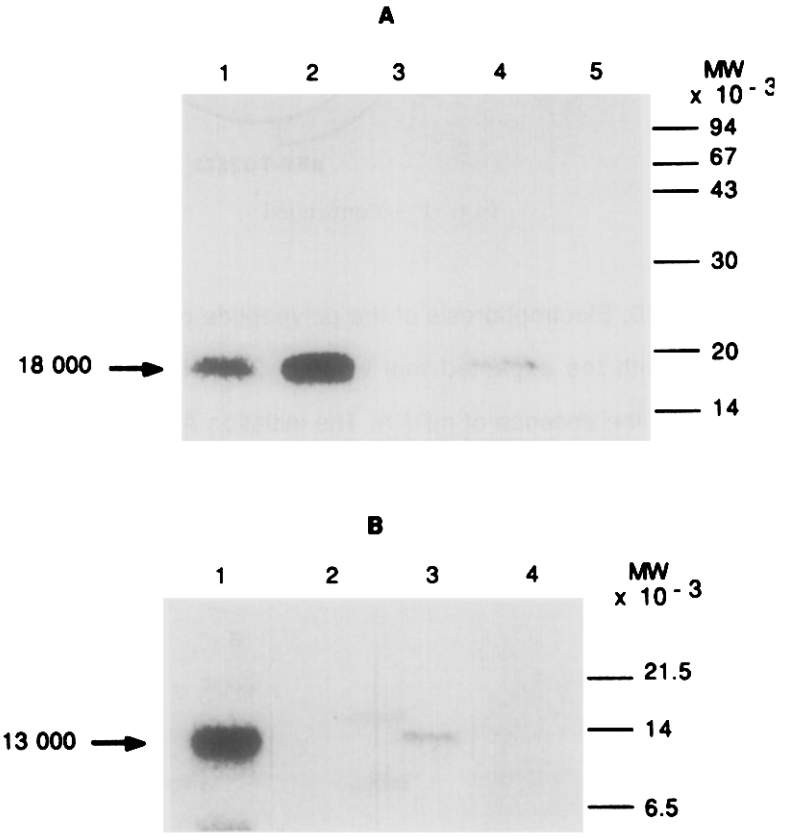

Figure 3 :

A - **Electrophoresis (0.1% SDS-15% polyacrylamide) of in vitro translation products of pBS-TG3522 linearized by BamHI.**
The restriction site BamHI is localized in the vWF cDNA insert 637 bp after the translation initiation site (ATG) (Fig. 1C).
Lane 1 : Translation products labeled with ^{3}H-leucine (150 000 cpm).
Lane 2 : Translation products immunoprecipitated with Ab 42-43 (IgG).
Lane 3 : Translation products treated with non-immune rabbit IgG.
Lane 4 : Translation products immunoprecipitated by MAb 418 (IgG).
Lane 5 : Translation products treated with MAb 345 (IgG).
MW : Molecular weight markers.

B - **Electrophoresis (0.1% SDS-18% polyacrylamide) of in vitro translation products of mRNA transcribed in vitro from pBS-TG3522 linearized by PvuI.**
The restriction site PvuI is localized in the vWF cDNA insert 538 bp after the translation initiation site (ATG) (Fig. 1C).
Lane 1 : Translation products immunoprecipitated with Ab 42-43 (IgG).
Lane 2 : Translation products treated with non-immune rabbit IgG.
Lane 3 : Translation products immunoprecipitated with MAb 418 (IgG).
Lane 4 : Translation products treated with MAb 345 (IgG).
MW : Molecular weight markers.

The in vitro synthesized fragment of vWF specifically reacted with both polyclonal and monoclonal antibodies to vWF but its reactivity was found to be higher with the polyclonal antibody than with MAb 418. Conversely, the P34 fragment corresponding to the N-terminal part of vWF, containing the binding domain of F.VIII (14), strongly reacted with MAb 418 and only weakly with the polyclonal antibody. Reasons for this discrepancy are not clear but could be related to a modification of the epitope conformation in the in vitro synthesized polypeptide.

In vitro transcription and translation of pBS-TG3522 linearized by BamHI and PvuI

The size of the vWF cDNA fragment insert in pBS-TG3522 was reduced by linearization of the plasmid with the enzyme BamHI. This cDNA fragment encodes AA 1 to 142 of the vWF subunit and the expected size of the synthesized polypeptide was about 18,000. As shown in Fig. 3A, a polypeptide of mol wt 18,000 was obtained following cell free translation. It was immunoprecipitated by Ab 42-43 as well as Mab 418 whereas it did not react with control antibodies (Fig. 3A).

The cDNA insert was reduced further in size by digestion with PvuI. The PvuI linearized plasmid encodes AA 1 to 106 and corresponds to a polypeptide of mol wt 13,000. The polypeptide was still specifically immunoprecipiated by MAb 418 (Fig. 3B) . Therefore the use of an approach based on in vitro transcription and translation allowed us to restrict the location of the epitope corresponding to MAb 418 from 272 (results previously shown with proteolytic digestion of vWF (14-15)) to 106 N-terminal amino acids of vWF suggesting that a binding site for FVIII is within this 13,000 mol wt fragment. Nevertheless the existence of this binding domain on the synthesized 13,000 mol wt polypeptide will need to be demonstrated by direct binding studies when sufficient material is available.

REFERENCES

1. Girma, J. P., Kalafatis, M., Piétu, G., Lavergne, J. M., Chopek, M. W., Edgington, T. S., Meyer, D.(1986) Blood 67, 1356-1366.
2. Fujimura, Y., Titani, K., Holland, L. Z., Russel, S. R., Roberts, J. R., Elder J.H., Ruggeri, Z. M., Zimmerman, T. S. (1986) J. Biol. Chem. 261, 381-385.
3. Sixma, J. J., Sakariassen, K. S., Stel, H. U., Houdijk, W. P. M., In der Maur, D. W., Hamer, R. J., de Groot, Ph. G., Van Mourik J. A. (1984) J.Clin. Invest. 74, 736-744.

4. Ruggeri, Z. M., Bader, R., De Marco, L. (1982) Proc. Natl Acad. Sci. USA 79, 6038-6041.
5. Pareti, F. I., Fujimura, Y., Dent, J. A., Holland, L. Z., Zimmerman, T., Ruggeri, Z. M. (1986) J. Biol. Chem. 261, 15310- 15315.
6. Kalafatis, M. , Takahashi, Y., Girma, J. P., Meyer, D. (1987) Blood 70, 1577-1583.
7. Fujimura, Y., Titani, K., Holland, L. Z. ,Roberts, J. R. , Kostel, P., Ruggeri, Z. M., Zimmerman, T. S. (1987) J. Biol. Chem. 262, 1734-1739.
8. Weiss, H. J., Sussman, I. I., Hoyer, L. W. (1977) J. Clin. Invest. 60, 390-404.
9. Pavirani, A., Meulien, P., Harrer, H., Schamber, F., Dott, K., Villeval, D., Cordier, Y., Wiesel, M.L., Mazurier, C., van de Pol, H., Piquet, Y., Cazenave, J.P., Lecocq, J.P. (1987) Biotechnology 5, 389-392.
10. Verweij, C. L., Diergaarde, P. J., Hart, M., Pannekoek, H. (1986) EMBO J. 5, 1839-1847.
11. Shelton-Inloes, B. B., Titani, K., Sadler, J. E. (1986) Biochemistry USA, 25, 3164-3177.
12. Bonthron, D., Orr, E. C., Mitsock, L. M., Ginsburg, D., Handin,R.I., Orkin, S. H. (1986) Nucl Acids Res. 14, 7125-7127.
13. Titani, K., Kumar, S., Takio, K., Ericsson, L.H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., Fujikawa, K. (1986) Biochemistry USA 25, 3171-3184.
14. Takahashi, Y., Kalafatis, M., Girma, J. P., Sewerin, K., Andersson, L.O., Meyer, D. (1987) Blood 70, 1679-1682.
15. Foster, P. A., Fulcher, C. A., Marti, T., Titani, K., Zimmerman, T. S. (1987) J. Biol. Chem 262, 8443-8446.
16. Wu, Q. Y., Bahnak, B., Coulombel, L., Kerbiriou-Nabias, D., Drouet, L., Piétu, G., Meulien, P., Pavirani, A., Caen, J. P., Meyer, D. (1988) Blood 71, 1341-1346.
17. Melton, D. A. , Krieg, P. A. , Rebagliati, M. R., Maniatis, T. , Zinn, K. , Green, M. R. (1984) Nucl. Acids Res. 12 ,7035 -7048.
18. Laemmli, U.K. (1970) Nature 227, 680-685.
19. Meyer, D., Obert, B., Piétu, G., Lavergne, J. M., Zimmerman, T. S. (1980) J. Lab. Clin. Med. 95, 590-602.
20. Meyer, D., Zimmerman, T.S., Obert, B., Edgington, T. S. (1984) Br. J. Haematol. 57, 597-608.
21. Fine, J. D., Steinbuch, M. (1970) Rev. Eur. Et. Clin. Biol., 15, 115-120.