Expression of the Blood-Clotting Factor-VIII cDNA Is Repressed by a Transcriptional Silencer Located in Its Coding Region

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Hemophilia A is caused by a deficiency of factor-VIII procoagulant (fVIII) activity. The current treatment by frequent infusions of plasma-derived fVIII concentrates is very effective but has the risk of transmission of blood-borne viruses (human immunodeficiency virus [HIV], hepatitis viruses). Use of recombinant DNA-derived fVIII as well as gene therapy could make hemophilia treatment independent of blood-derived products. So far, the problematic production of the fVIII protein and the low titers of the fVIII retrovirus stocks have prevented preclinical trials of gene therapy for hemophilia A in large-animal models. We have initiated a study of the mechanisms that oppose efficient fVIII synthesis. We have established that fVIII cDNA contains sequences that dominantly inhibit its own expression from retroviral as well as from plasmid vectors. The inhibition is not caused by instability of the fVIII mRNA (t1/2, ≥6 hours) but rather to repression at the level of transcription. A 305-bp fragment is identified that is involved in but not sufficient for repression. This fragment does not overlap the region recently identified by Lynch et al (Hum Gene Ther 4:259, 1993) as a dominant inhibitor of RNA accumulation. The repression is mediated by a cellular factor(s) and is independent of the orientation of the element in the transcription unit, giving the repressor element the hallmarks of a transcriptional silencer.

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MATERIALS AND METHODS

Cell lines and virus preparation. Cell lines were grown in high-glucose (4.5 g/L) Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum in a 5% CO2 atmosphere at 37°C. Generation of virus-producing cell lines and retrovirus harvesting, infection, and titration were performed as described.7 Polyclonal cell lines containing a single copy of the provirus were generated by infection of near-confluent cell cultures with a low multiplicity of infection (moi; <0.01) and selection in 400 pg G418 per milliliter. The resulting G418® polyclonal cell populations were maintained in medium containing 200 µg G418 per milliliter until use. In all instances that are more amenable to manipulation, such as from the skin,9 endothelium, bone marrow,4 and muscle.10 To date, retrovirus-mediated gene transfer is the most tested system in clinical trials of gene therapy.6,11 To accommodate factor VIII cDNA into the retroviral vectors, we and others have used shortened cDNA clones from which the region encoding the nonessential internal B-domain has been deleted.7,8,12 Such vectors have been successfully used to produce functional fVIII in primary human cells.7,9 However, the relatively low titer of fVIII retroviruses and the disappointingly low protein yields have hampered the initiation of preclinical trials in large-animal models. Several mechanisms have been identified that thwart the production of fVIII. Accumulation of fVIII mRNA is inhibited, purportedly as the result of some posttranscriptional event,8,13 the intracellular transport of fVIII is very inefficient,6,14 and, once secreted, the protein is extremely susceptible to proteolytic degradation and needs to be stabilized by the von Willebrand factor (VWF) protein.4

We report evidence for the presence of a dominant transcriptional silencer element in the fVIII cDNA and map the fVIII cDNA sequences involved. Characterization of this element will aid the development of more efficiently expressed fVIII cDNA clones. Increased expression would not only advance the development of gene therapy protocols but could also increase yields of recombinant-DNA-derived fVIII and, thus, will reduce the currently high price of this product.

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experiments, polyclonal cultures were used at low passage number. The packing lines Ps1 and 8A317 producing the M5neo, M5F8dB2.6, and XT-HER35 viruses have been described previously. The XT-HER vector contains the human epidermal growth factor-receptor (EGF-r) cDNA, driven by an internal herpes simplex virus-thymidine kinase gene promoter. In addition XT-HER contains the neomycin-resistance gene (neo) driven by the long terminal repeat (LTR) promoter. In the infected cells, mRNAs can be detected of 8.3 to 7.8 kb (with a neo+ probe) and 5 kb (only with an EGF-r probe). The M5FIX retroviral vector was constructed by insertion of a 1.4-kb BamHI containing the complete coding region of the human factor IX cDNA (provided by Dr. E. Davies, University of Washington, Seattle) into the unique BamHI site of plasmid M5neo. Polyclonal cultures of RAT2 cells with chromosomically-acetyl transerase (CAT)-reporter plasmids integrated in their genome were generated by cotransfection of 1 µg pRSVneo.46 2 µg pMulV-CAT (or equimolar amounts of its derivative plasmids carrying fVIII cDNA fragments), and 7 µg salmon-sperm DNA per 10-cm tissue culture dish. The resulting G418+ polyclonal cell population (consisting of greater than 50 transfectants) was maintained in G418-containing (200 µg/mL) medium and used at low passage number.

RNA analyses. Total cellular RNA was isolated from near-confluent cell cultures by the LiCl-urea method,17 and Northern blotting analyses were performed on nitrocellulose. To study the stability of the mRNA, actinomycin D (ActD; Sigma, St. Louis, MO) was added to near-confluent cultures at zero time to a final concentration of 5 µg/mL. RNA was isolated at the desired timepoints and analyzed by Northern analysis.

Probes. The neo6-specific probe used for the Northern analysis was the 1.3-kb HindIII-SmaI fragment of pRSVneo.46 The fVIII-specific probe was the 4.8-kb Sal I-Sal I fragment from pUC-F8dB2.6. The glyceraldehyde-3-phosphate (GAPDH)-specific probe was the 1.3-kb Pst I fragment from plasmid prGAPDH-13,18 representing the entire rat cDNA. The c-myc-specific probe used was the 1.3-kb Cla I-EcoRI fragment of the human c-myc gene.20 Probes were radiolabeled with [α-32P]dThidiposphate (UTP) in 200 µL. Subsequently, cold UTP was added to 100 µm/L, and the incubation was continued for 10 minutes. The samples were treated with RNase-free DNase (0.25 U/mL) and, subsequently, with proteinase K (100 µg/mL). The labeled RNA was precipitated and dissolved in hybridization buffer (40% [vol/vol] formamide, 20 mmol/L PIPES [pH 7.5], 0.2% sodium dodecyl sulfate [SDS], 0.4 mmol/L NaCl, 200 µg/mL yeast RNA, 1× Denhardt’s solution). Aliquots were counted, and identical amounts of radiolabeled RNA were added to each probe filter and hybridized for 48 hours at 42°C. The probe filters were prepared by spotting 2 µg of the single-stranded plasmid pM13 clones onto 0.22-µm nitrocellulose membranes. After the hybridization, the filters were washed in 2× standard sodium citrate (SSC), 0.5% SDS at 50°C and subsequently incubated in 10 mmol/L Tris-HCl (pH 7.6), 300 mmol/L NaCl, and 10 µg/mL RNase A (DNase-free); washed in 2× SSC, 0.1% SDS at 42°C; and left to expose Kodak XAR-5 films (Eastman-Kodak, Rochester, NY).

RESULTS

The fVIII protein consists of three types of structural domains that are arranged in the order A1:A2:B:A3:C1:C2. The unique B domain delimited by amino acids 740 and 1648 is removed during the proteolytic processing and activation and is dispensable for coagulation activity in vivo and in vitro.24,25 To accommodate the fVIII cDNA into a retroviral vector without exceeding the packaging capacity of the vector, we used a fVIII cDNA from which we had deleted virtually all codons for the B domain. We have previously shown that the resulting B-domain-deleted fVIII is fully functional.21 The vector used relies on differential splicing for the expression of the fVIII cDNA and the neo6 gene (Fig 1A). The titers of the fVIII vectors (≥3 × 104 fG18E colony-forming units [CFU]/mL) are much lower than those routinely obtained with other vectors (105 to 107 CFU/mL), as is the amount of fVIII protein secreted. For example, the amount of fVIII protein is at least 200-fold lower on a molar basis than that of coagulation factor IX from cells infected with a retroviral vector carrying fIX cDNA (Table 1). Our attempts to increase the titer by using other vectors and by
including an extended packaging signal\(^{26,27}\) into the M5neo vector did not yield any high-titer virus producer. In contrast, the vectors invariably lost (parts of) the fVIII cDNA (data not shown).

Sequences in the fVIII cDNA repress the accumulation of its RNA. It has been reported that sequences in the fVIII cDNA prevent accumulation of fVIII mRNA,\(^8\) supposedly by destabilizing the fVIII transcripts.\(^{15}\) This could explain both the low virus production by the packaging cells and the low amounts of fVIII protein secreted by the fVIII virus-infected cells. A Northern analysis of a G418\(^{8}\) population of Rat2 fibroblasts that contain the M5F8dB2.6 vector confirmed a low abundance of vector-specific RNA compared with cells infected with the parental M5neo vector (Fig IB), in which both the unspliced and spliced mRNAs are distinct. This is not due to the large size of the fVIII cDNA insert, as is shown by the amounts of 8.3- to 7.8-kb EGF-r mRNA in cells harboring a vector carrying the human EGF-r cDNA, which is similar in size to the B domain-deleted fVIII cDNA. The absence of rearrangements in the M5F8dB2.6 vector was confirmed by a Southern blot assay on DNA extracted from the infected Rat2 cell population (data not shown). The integrity of the vector was further evidenced by the appearance of fVIII RNA of the expected size after treatment of the cells with sodium butyrate (Fig 1B, compare lanes 2 and 4; in lane 4, both the unspliced 8.1-kb and the spliced 2.5-kb messengers are visible). The repression of fVIII mRNA accumulation was not unique for RAT2 cells but has been found in all cell lines tested; eg, Swiss3T3 cells, Psi-2 cells, murine endothelioma cells, and human skin fibroblasts (data not shown).

Factor VIII mRNA is stable in the packaging cells. To study the stability of the fVIII transcripts in the packaging cell line Psi2:M5F8dB2.6, transcription was blocked with ActD, an inhibitor of RNA polymerase II. At various times, RNA was extracted from the cell cultures and analyzed by Northern blotting (Fig 2). No significant decrease was observed up to 6 hours after addition of the ActD, suggesting that the fVIII transcripts are rather stable in these cells.

Rehybridization of this blot with a c-myc–specific probe showed the expected rapid decay of the c-myc transcripts [half-life (\(t_{1/2}\)), approximately 15 minutes\(^{29}\)], confirming the effectivity of the ActD block. The amount of RNA loaded was verified by reprobing the blot with a GAPDH-specific probe, an mRNA known to have a long half-life.\(^{18}\) From these data we conclude that the steady-state fVIII mRNA is stable \(t_{1/2}, \approx 6\) hours. The fact that not only accumulation of the unspliced messenger is inhibited in the M5F8dB2.6-infected cells, but also that of the spliced neo\(^8\) messenger, which does not contain any fVIII, suggests that repression acts before splicing occurs, possibly at the level of transcription. The fVIII viruses produced by the Psi2: M5F8dB2.6 cell line have been used to generate the fVIII-producing rodent cell lines described here, thus excluding the possibility that the fVIII vector in this cell line contains RNA-stabilizing mutations.
Expression is repressed at the level of transcription. A nuclear run-on analysis was performed to measure the transcription rate of the integrated fVIII provirus in both G418R Rat2:M5F8dB2.6 cells and G418R Rat2:M5neo cells. As shown in Fig 3, the neoR probe detects a signal only in RNA isolated from the M5neo-infected cells. In the Rat2: M5F8dB2.6 cells, neither the neoR probe nor the fVIII-specific probe shows any transcription of the provirus. The absence of signal with the antisense neoR probe confirms the specificity of the hybridization. The equality of labeling efficiencies was verified by a probe specific for the cellular GAPDH transcripts. Thus, insertion of the fVIII cDNA into the M5neo vector reduces the transcription of the integrated provirus to very low levels.

Factor VIII cDNA fragments repress expression of a heterologous gene. To study whether fVIII cDNA sequences can repress expression of a heterologous gene, fVIII cDNA fragments were cloned in the pMuLV-CAT expression vector (see Fig 5). In this vector, expression of the CAT reporter gene is driven by the Mo-MuLV LTR.20 To maximally mimic the natural configuration of the repressor elements, the fVIII cDNA fragments were introduced into the unique HpaI site of the expression plasmid. This site is located within the transcription unit but 3' of the CAT-encoding open reading frame so as not to influence translation of the CAT message. Two fragments were tested: SX, encompassing domains A1 and A2, and XS, which encodes domains A3, C1, and C2 (see Fig 5). Initially, both fragments were tested in the orientation they had in the retroviral vector. Insertion of the XS fragment did not cause a lower CAT activity than the parental pMuLV-CAT vector, whereas insertion of the SX fragment decreased CAT activity dramatically (Fig 4, left panel). A similar decrease of CAT activity was observed when the SX fragment was tested in the reverse orientation. In stably transfected polygonal cell cultures too, the SX fragments repressed expression of the MuLV-driven CAT-reporter gene (Fig 4, center panel). Northern blot analysis of RNA extracted from these cultures showed a vastly reduced amount of CAT mRNA in cells carrying the pMuLV-CAT/SX plasmid, which confirms that the decreased CAT activity in the SX fragment-containing cell population is reflecting the reduced amounts of CAT mRNA (data not shown). From these data we conclude that the repressor element that resides in the fVIII cDNA inhibits the expression of integrated as well as unintegrated heterologous genes and that this repression is independent of the orientation of the fVIII cDNA insert.

To map the sequences responsible for this repression in more detail, various subfragments have been tested for their capacity to reduce CAT activity (Fig 5). The AH fragment was a far more potent repressor than SA. The smallest fragment that could bring about repression was the PP fragment. Smaller fragments derived from the AH fragment, eg, HB, BH, XmB, and XmBm, did not decrease CAT activity. However, when the 305-bp XmB fragment, which by itself does not repress expression, was deleted from AH, the resulting clone AHdxmB was unable to repress expression. Deletion of the XmB fragment from the SX fragment, too, disrupted the repressor, as witnessed by the decreased CAT activity after transient expression (Fig 5) and in stably transfected polygonal cell populations (Fig 4, center panel). From these data, we conclude that sequences in the 305-bp XmB fragment are part of the repressor element, but are not sufficient to mediate repression.

The repression can be relieved by competitor DNA. In a competition experiment, the repression induced by the SX and the SXdxmB fragments were compared in the absence and in the presence of a 40-fold excess of the competitor plasmid, pUC-AH. The SX fragment represses expression...
of the CAT gene, in contrast to the SXdXmB construct (Fig 4, right panel). However, although cotransfection of the pUC-AH construct does not increase the expression of the reporter gene with the SXdXmB fragment inserted, the SX fragment-induced repression was relieved, and the CAT level was similar to the SXdXmB fragment. These data indicate that the repression is not caused by intrinsic properties of the DNA (ie, an unfavorable conformation), but that the repression is mediated by a cellular factor (or factors) that can be titrated off.

**DISCUSSION**

This report describes the identification of sequences in the coding region of human FVIII cDNA that repress expression at the level of transcription. This repressor downmodulates the expression of FVIII retroviral vectors, which results in inferior FVIII protein synthesis and reduces the production of recombinant retroviruses by at least two orders of magnitude. The scarcity of the vector-specific mRNA in the packaging lines can fully explain the low titers of the vector in the packaging lines and the difficulty of producing high titer retroviral vectors. This is in agreement with the observation that amplification of the FVIII vector in the packaging cell lines increases virus production. Furthermore, the amount of neo* transcripts in the target cells may be too low to allow the cell to survive selection by G418, lowering the apparent viral titer. Our observations confirm and extend the description by Lynch et al of the inhibition of mRNA accumulation by FVIII cDNA sequences [nucleotides (nt) 1681 to 2277, followed by 5002 to 5582]. However, we found the repression to be dependent on the presence of the Xmn I-Bgl II fragment (nt 1375 to 1680), which is located outside the region identified by these investigators. The cause of this discrepancy is not clear. Whereas the above mentioned study relied on virus production as a parameter for repression, we exploited a more direct assay based on the transient expression of a CAT gene, thereby circumventing the problems with the instability (deletion of FVIII sequences) of the viral vectors.

Kaufman et al studied RNA levels in Chinese hamster ovary (CHO) cells containing multiple (>100) FVIII cDNA copies and vWF cDNA expression vectors and observed 40-fold lower amounts of FVIII RNA than of vWF RNA when corrected for expression vector copy number. The transcription rates of both genes were found to be similar in a nuclear run-on analysis, which suggested that the cause of repression was posttranscriptional. In contrast, our study suggests the inhibition to be caused by a transcriptional mechanism. Our conclusion is based on several lines of evidence. First, the difference in steady-state amount of vector-specific RNA observed in cells infected with the M5neo vector and its M5F8dB2.0 derivative reflects the transcription rates as measured in a nuclear run-on assay. Second, the subgenomic neo* RNA, which does not contain any FVIII sequences, is repressed in cells containing the FVIII vector, suggesting a mechanism that operates before splicing occurs. Third, the relative stability of the FVIII mRNA in the packaging lines excludes cytoplasmic stability as a mechanism. This is further supported by the observation that insertion of the SX
Fig 5. Inhibition of heterologous gene expression by VIII cDNA fragments. The top lines depict the domain structure of the VIII protein and its B-domain-deleted derivative. Below this scheme, the various cDNA fragments tested for their capacity to repress expression of the linked CAT-reporter gene are indicated. Assays were performed as described in the legend to Fig 4. All VIII cDNA fragments are described in Materials and Methods. Fragments that severely inhibited CAT expression (greater than 10-fold reduction; ++) are represented as solid bars; the fragment that only moderately affected CAT activity (twofold to threefold reduction; +/−) is represented by a shaded bar; and fragments that affected CAT activity hardly, if at all, (less than 50% reduction; −) are hatched. All fragments were tested in the same orientation they had in the retroviral vector. The inset shows the CAT-reporter plasmid pMuLV-CAT used in this study. The Hpa I site was used to insert the VIII cDNA fragments; sd and sa, splice-donor and splice-acceptor sites, respectively; pA, polyadenylation signals.
the autonomously replicating sequences (ARS) of yeast.\textsuperscript{32,33} The ARS elements allow extrachromosomal maintenance of plasmids in yeast and have been implicated as functioning as chromosomal replication origins and as nuclear-matrix attachment regions (MARs), as well as modulating the activity of transcriptional enhancers and silencers.\textsuperscript{32-36} In MARs of higher eukaryotes, similar consensus sequences have been found.\textsuperscript{37} Thus, an MAR or ARS in the FVIII cDNA could explain the repressing effects of the FVIII cDNA on transcription. In addition, the observation that deletion of the Xmn I-Bgl II region relieves repression while the fragment itself is not sufficient to decrease expression fully complies with the known modular structure of MARs as well as ARS elements.\textsuperscript{32,34} Removal or mutation of some of the ARS elements decreases its function, but does not abolish it completely.\textsuperscript{32,34} We have evidence that the FVIII-derived WT TT AY R T T T T T W elements can repress expression of heterologous genes if inserted in the transcription unit,\textsuperscript{37} showing the potential of these elements in the FVIII cDNA to repress expression. If the expression of FVIII is inhibited by the fortuitous presence of ARS or MARs in the FVIII cDNA, multiple mutations will be required to completely abolish the repressor. Deletion of the 305-bp Xmn I-Bgl II fragment from the expression vectors is not an option. This region of the molecule is essential for FVIII function, as eight missense mutations have been identified in this region that result in hemophilia A.\textsuperscript{38} Currently, we are trying to elucidate the precise mechanism of repression and to define the sequences involved; eg, by identifying protein-binding elements in the 305-bp Xmn I-Bgl II fragment. Identification of the responsible elements will permit elimination of the binding sites by the introduction of silent mutations. As an alternative approach, introns could be introduced to separate and potentially inactivate various repressor elements. The generation of mutated FVIII cDNAs that are expressed more efficiently will increase the amount of FVIII protein produced in the cells and will improve the titer of FVIII virus stocks. This, of course, will not only improve the feasibility of gene therapy for hemophilia A, but will also be of considerable importance for the production of recombinant-DNA--derived FVIII for transfection.

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