Factor VIII expression and regulation in health and disease
Hollestelle, M.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 2

Tissue distribution of factor VIII gene expression

in vivo – a closer look

Martine J. Hollestelle¹, Terri Thinnes², Karen Crain², Ann Stiko², Johan K. Kruijt³, Theo J.C. van Berkel³, David J. Loskutoff², and Jan A. van Mourik¹,⁴

From the ¹Department of Blood Coagulation and Department of Plasma Proteins, CLB, Amsterdam, The Netherlands, the ²Department of Vascular Biology (VB-3), The Scripps Research Institute, La Jolla, CA, the ³Division of Biopharmaceutics, Leiden-Amsterdam Center for Drug Research, Sylvius Laboratories, Leiden, The Netherlands and ⁴Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam.

ABSTRACT

Previous studies have shown that factor VIII (FVIII) is expressed by multiple tissues. However, little is known about its cellular origin or its level of expression in different organs. In the present study, we examined FVIII gene expression in different tissues on a quantitative basis. Most of the tissues, especially liver and kidney, expressed high levels of FVIII mRNA compared to their level of expression of other hemostatic proteins, including von Willebrand factor (VWF). This was unexpected since FVIII is a trace protein. In situ hybridization analysis confirmed that liver and kidney were rich in FVIII mRNA. In the liver, a clear hybridization signal was detected in cells lining the sinusoids. FVIII mRNA analysis of purified liver cells confirmed the expression of FVIII mRNA by sinusoidal endothelial cells and Kupffer cells. Low but significant levels of FVIII mRNA were also detected in the hepatocytes. VWF mRNA was not detectable in these cells. Similarly, immunohistochemical staining of liver tissue revealed that FVIII protein is primarily associated with sinusoidal cells. VWF protein was predominantly located in the endothelium of larger vessels. In the kidney, FVIII synthesis was localized to the glomeruli and to tubular epithelial cells. Taken together, these results suggest that besides hepatocytes, non-parenchymal cells (e.g. sinusoidal endothelial cells) contribute to FVIII synthesis. VWF synthesis is primarily confined to extra-hepatic tissues.

INTRODUCTION

Factor VIII (FVIII) is a plasma protein that plays an essential role in the hemostatic system. In the intrinsic coagulation cascade it acts as a cofactor for the factor X-activating complex consisting of activated FVIII, the serine protease factor IXa, calcium ions and phospholipid membranes (1). FVIII is deficient or defective in the bleeding disorder hemophilia A. FVIII is synthesized as a single chain protein and, because of endoproteolytic processing, circulates as a heterodimeric protein. In addition, FVIII forms tight, non-covalently linked complexes with von Willebrand factor (VWF), its carrier protein. This interaction protects FVIII against proteolytic inactivation and premature clearance (2,3).

A number of reports suggest that the liver is the principal site of FVIII synthesis (4-9). This hypothesis is consistent with the observation that plasma FVIII levels in hemophilia A patients are restored upon liver transplantation (10). To date, it is unclear which cell type in the liver produces FVIII. Both hepatocytes and sinusoidal endothelial cells have been implicated, although studies on the identification of the specific cells in the liver responsible for FVIII synthesis are somewhat controversial (4-6). Moreover, non-hepatic cells may also produce FVIII. For example FVIII mRNA was detected in a variety of extra-hepatic tissues, including kidney, spleen and lymph node (5,7-9). However, neither the cell types that are responsible for this extra-hepatic FVIII synthesis, nor their relative level of FVIII gene expression have been established. Knowledge of these features may help to understand the potential role of FVIII in controlling local and systemic hemostatic processes.
In this report, a quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay was used to determine FVIII mRNA levels in various tissues of the mouse. In situ hybridization was employed to identify FVIII-producing cells in these tissues. In addition, we measured FVIII mRNA levels in purified liver cells. The presence of FVIII antigen in liver was studied by immunohistochemical staining. High levels of FVIII mRNA were detected both in the liver and the kidney, with lower but significant levels in the spleen, lung, brain and testis. The in situ hybridization studies of liver sections and RT-PCR analysis of purified liver cells demonstrated FVIII mRNA primarily in cells lining the sinusoids. In the kidney FVIII mRNA was most predominant in glomerular and peritubular cells. Similarly, FVIII antigen was only detected in the sinusoidal endothelial cells and Kupffer cells. On the other hand, VWF mRNA concentration in the liver and kidney was low relative to FVIII mRNA. VWF antigen was primarily located in larger vessels.

MATERIALS AND METHODS

RNA and tissue preparation

Two-month-old male and female C57Bl/6 mice (Scripps Research Institute Rodent Breeding Colony, La Jolla, CA and CLB, Amsterdam, The Netherlands), were used for all experiments. The mice were sacrificed and various tissues were removed surgically and immediately frozen in liquid nitrogen. Total RNA was isolated from the frozen tissues by acid guanidium thiocyanate-phenol-chloroform extraction as previously described (11), and then treated with RNase-free DNase (Promega Corp., Madison, WI). RNA was quantified by measuring absorption at 260 nm. Tissues to be used for in situ hybridization were rapidly removed and then fixed for 4 hours by immersion in zinc formalin, transferred to 70% ethanol overnight and embedded in paraffin. Tissue sections (2-5 μm) were used for analysis.

Isolation of liver cells

Male C57Bl/6 mice (4-month-old; Sylvius Laboratories, Leiden, The Netherlands) were anesthetized and the liver cells were isolated as described earlier (12). After collagenase perfusion, a liver lobule was tied off, excised and stored in liquid nitrogen. Hepatocytes were separated from nonparenchymal cells by differential centrifugation and the viability was determined by counting in a Bürker chamber after incubation with trypan blue. The viability of the cell preparations varied from 70% to 95%. Nonparenchymal cells were isolated by density-gradient centrifugation with Nycodenz. Then the sinusoidal endothelial cells were separated from the Kupffer cells by centrifugal elutriation. The purity of the sinusoidal endothelial cells and Kupffer cells was determined by staining with 3,3'-diaminobenzidine for endogenous peroxidase activity. The sinusoidal endothelial cells were more than 95% pure. The purity of the Kupffer cells was 70-90%. Total RNA from liver lobule, hepatocytes, sinusoidal endothelial cells and Kupffer cells was isolated as
Tissue distribution of factor VIII

described above. The identity of hepatocytes was also confirmed by factor V (FV) mRNA analysis (see below).

Quantitative PCR

A synthetic DNA template composed of oligomers specific for murine FVIII and a number of other hemostatic genes, was constructed by cloning fragments containing either 5’ or 3’ primers into pCR 2.1 (Invitrogen, The Netherlands). Briefly, the primer cassette described previously (13,14) was cut out of the vector with XbaI and Kpnl. Phosphorylated 5’ and 3’ primers containing a part of the FVIII sequence and a part of the PAI-1 sequence were mixed with this cassette and amplified by PCR. The PCR product was then cloned into the TA cloning site of pCR 2.1. In addition, parts of the murine FV sequence were inserted in the cassette by this procedure. The sequence of the final fragment was confirmed by DNA sequence analysis.

The plasmid described above was linearized by digestion with Kpnl. A cRNA standard was then prepared (using the linearized plasmid as template) by in vitro transcription from the T7 promoter using the Riboprobe in vitro transcription system according to the manufacturer’s instruction (Promega). Finally, the purified cRNA standard (669 nucleotides) was diluted to a concentration of \(10^{12}\) molecules/\(\mu\)l.

The concentration of various mRNAs (i.e. FVIII; FV; VWF; urokinase-type plasminogen activator, uPA; and tissue factor, TF) was determined using a quantitative RT-PCR method as described previously (13,14). Total tissue RNA (1 \(\mu\)g) and a fixed concentration of the cRNA standard (varying from \(10^4\) to \(10^7\) molecules) were mixed and reverse transcribed. Serial two-fold dilutions of the cDNA mixture were each amplified using specific primers for both the target and the standard products, and containing \(1 \times 10^6\) cpm of \(^{32}\)P-end labeled forward primer. The amount of incorporated \(^{32}\)P was determined by first running 36 \(\mu\)l of the PCR mixture on a 1.5% agarose gel, in the presence of ethidium bromide. The appropriate bands corresponding to the internal standard cRNA product and the target mRNA product (for FVIII measurements, 485 bp and 359 bp, respectively) were then cut out from the gel and radioactivity was determined by Cerenkov counting. The radioactivity in the PCR product was plotted against the number of standard cRNA molecules, using a double-logarithmic scale. This standard curve was used to extrapolate the number of target mRNA molecules per \(\mu\)g total RNA, according to the procedure described by Wang et al. (15). The estimation of the concentration of endogenous target mRNA was calculated as follows:

\[
\frac{\text{pg target mRNA}}{\mu\text{g of total tissue RNA}} = \frac{\text{No. of molecules of target mRNA}}{\mu\text{g of total tissue RNA}} \times \frac{\text{No. of nucleotides of full-length mRNA for target gene}) \times (321)}{(10^{12})} \times 6.023 \times 10^{23}
\]
where the number of nucleotides in full-length mRNA is 7,493 for FVIII (9); 6,585 for FV (16); 1,800 for TF (13); 2,300 for uPA (13) and 8,800 for VWF (14); where 321 is the average molecular weight of a nucleotide; and where $6.023 	imes 10^{23}$ is Avogadro’s number. The possibility that PCR products were generated from contaminating genomic DNA was ruled out because the upstream [5'-GAGGAACCACCGTCAAGCTTCATT'-3', position 1301-1324 (9)] and downstream [5'-CTGAAGGTGCATAGTCCCAGTCTT'-3', position 1636-1659] primers for FVIII anneal in two different exons. For FV mRNA derived from tissues the PCR product generated using the upstream [5'-ATCAGAGCCAGGTACAGAGA-3', position 1697-2323] and downstream [5'-GAGTGCCCAGTGAAGTGGAT-3', position 1301-1324] FV primers resulted in a fragment of 620 bp corresponding to the target mRNA and a band of 525 bp corresponding to the standard cRNA.

Riboprobe preparation

A 627 bp murine FVIII cDNA fragment consisting of part of the A2 domain of FVIII (position 1697-2323) was subcloned into the vector pGEM-5Zf(+) (Promega). The vector was linearized and used as a template for in vitro transcription of radiolabeled antisense or sense riboprobes using SP6 or T7 polymerase (Promega), respectively, in the presence of [$^{35}$S]UTP (>1,200 Ci/mmol; Amersham Corp, Arlington Heights, IL). Vector DNA was removed by digestion with RQ1 DNase (Promega) for 15 minutes at 37°C, and the riboprobes were purified by phenol extraction and ethanol precipitation. In addition to FVIII, a 596-bp mouse factor VII (FVII) cDNA fragment containing nucleotides 500-1095 (17) was subcloned into the vector pCRII (Invitrogen). This vector was linearized and used as template for in vitro transcription of radiolabeled antisense or sense riboprobes using SP6 or T7 RNA polymerase, respectively, in the presence of [$^{35}$S]UTP.

In situ hybridization

In situ hybridizations were performed as described previously (18). After hybridization, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 M NH₄Ac, dried overnight, dipped in NTB2 emulsion (Kodak, Rochester, NY; 1:2 in water), and exposed in the dark at 4°C for 4 to 12 weeks. Slides were developed for 2 minutes in D19 developer (Kodak), fixed, washed in water, and counterstained with hematoxylin and eosin. The amount of non-specific hybridization in each experiment was detected in parallel sections using $^{35}$S-labeled sense probes.

Immunohistochemistry

A polyclonal antibody against FVIII light chain was obtained by immunizing rabbits according to standard procedures with FVIII light chain purified as described previously (19). Acetone-fixed frozen liver sections were incubated with primary rabbit antibodies 1:10
dilution of anti-human FVIII light chain, preabsorbed with recombinant VWF (kindly provided by Dr. H.P. Schwarz, Immuno/Baxter, Vienna) or 1:100 dilution of anti-human VWF IgG (Dakospatts, Glostrup, Denmark). The slides were then washed at 37°C with PBS and sequentially treated with FITC labeled horse anti-rabbit IgG (CLB, Amsterdam) for 30 minutes. After washing the slides at 37°C with PBS, they were mounted with Vectashield mounting medium (Vector Laboratories, Inc, Burlingame, CA) and examined by confocal laser scanning microscopy.

RESULTS

Distribution of FVIII mRNA in murine tissues

To examine the expression of FVIII in vivo, total RNA was isolated from tissues of adult C57Bl/6j mice, and the concentration of FVIII mRNA was determined (see Materials and Methods). In the initial experiments, the level of FVIII mRNA was determined semi-quantitatively by RT-PCR amplification of serial, two-fold dilutions of the cDNA mixture in the presence of different concentrations of the cRNA standard (Fig.1). This pilot experiment clearly shows that the target band for FVIII in the liver was the most prominent, while skeletal muscle had the least intense band. This observation was confirmed using the quantitative RT-PCR assay (Fig.2). The liver and the kidney contained similar amounts of FVIII mRNA (257 pg/μg and 209 pg/μg total RNA, respectively). Significantly less (p<0.001) FVIII mRNA was detected in testis (48 pg/μg) and spleen (47 pg/μg), while relatively low levels of FVIII mRNA were present in heart (8.6 pg/μg) and skeletal muscle (3.5 pg/μg). Large inter-individual variations of FVIII mRNA levels were apparent in the different tissues. The variation coefficient of the analysis of individual samples was about 30% (n = 5), whereas inter-individual variation was about 65% (SD, n = 14, Fig.2). Thus, the observed variation between individual mice seems to reflect differences in endogenous transcription levels rather than experimental variation.

Figure 1. FVIII mRNA expression in murine tissues. One μg of RNA from each tissue and a fixed concentration of cRNA standard (the number of molecules of standard used is depicted above the picture) were mixed and transcribed. Serial two-fold dilutions of the cDNA mixture were amplified using FVIII-specific primers. The top band (485 bp) represents the cRNA standard and the bottom band (359 bp) is the PCR product derived from endogenous FVIII mRNA. Differences in FVIII expression levels can be visualized by comparing the intensity of both bands between the different tissues.
The concentration of FVIII mRNA in various tissues was 10-100 times higher than the concentration of a number of other hemostatic genes, such as TF, uPA and VWF (Table 1). The mRNA levels of these genes were similar to those determined previously (13,14). Thus, it appears that in all tissues examined, FVIII mRNA levels are considerably higher (10- to 100-fold) than the mRNA levels of the other hemostatic genes examined.

Figure 2. Expression levels of FVIII mRNA in murine tissues. The concentration of FVIII mRNA was determined using a quantitative RT-PCR assay (Materials and Methods). The mean values are indicated and are the results from two or more separate analyses of each sample. The number between brackets shows the number of mice analyzed for FVIII mRNA levels. *, results of individual mice. Li, liver; Ki, kidney; Te, testis; Sp, spleen; Ov, ovary; Lu, lung; Br, brain; He, heart; Mu, muscle.

Table 1. Quantitative analysis of FVIII, VWF, uPA and TF gene expression in murine tissues

<table>
<thead>
<tr>
<th></th>
<th>RNA</th>
<th>FVIII</th>
<th>VWF</th>
<th>uPA</th>
<th>TF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (a)</td>
<td>mRNA (b)</td>
<td>mRNA (b)</td>
<td>mRNA (b)</td>
<td>mRNA (b)</td>
</tr>
<tr>
<td>(\mu g/\text{tissue})</td>
<td>(x 10^{-3} \text{ fmol}/\mu g)</td>
<td>fmol/\text{tissue})</td>
<td>(x 10^{-3} \text{ fmol}/\mu g)</td>
<td>fmol/\text{tissue})</td>
<td>(x 10^{-3} \text{ fmol}/\mu g)</td>
</tr>
<tr>
<td>Liver</td>
<td>7.294</td>
<td>60.0 ± 10.3 (4)</td>
<td>438</td>
<td>0.002 ± 0.001 (5)</td>
<td>0.017</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.219</td>
<td>53.0 ± 11.5 (5)</td>
<td>73</td>
<td>0.003 ± 0.001 (3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Lung</td>
<td>320</td>
<td>13.0 ± 3.0 (4)</td>
<td>19</td>
<td>0.165 ± 0.100 (4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Brain</td>
<td>621</td>
<td>8.6 ± 2.3 (5)</td>
<td>37</td>
<td>0.045 ± 0.024 (5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart</td>
<td>220</td>
<td>8.8 ± 2.3 (4)</td>
<td>13</td>
<td>0.014 ± 0.008 (4)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

\(a\)Average of 3 experiments.  
\(b\)Results are the mean ± SEM. Number of observations is given in parentheses. (nd, not determined)
Localization of FVIII mRNA in murine tissues

To identify the cellular sites of FVIII mRNA expression in murine tissue in vivo, the isolated and paraffin-embedded tissues were analyzed by in situ hybridization using a $^{35}$S-labeled A2 probe of FVIII. Representative sections from the liver and kidney are shown in Fig.3. The hybridization signal was primarily confined to non-parenchymal cells, probably sinusoidal endothelial cells and/or Kupffer cells (Fig.3A). In these experiments, no or little FVIII mRNA could be detected in hepatocytes. This pattern was distinct from that of FVII mRNA, which was most prominent within the hepatocytes (Fig.3B). These latter cells are easily identified by their large, round and centrally located nuclei which contained scattered clumps of chromatin. FVIII mRNA was also readily detected in isolated liver cell fractions by RT-PCR (Fig.4). By this technique FVIII mRNA was not only detected in the sinusoidal endothelial cells, but also in the hepatocyte and Kupffer cell fraction. Very low levels of VWF mRNA were detected in total liver relative to FVIII mRNA. No VWF mRNA was detectable in the purified cells of the liver, neither the sinusoidal endothelial cells, nor hepatocytes and Kupffer cells (Fig.4). There was no detectable mRNA for FV, a hepatocyte marker, in the sinusoidal endothelial cell or Kupffer cell fraction (Fig.4).

In the kidney, FVIII mRNA was primarily detected in the glomeruli, in epithelial cells of the collecting tubules and occasionally in cells lining veins or arteries (Fig.3C). FVIII mRNA was also detected in the germinal cells of the testis and ovary, in the oviduct, and in the adrenal and thymus (data not shown). No specific signal for FVIII mRNA could be detected in the lung, aorta, skeletal muscle, gut, and adipose tissue. Although FVIII mRNA was detected within the spleen by quantitative RT-PCR, we failed to detect a specific FVIII mRNA signal in this tissue by in situ hybridization (data not shown).

FVIII and VWF antigen in liver

Immunohistochemical analysis of FVIII and VWF antigen in the liver revealed two different patterns. FVIII antigen was predominantly observed in sinusoidal endothelial cells or possibly Kupffer cells (Fig.5A), whereas VWF antigen was primarily present in larger vessels (Fig.5B). Also the boundaries of the hepatocytes stained positive for FVIII, though weakly. No FVIII antigen was demonstrated in endothelial cells of larger vessels. Only weak staining of VWF was observed in microvascular endothelial cells.
Figure 3. High-resolution in situ hybridization analysis of FVIII and FVII mRNAs in different tissues of healthy mice. In the liver (A) FVIII mRNA is primarily detected in the sinusoidal cells indicated with arrows, while FVII mRNA (B) is predominantly detected in the hepatocyte fraction of the liver. Arrowheads denote the hepatocytes. In the kidney (C) FVIII is expressed in the glomeruli (open arrow). Solid arrows denote tubular epithelial cells; arrowhead indicates cells lining a vein. All slides were exposed for 12 weeks at 4°C, except the FVII in situ hybridization slides, which were exposed for 8 weeks. (Magnification x400)

Figure 5. Immunofluorescent staining analysis of FVIII and VWF antigens in murine liver sections. FVIII antigen (A) was mainly localized to the sinusoidal endothelial cells, while VWF antigen (B) was predominantly observed in relatively large vessels. Solid arrows denote sinusoidal endothelial cells; arrowhead indicates large vessels. Scale bar = 20 μm
Figure 4. **Cellular distribution of FVIII, VWF and FV mRNA in liver tissue.** (A) One μg of RNA from each tissue and 1 x 10^5 molecules of cRNA standard were mixed, transcribed and subjected to PCR. For FVIII measurements the top band (485 bp) represents the cRNA standard and the bottom band (359 bp) is the PCR product derived from endogenous FVIII mRNA. In the VWF and FV assay the top band (respectively 1130 and 620 bp) represents the (target) mRNA band and the bottom band (respectively 172 and 525 bp) is the PCR product derived from the cRNA standard. Differences in factors V, VIII and VWF expression levels can be visualized by comparing the intensity of both bands between the different cell types. (B) The results presented in A were used to optimize the relative concentration of cRNA standard to be used in the more quantitative assay using ³²P-labeled primers as described in Materials and Methods. The mean concentration is calculated from four different mice for the liver lobule (Li) and hepatocytes (Hp), and from two different preparations for the sinusoidal endothelial cells (EC) and Kupffer cells (KC). The standard error of the mean is indicated by error bars. The levels of FV mRNA in the sinusoidal endothelial cells and Kupffer cells fractions were less than 1 pg/μg total tissue RNA; VWF mRNA levels in sinusoidal endothelial cells, hepatocytes, and Kupffer cells were less than 0.01 fg/μg total tissue RNA.
DISCUSSION

This study addresses two important questions that have been the subject of many studies in the past, but have not been answered satisfactorily. First, which tissues or organs play a significant role in expressing the FVIII gene and, secondly, which cell(s) within them produce FVIII and at what level? The unique use of quantitative RT-PCR, in situ hybridization and cell fractionation techniques described here has allowed the first direct analysis of FVIII mRNA levels on a quantitative basis. These studies confirm and extend the observations that FVIII is expressed in a variety of tissues (5,7-9). In particular, relatively high levels of FVIII mRNA were detected in the liver and kidney by RT-PCR, and significant, though less prominent expression was observed in a number of other tissues (Fig.2). Clearly FVIII gene expression is not tissue-specific. However, because of their relatively large size and high steady-state levels of FVIII mRNA expression, the liver and kidney most likely represent the major sources of plasma FVIII. Comparing the volume and specific FVIII mRNA contents of the liver and kidney, the data suggest that the liver may account for about 75% of plasma FVIII and the kidney for 12.5% (Table 1). This suggests that hepatic rather than renal synthesis primarily contributes to the production of plasma FVIII. Indeed, it has been reported that FVIII deficiency cannot be restored by kidney transplantation (20,21). On the other hand, liver transplantation in patients with severe FVIII deficiency clearly corrected the inborn metabolic error (10). Paradoxically, in patients with severe liver failure, FVIII levels are frequently substantially elevated (22), suggesting a role for extra-hepatic sites of FVIII synthesis under these conditions. Indeed, organ transplant studies raise the possibility that the spleen (23), lung (24) and lymphatic tissue (25) may compensate for aberrant production of FVIII by the liver. Alternatively, it is possible that certain subsets of cells responsible for FVIII synthesis in the liver (e.g. sinusoidal endothelial cells, see below) are still capable of producing FVIII, even under conditions of severe liver insufficiency.

A prominent feature of the present study is the apparent high steady state level of FVIII mRNA in the liver, kidney, and other tissues examined compared to the mRNA levels of a number of other hemostatic genes (Fig.2, Table 1). For example, FVIII mRNA levels were 10 to 100 times higher, both in hepatic and in extra-hepatic tissues compared to VWF, uPA and TF (13,14) (Table 1). These observations indicate that the FVIII gene is efficiently transcribed. The relatively high levels of FVIII mRNA in tissues is in sharp contrast with the relatively low levels of plasma FVIII. This is unexpected. Compared with other hemostatic proteins such as protein C, vitronectin, or VWF, there seems to be a lack of correspondence between mRNA and protein levels. It is estimated that the plasma FVIII concentration is 100- to 10,000-fold lower than expected based on of the respective mRNA and corresponding blood levels and biological half-lives of these proteins (13,14,26,27). Apparently, FVIII mRNA is not efficiently translated or, alternatively, FVIII is poorly transported to the outside of the cell (28). In addition, it should be noted that there was considerable inter-individual variation in FVIII mRNA level in all tissues examined (Fig.2).
This observation suggests that the transcriptional activity of the FVIII gene is not a critical factor in controlling FVIII plasma levels.

The cellular origin of FVIII is one of the most controversial issues in this field. The data presented here demonstrate for the first time, the localization of FVIII mRNA in cells by in situ hybridization. Within the detection limit of this approach, expression of FVIII mRNA was localized to cells lining the micro-vasculature of the liver, most likely sinusoidal cells and/or Kupffer cells (Fig.3A). We failed to detect a specific signal for FVIII mRNA within hepatocytes, even though FVIII mRNA was detected in purified hepatocytes by RT-PCR (Fig. 4). Apparently, the level of FVIII mRNA in hepatocytes is below the sensitivity of the in situ hybridization method. Also previous studies have shown that the sensitivity of in situ hybridization appears to be lower than mRNA levels measured by quantitative RT-PCR (26,27). This notion could also explain why spleen FVIII mRNA was only detectable by RT-PCR and not by in situ hybridization. In any case, the in situ hybridization pattern for FVIII was clearly distinct from the cellular distribution pattern of FVII mRNA, a typical gene product of liver parenchymal cells (5). Unlike FVIII mRNA, the FVII transcript was detected only within hepatocytes by this technique (Fig.3B).

In purified sinusoidal endothelial cells, the FVIII mRNA concentration is about 3 fold higher than the FVIII mRNA level in hepatocytes (Fig.4). There was almost no detectable (<1 pg/μg total mRNA) mRNA for FV, a typical hepatocyte marker, in the endothelial cell- and Kupffer cell preparation, indicating that these latter fractions were not contaminated with hepatocytes. It should be noted, however, that, unlike FV mRNA levels, the steady state FVIII mRNA levels of the purified cells were lower than expected on the basis of the mRNA analysis of total liver lobule extracts (Fig.4). These results suggest that, FVIII mRNA may be less stable in the isolated cell fractions than in intact tissue, notably cells that are obtained only a few hours after initiation of the liver perfusion and cell elutriation procedure (i.e. endothelial cells and Kupffer cells). This complicates the interpretation of the quantitative RT-PCR data on the FVIII mRNA levels in the purified cells. In any case, our results clearly demonstrate that FVIII mRNA is expressed both in hepatocytes and sinusoidal endothelial cells, and possibly also Kupffer cells. Similarly, a recent report showed that FVIII mRNA levels are readily detectable in purified murine hepatocytes as well as in sinusoidal endothelial cells (29). Though different liver cell types differ in specific FVIII mRNA content, this does not necessarily reflect differences in translational efficiency and/or protein export from the cell. On the other hand, our immunohistochemical analysis and previous studies on the cellular localization of FVIII protein (4,6) show that FVIII is primarily localized to microvascular cells, most likely sinusoidal endothelial cells and Kupffer cells (Fig.5A). Also FVIII staining of parenchymal cells could be observed, although the signal was relatively weak. These observations suggest that the sinusoidal endothelium of the liver contributes to FVIII synthesis.

Although the kidney was previously recognized as a site of FVIII gene expression (7-9) (see also Fig.2), the cellular sites of FVIII expression had not been determined. The present study localizes FVIII mRNA to distinct cell types within this organ (Fig.3C). For example, the in situ hybridization signal for FVIII mRNA is apparent in glomerular cells,
and podocytes, endothelial cells, and macrophages are potential candidates. A more detailed analysis using specific cellular markers is required to identify the cell type (or types) responsible for FVIII expression in this organ. As outlined above, the kidney apparently does not contribute substantially to the production of circulating FVIII in humans. Whether it does so in the mouse remains to be determined. In any case, our observations raise the possibility that FVIII produced by the kidney, serves a role in controlling local hemostasis.

It is of interest to note that compared to FVIII, the liver is apparently not a significant site of VWF synthesis, at least at the mRNA level (Table 1, Fig.4) (14). Similarly, purified hepatocytes, sinusoidal endothelial cells and Kupffer cells do not express detectable amounts of VWF mRNA, though FVIII mRNA is readily detectable in these cell types. In addition the presence of VWF antigen is mainly confined to the endothelial cells of large vessels (Fig.5B), while FVIII antigen is predominantly expressed in the sinusoidal endothelial cells (Fig.5A). Because of the distinct differences in both tissue- and cell-specific expression of each protein, it is possible that FVIII-VWF complex formation is an event that not only occurs at cellular sites that are in close proximity but also in the blood stream after release from different tissues.

ACKNOWLEDGMENTS

We thank Peter van Mourik, Ruud van Aalst and Anyal de Jonge for their assistance in isolating mouse tissue and Dr. Riekie Geertzen and Ron van der Meijden for their help in immunohistochemistry analysis. We also thank Dr. Koji Yamamoto for his help in initiating this study and Dr. Fahumiya Samad and Dr. Jan Voorbergh for helpful discussions. This work was supported in part by National Institutes of Health grant HL-47819 (D.J. Loskutoff).

REFERENCES

<table>
<thead>
<tr>
<th>Reference</th>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
<th>Year</th>
</tr>
</thead>
</table>


