2 Naylor J, Brinke A, Hassock S, Green PM, Giannelli F. Characteristic mRNA abnormality found in half the patients with severe haemophilia A is due to large DNA inversions. *Hum Mol Genet* 1993; **2**: 1773–8.
3 Naylor JA, Buck D, Green P, Williamson H, Bentley D, Giannelli F. Investigation of the factor VIII intron 22 repeated region (int22h) and the associated inversion junctions. *Hum Mol Genet* 1995; **4**: 1217–24.
4 Bagnall RD, Giannelli F, Green PM. Polymorphism and hemophilia A causing inversions in distal Xq28: a complex picture. *J Thromb Haemost* 2005; **3**: 2598–9.
5 Bagnall RD, Giannelli F, Green PM. Int22h-related inversions causing hemophilia A: a novel insight into their origin and a new more discriminant PCR test for their detection. *J Thromb Haemost* 2006; **4**: 591–8.
6 Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood* 2002; **99**: 168–74.
7 Schroder W, Wehnert M, Herrmann F. Intron 22 of factor VIII gene – a hot spot for structural aberrations causing severe hemophilia A [letter]. *Blood* 1996; **87**: 3067–8.
8 Andrikovics H, Klein I, Bors A, Nemes L, Marosi A, Varadi A, Tordai A. Analysis of large structural changes of the factor VIII gene, involving intron 1 and 22, in severe hemophilia A. *Haematologica* 2003; **88**: 778–84.
9 Abou-Elew H, Ahmed H, Raslan H, Abdelwahab M, Hammoud R, Mokhtar D, Arnaout H. Genotyping of intron 22-related rearrangements of F8 by inverse-shifting PCR in Egyptian hemophilia A patients. *Ann Hematol* 2011; **90**: 579–84.
10 Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* 2006; **12**: 15–22.
11 Sugihara T, Takahashi I, Kojima T, Okamoto Y, Yamamoto K, Kamiya T, Matsushita T, Saito H. Identification of plasma antibody epitopes and gene abnormalities in Japanese hemophilia A patients with factor VIII inhibitor. *Nagoya J Med Sci* 2000; **63**: 25–39.
12 Kojima T, Tanimoto M, Kamiya T, Obata Y, Takahashi T, Ohno R, Kurachi K, Saito H. Possible absence of common polymorphisms in coagulation factor IX gene in Japanese subjects. *Blood* 1987; **69**: 349–52.
13 Liu Q, Nozari G, Sommer SS. Single-tube polymerase chain reaction for rapid diagnosis of the inversion hotspot of mutation in hemophilia A. *Blood* 1998; **92**: 1458–9.
14 Rossetti L, Radic C, Larripa I, De Brasi C. Developing a new generation of tests for genotyping hemophilia-causative rearrangements involving *int22h* and *int1h* hotspots in the factor VIII gene. *J Thromb Haemost* 2008; **6**: 830–6.
15 Tsukahara A, Yamada T, Takagi A, Murate T, Matsushita T, Saito H, Kojima T. Compound heterozygosity for two novel mutations in a severe factor XI deficiency. *Am J Hematol* 2003; **73**: 279–84.
16 Rossetti L, Radic C, Abelleyro M, Larripa I, De Brasi C. Eighteen years of molecular genotyping the hemophilia inversion hotspot: from southern blot to inverse shifting-PCR. *Int J Mol Sci* 2011; **12**: 7271–785.
17 Daley JM, Palmbos PL, Wu D, Wilson TE. Nonhomologous end joining in yeast. *Annu Rev Genet* 2005; **39**: 431–51.
18 Lieber MR, Ma Y, Pannicke U, Schwarz K. The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination. *DNA Repair* 2004; **3**: 817–26.
19 Symington L. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* 2002; **66**: 630–70.
20 Sheen CR, Jewell UR, Morris CM, Brennan SO, Férec C, George PM, Smith MP, Chen J-M. Double complex mutations involving F8 and FUNDC2 caused by distinct break-induced replication. *Hum Mutat* 2007; **28**: 1198–206.
21 Lieber MR. The Mechanism of Human Nonhomologous DNA End Joining. *J Biol Chem* 2008; **283**: 1–5.
22 Rossiter JP, Young M, Kimberland ML, Hutter P, Ketterling RP, Gitschier J, Horst J, Morris MA, Schaid DJ, de Moerloose P, Sommer SS, Kazazian HH, Antonarakis SE. Factor VIII gene inversions causing severe hemophilia A originate almost exclusively in male germ cells. *Hum Mol Genet* 1994; **3**: 1035–9.
23 Mühle C, Schulz-Drost S, Khrenov AV, Saenko EL, Klinge J, Schneider H. Epitope mapping of polyclonal clotting factor VIII-inhibitory antibodies using phage display. *Thromb Haemost* 2004; **91**: 619–25.
24 Mühle C, Zenker M, Chuzhanova N, Schneider H. Recurrent inversion with concomitant deletion and insertion events in the coagulation factor VIII gene suggests a new mechanism for X-chromosomal rearrangements causing hemophilia A. *Hum Mutat* 2007; **28**: 1045.
25 Miskinyte S, Butler MG, Hervé D, Sarret C, Nicolino M, Petralia JD, Bergametti F, Arnould M, Pham VN, Gore AV, Spengos K, Gazal S, Woimant F, Steinberg GK, Weinstein BM, Tournier-Lasserve E. Loss of BRCC3 deubiquitinating enzyme leads to abnormal angiogenesis and is associated with syndromic moyamoya. *Am J Hum Genet* 2011; **88**: 718–28.

BJ Gene

Biochem. J. (2013) **449**, 231–239 (Printed in Great Britain) doi:10.1042/BJ20121286

online data

Ribavirin-induced intracellular GTP depletion activates transcription elongation in coagulation Factor VII gene expression

Atsuo SUZUKI*, Yuhri MIYAWAKI*, Eriko OKUYAMA*, Moe MURATA*, Yumi ANDO*, Io KATO*, Yuki TAKAGI*, Akira TAKAGI*, Takashi MURATE*, Hidehiko SAITO† and Tetsuhito KOJIMA*[1]

*Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya 461-8673, Japan, and †National Hospital Organization-Nagoya Medical Center, Nagoya 460-0001, Japan

Coagulation FVII (Factor VII) is a vitamin K-dependent glycoprotein synthesized in hepatocytes. It was reported previously that FVII gene (*F7*) expression was up-regulated by ribavirin treatment in hepatitis C virus-infected haemophilia patients; however, its precise mechanism is still unknown. In the present study, we investigated the molecular mechanism of ribavirin-induced up-regulation of *F7* expression in HepG2 (human hepatoma cell line). We found that intracellular GTP depletion by ribavirin as well as other IMPDH (inosine-5′-monophosphate dehydrogenase) inhibitors, such as mycophenolic acid and 6-mercaptopurine, up-regulated *F7* expression. FVII mRNA transcription was mainly enhanced by accelerated transcription elongation, which was mediated by the P-TEFb (positive-transcription elongation factor b) complex, rather than by promoter activation. Ribavirin unregulated ELL (eleven-nineteen lysine-rich leukaemia) 3 mRNA expression before *F7* up-regulation. We observed that ribavirin enhanced ELL3 recruitment to *F7*, whereas knockdown of ELL3 diminished ribavirin-induced FVII mRNA up-regulation. Ribavirin also enhanced recruitment of CDK9 (cyclin-dependent kinase 9) and AFF4 to *F7*. These data suggest that ribavirin-induced intracellular GTP depletion recruits a super elongation complex containing P-TEFb, AFF4 and ELL3, to *F7*, and modulates FVII mRNA transcription elongation. Collectively, we have elucidated a basal mechanism for ribavirin-induced FVII mRNA up-regulation by acceleration of transcription elongation, which may be crucial in understanding its pleiotropic functions *in vivo*.

Key words: coagulation Factor VII, eleven-nineteen lysine-rich leukaemia 3 (ELL3), gene up-regulation, positive-transcription elongation factor b (P-TEFb), ribavirin, transcription elongation.

INTRODUCTION

FVII (Factor VII), a vitamin K-dependent plasma glycoprotein, is synthesized in the liver and secreted into the blood as a single-chain zymogen at a concentration of 500 ng/ml in plasma [1,2]. Upon vascular injury and in the presence of calcium, FVII forms a one-to-one stoichiometric complex with its cell-surface receptor and cofactor TF (tissue factor). Once in a complex with TF, FVII is rapidly cleaved to its active form FVIIa, and converts zymogen Factor IX and Factor X into active enzymes [3,4]. The formation of an active complex between TF and FVIIa is widely thought to be the primary stimulus for blood coagulation.

Haemophilia is an X-linked recessive bleeding disorder caused by a deficiency in the activity of coagulation Factor VIII or IX. One of the treatments for haemophilia is replacement of lacked coagulation Factor VIII (haemophilia A) or IX (haemophilia B); however, the development of an alloimmune antibody inhibiting Factor VIII or IX currently represents the most serious complication, particularly if the antibody is classified as highly responding, where substitution with Factor VIII or IX fails to provide adequate haemostasis [5]. In haemophilia patients with high responding inhibitors, haemostatic treatments include clotting Factor VIII- or IX-bypassing agents, such as a prothrombin complex concentrate, plasma-derived activated prothrombin complex or rFVIIa (recombinant FVIIa) [6]. In particular, rFVIIa eliminates the risk of human blood-transmitted diseases and acts by enhancing the natural coagulation pathway through activation of prothrombinase complex formation and has a local action only in areas where TF and/or phospholipids are exposed [3,7].

In 2006, Yamamoto et al. [8] reported that the anti-HCV (hepatitis C virus) agent ribavirin elevated the activity of FVII, leading to reduced doses of clotting factors that are used in haemostatic therapy for patients with chronic hepatitis C. They demonstrated increased mRNA expression of the *F7* (FVII) gene by ribavirin treatment in HepG2 (human hepatoma cell line) and in cultured human hepatocytes; however, the detailed molecular mechanisms for this phenomenon remain unknown.

Ribavirin is a purine nucleoside analogue used in anti-HCV therapy. The proposed mechanisms for the antiviral actions of ribavirin include inducing error catastrophe, inhibiting the activity of RNA-dependent RNA polymerase of HCV, inhibiting the activity of IMPDH (inosine-5′-monophosphate dehydrogenase) to decrease the GTP pool and modulating the immune system [9]. Although the actions of ribavirin are partially understood, the mechanisms are not completely clear.

The aim of the present study was to investigate further the molecular mechanisms of increased FVII mRNA expression by ribavirin treatment in HepG2 cells. To date, there have been few reports on the effects of ribavirin on endogenous gene

Abbreviations used: 6-MP, 6-mercaptopurine; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CTD, C-terminal domain; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; ELL, eleven-nineteen lysine-rich leukaemia; EU, 5-ethynyl uridine; FBS, fetal bovine serum; FVII, Factor VII; HCV, hepatitis C virus; IFN, interferon; IMPDH, inosine-5′-monophosphate dehydrogenase; ISG, IFN-stimulated gene; MPA, mycophenolic acid; NS, non-specific; Pol II, RNA polymerase II; P-TEFb, positive-transcription elongation factor b; pSer, phosphorylated serine; qRT-PCR, quantitative real-time PCR; rFVIIa, recombinant FVIIa; RMP, ribavirin monophosphate; RNAi, RNA interference; SEC, super elongation complex; siRNA, small interfering RNA; TF, tissue factor.

[1] To whom correspondence should be addressed (email kojima@met.nagoya-u.ac.jp).

expression in mammalian cells. It is important to elucidate the molecular mechanisms of ribavirin before understanding its *in vivo* functions.

EXPERIMENTAL

Cell culture and reagents

The HepG2 cell line was purchased from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured as described previously [10]. HepG2 cells were incubated with ribavirin, MPA (mycophenolic acid) or 6-MP (6-mercaptopurine) (Sigma) in DMEM (Dulbecco's modified Eagle's medium) (Wako) supplemented with 10% (v/v) FBS (fetal bovine serum) (JRH Biosciences) and 5 μg/ml vitamin K_1 (Isei). DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside) was purchased from Sigma.

Total RNA isolation and qRT-PCR (quantitative real-time PCR)

RNA preparation and qRT-PCR were performed as described previously with minor modifications [10]. Total RNA was extracted from the cells using the RNeasy mini kit (Qiagen) and the first strand cDNA was prepared from 1 μg of total RNA using the PrimeScript™ RT reagent kit (TaKaRa Bio). qRT-PCR was performed with SYBR® Premix ExTaq II to detect GusB, HPRT1, ELL (eleven-nineteen lysine-rich leukaemia) 3 and TCEB3 mRNA, and with SYBR® Premix Dimer Eraser to detect FVII and ELL2 mRNA, using the primers described in Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490231add.htm. The level of mRNA expression in all experiments was calculated as relative values of the respective mRNAs normalized to GusB mRNA.

Protein extraction and Western blotting

Nuclear protein was extracted from HepG2 cells treated with or without 100 μg/ml ribavirin using the CelLytic™ NuCLEAR™ Extraction Kit (Sigma) according to the manufacturer's protocol.

Western blotting was performed as reported previously [10]. Membranes were blocked with excess protein (1%, w/v, non-fat dried skimmed milk powder) in PBS supplemented with 0.05% Tween 20. Primary antibodies against CDK (cyclin-dependent kinase) 9, histone H1 (Santa Cruz Biotechnology), Pol II (RNA polymerase II) 8WG16 (Covance), Pol II CTD (C-terminal domain) $pSer^2$ (pSer is phosphorylated serine), Pol II CTD $pSer^5$ (Abcam), ELL3 (Abnova) or α-tubulin (Sigma) were used. When using anti-pSer antibodies, membranes were blocked with 0.5% BSA in Tris-buffered saline supplemented with 0.05% Tween 20. Signals were visualized using Immobilon-Western chemiluminescent substrate (Millipore).

Luciferase reporter assay

The human *F7* promoter region was prepared by PCR amplification from human genomic DNA. The PCR products from -1593 to -1 or -722 to -1 (with A of the ATG translation initiation codon as $+1$) were inserted into the pGL4.10 vector (Promega) (F7–1593/pGL4 or F7–722/pGL4 respectively). The *PROS1* (protein S) gene promoter region was cloned previously [10], and the *PROS1* promoter fragments were recombined into pGL4.10 vectors.

HepG2 cells were seeded into 35-mm-diameter dishes at a concentration of 10^5 cells in DMEM supplemented with 10% FBS. After 16 h, the appropriate *F7* luciferase reporter plasmids (200 ng) or empty vector and 50 ng of pGL4.74 vector (Promega) were transiently co-transfected using the Lipofectamine 2000™ reagent (Invitrogen), according to the manufacturer's protocol. Following the 4-h transfection period, the culture medium was exchanged for fresh DMEM supplemented with 10% FBS and vitamin K_1, with or without 100 μg/ml of ribavirin. The cells were treated with ribavirin and harvested at 24 h. Luciferase activities were subsequently determined with a luciferase assay system (Promega) according to the manufacturer's protocol, with luciferase activity normalized to the activity of co-transfected *hRluc* (human-optimized *Renilla* luciferase) as an internal control for transfection efficiency.

Nuclear run-on assay

The nuclear run-on assay was performed as reported previously with minor modifications [11]. HepG2 cells were grown in 100-mm-diameter dishes and cultured with or without ribavirin (100 μg/ml) or MPA (100 μM) for 24 h. The cells were washed twice with chilled PBS and intact nuclei were collected. Total RNA was isolated using the RNeasy mini kit. The biotin-labelled RNA-binding pull-down assay was performed using Dynabeads® M-280 Streptavidin (Invitrogen). The RNA-bound beads were suspended in 10 μl of RNase-free water, and the bound RNA was used as a template for reverse transcription and subjected to qRT-PCR as described above.

Nascent RNA capturing

Newly synthesized RNA transcripts were determined by the Click-iT® Nascent RNA Capture Kit (Invitrogen) according to the manufacturer's protocol. HepG2 cells were cultured with or without ribavirin (100 μg/ml) for 16 h and subsequently pulsed with 0.2 mM EU (5-ethynyl uridine) at 37 °C for 8 h. The cells were washed and collected, and total RNA was extracted. A Click reaction was performed using 5 μg of EU-labelled RNA and 0.5 mM biotin azide; the mixture was incubated at room temperature (25 °C) for 30 min. Following RNA precipitation, the RNA was dissolved in 50 μl of RNase-free water. Biotin-labelled EU–RNA-binding pull-down assay was performed using 50 μl of Dynabeads® MyOne™ Streptavidin and the bound RNA was washed and used as a template for reverse transcription. The captured nascent RNA was analysed using qRT-PCR as described above.

We also used the Click-iT® Nascent RNA Capture kit to determine mRNA decay. HepG2 cells were treated with ribavirin (100 μg/ml) and 5-EU (0.2 mM) at 37 °C for 24 h, and the medium was replaced with growth medium without 5-EU. Total RNAs were isolated at several time points (0, 12 and 24 h after exchanging the medium) and subjected to the Click reaction. The reaction was performed using 1 μg of EU-labelled RNA and 0.25 mM biotin azide. A biotin-labelled EU–RNA pull-down assay was performed using 15 μl of Dynabeads® MyOne™ Streptavidin.

Microarray

Gene expression profiles of HepG2 cells treated or untreated with ribavirin were obtained by microarray analysis using the Human 3D-Gene Oligo chip 25K from outsourcing to Toray.

RNAi (RNA interference)

HepG2 cells were transfected with ELL3-specific siRNA (small interfering RNA) (siGENOME SMARTpool siRNA, human ELL3; Dharmacon) or NS (non-specific) siRNA (siGENOME

Non-Targeting siRNA#3; Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) by the reverse transfection protocol according to the manufacturer's protocol. The siRNAs (20 μM) were combined with Opti-MEM (Invitrogen) and mixed with Lipofectamine RNAiMAX reagent. After incubation at room temperature for 20 min, 2.5×10^5 cells were seeded into 60-mm-diameter dishes and cultured for 24 h. The cells were then treated with ribavirin (100 μg/ml) for 24 h and nuclear protein or total RNA was extracted as described above.

ChIP (chromatin immunoprecipitation)

ChIP was performed as described previously with minor modifications [10]. HepG2 cells were cultured with or without ribavirin (100 μg/ml) for 18 h, and fixed with 1 % formaldehyde at 37 °C for 10 min. The cross-linking reaction was stopped by the addition of glycine, at a final concentration of 0.125 M. The chromatin supernatants were precleared with Dynabeads® Protein G and immunoprecipitated with respective antibodies specific to Pol II 4H8, Pol II CTD pSer^2, Pol II CTD pSer^5 (Abcam), CDK9, AFF4, ELL3 and NS normal rabbit IgG (Santa Cruz Biotechnology) at 4 °C for 3 h. The protein–antibody complexes were incubated with Dynabeads® Protein G at 4 °C for 1 h. The beads were extensively washed and the immune complexes were extracted from the beads using elution buffer, and the protein–DNA cross-linking was reversed by incubation at 65 °C for overnight. Bound DNA was purified with a High Pure PCR Cleanup Micro Kit (Roche Applied Science) and used as a template for subsequent quantitative PCR performed with a SYBR® Premix ExTaq II reagent. The primers used for quantitative PCR are listed in Supplementary Table S2 at http://www.biochemj.org/bj/449/bj4490231add.htm.

Statistical analysis

Data are presented as means ± S.D. and are representative of at least three independent experiments. Significant differences ($P < 0.05$) between experimental groups in the qRT-PCR and luciferase assays were observed using Student's t test.

RESULTS

Effects of ribavirin treatment on FVII mRNA expression in HepG2 cells

In the present study, we investigated further the molecular mechanisms of up-regulated *F7* expression by ribavirin treatment. We first examined FVII mRNA levels in HepG2 cells treated with various concentrations of ribavirin (for 24 h) and observed increased FVII mRNA expression in a dose-dependent manner (Figure 1A). We also analysed the effects of ribavirin treatment (100 μg/ml) at various time points in HepG2 cells and observed increased FVII mRNA levels in a time-dependent manner (Figure 1B). The referenced GusB gene expression was slightly increased by ribavirin treatment, but not in a dose-dependent and time-dependent manner (Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490231add.htm). Therefore we used ribavirin at a concentration of 100 μg/ml for 24 h in the following experiments.

Effects of other IMPDH inhibitors on *F7* expression

Ribavirin is metabolized in the cytoplasm to RMPs (ribavirin monophosphates), which are known to inhibit IMPDH in *de novo* synthesis of purine metabolisms. As the immunosuppressants

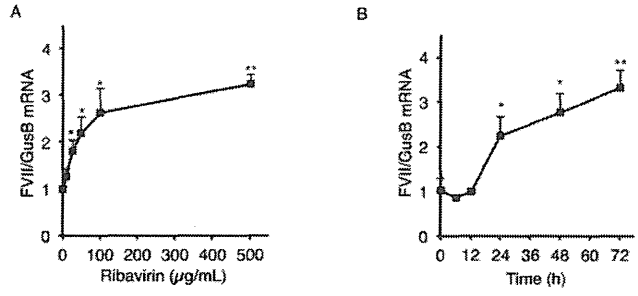


Figure 1 Quantification of FVII mRNA in HepG2 cells treated with or without ribavirin

qRT-PCR was performed to determine FVII mRNA levels in HepG2 cells. (**A**) Dose-dependent increase in FVII mRNA expression in HepG2 cells treated with ribavirin for 24 h. The cells were treated with several different concentrations of ribavirin (10, 25, 50, 100 or 500 μg/ml). (**B**) Time-course analysis of FVII mRNA levels under ribavirin treatment in HepG2 cells. The cells were treated with 100 μg/ml ribavirin for 6, 12, 24, 48 or 72 h and total RNA from the cells were isolated as described in the Experimental section. The expression levels of FVII mRNA normalized to GusB mRNA were shown as relative values to those of untreated cells. *$P < 0.05$ compared with untreated cells. **$P < 0.01$ compared with untreated cells.

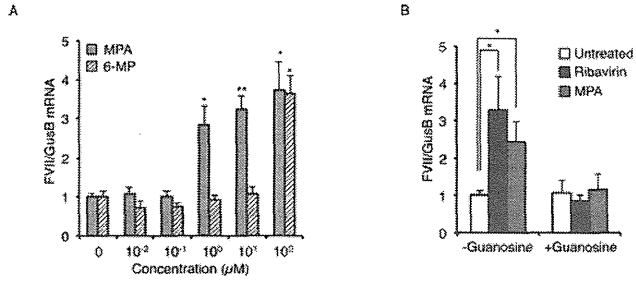


Figure 2 Induction of FVII mRNA expression in HepG2 cells by treatment with MPA and retraction by treatment with guanosine

(**A**) HepG2 cells were treated with MPA or 6-MP (0–100 μM) for 24 h and total RNA was isolated from the cells. FVII mRNA levels were determined by qRT-PCR and expressed as relative values to that of untreated HepG2 cells. (**B**) FVII mRNA induced by ribavirin or MPA treatment was retracted by the addition of guanosine in HepG2 cells. The expression levels of FVII mRNA normalized to GusB mRNA were shown as relative values to those of untreated cells. *$P < 0.05$ compared with untreated cells. **$P < 0.01$ compared with untreated cells.

MPA and 6-MP are also known to be IMPDH inhibitors, we examined the effects of MPA and 6-MP on *F7* gene expression in HepG2 cells (Figure 2A). MPA and 6-MP were used at concentrations of up to 100 μM to treat HepG2 cells for 24 h. We found that these IMPDH inhibitors induced an approximate 3–4-fold increase in FVII mRNA levels at concentrations ranging from 1 to 100 μM.

As treatment with the IMPDH inhibitors resulted in reduced intracellular guanine nucleoside pools, we tested whether the addition of guanosine would reverse FVII mRNA up-regulation (Figure 2B). The increased FVII mRNA expression in the HepG2 cells was retracted following supplementation of guanosine (100 μM) in the presence of ribavirin or MPA. These results confirmed that IMPDH inhibitors induced intracellular GTP depletion and increased FVII mRNA expression, which was retracted by the addition of guanosine by retrieving the guanine nucleotides.

Ribavirin-induced FVII mRNA up-regulation through transcription elongation

To investigate how ribavirin contributes to FVII mRNA up-regulation in HepG2 cells, we analysed the effects of ribavirin

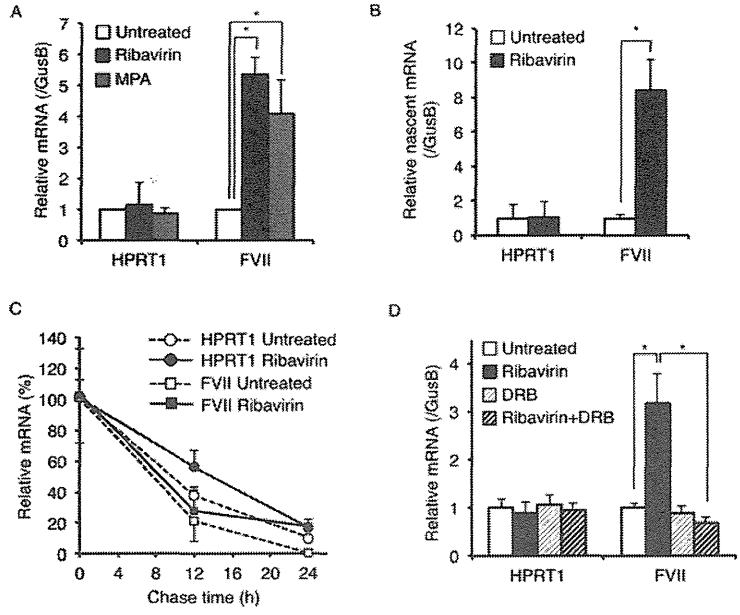


Figure 3 ***De novo*** **synthesis of FVII mRNA in ribavirin-treated HepG2 cells**

The mechanisms of ribavirin-induced FVII mRNA up-regulation in HepG2 cells were investigated. (**A**) Nuclear run-on assay was performed using HepG2 cells treated with or without ribavirin and MPA for 24 h, and intact nuclei were isolated. Newly synthesized transcripts were labelled with biotin-16-UTP and collected using Streptavidin-conjugated magnet beads. Relative levels of HPRT1 and FVII mRNAs normalized to GusB mRNA were determined *in vitro* by qRT-PCR. (**B**) Measurement of nascent mRNA in HepG2 cells treated with ribavirin was conducted as described in the Experimental section. Relative expression levels of HRPT1 and FVII mRNAs were normalized to GusB mRNA. (**C**) FVII and HRPT1 mRNA stability in HepG2 cells treated with or without ribavirin. The mRNA stability was determined using Click-iT® Nascent RNA Capture kit as described in the Experimental section. The results show mRNA decay of FVII and HPRT1 in HepG2 cells. (**D**) Inhibition of transcription elongation by DRB. HepG2 cells were treated with 50 μM DRB for 8 h before exposure with ribavirin (100 μg/ml) for 16 h. DMSO was added as the vehicle control for DRB. Relative expression levels of HPRT1 and FVII mRNAs were normalized to GusB mRNA. $^*P < 0.05$ compared with untreated cells.

treatment on *F7* transcription activity. We assessed newly synthesized FVII mRNA transcripts with the nuclear run-on assay under ribavirin treatment (Figure 3A). The nuclear run-on assay demonstrated that *in vitro F7* transcriptants in ribavirin-treated HepG2 cells were five times more abundant than those in the untreated cells. We measured HPRT1 mRNA as a ribavirin-insensitive control, which showed no significant increase by ribavirin treatment. We also observed that MPA augmented newly synthesized FVII mRNA in HepG2 cells. This observation was confirmed by measuring the nascent FVII mRNAs using the Click-iT® Nascent mRNA Capturing Kit. Nascent FVII mRNA synthesis was increased ~8-fold in HepG2 cells treated with ribavirin compared with untreated cells (Figure 3B), whereas HPRT1 mRNA synthesis did not show any significant changes. We performed luciferase-reporter analyses, and observed a small increase (~1.5-fold) in *F7* promoter activity by ribavirin treatment (Supplementary Figure S2A at http://www.biochemj.org/bj/449/bj4490231add.htm); however, we also observed a similar increase (~1.3-fold) in ribavirin-insensitive *PROS1* promoter activity (Supplementary Figures S2B and S2C). These results suggested that the up-regulation of *F7* promoter activity by ribavirin might be caused mainly by a background effect, probably as a result of the pGL4.10 vector itself, in the present study. Meanwhile we could not find significant changes in FVII mRNA stability (Figure 3C).

To assess the impact of ribavirin at the late stages of transcription, we examined the effects of the transcription elongation inhibitor DRB on FVII mRNA up-regulation in ribavirin-treated HepG2 cells (Figure 3D). DRB is known to inhibit the activity of CDK9, which is a major component of P-TEFb [12]. In untreated HepG2 cells, DRB (50 μM) caused only a slight decrease in FVII mRNA expression. By contrast, DRB abolished the increased FVII mRNA expression in HepG2 cells treated with ribavirin.

Microarray analyses for gene expression profiles induced by ribavirin treatment in HepG2 cells

To investigate how ribavirin modulates *F7* transcript elongation, we used cDNA microarray analysis to search for candidate molecules associated with increased FVII mRNA expression in HepG2 cells. Microarray analysis screened 25 394 human genes and found that 842 genes were up-regulated following ribavirin treatment in HepG2 cells. As shown in Table 1, the transcription elongation factors ELL2 gene (*ELL2*), ELL3 gene (*ELL3*) and elongin A gene (*TCEB3*) were found as putative up-regulated genes related to 'transcription and translation'. We examined the expression levels of these three genes by qRT-PCR and found no significant increase in ELL2 and elongin A mRNA expression, whereas ELL3 mRNA expression was significantly increased in HepG2 cells treated with both ribavirin and MPA.

Ribavirin induced ELL3 mRNA expression before up-regulation of FVII mRNA expression

Next, we determined ELL3 expression levels at several time points in ribavirin-treated HepG2 cells. We found an approximate 3-fold increase in ELL3 mRNA expression at 3–12 h following ribavirin treatment, which increased 8-fold at 24 h post-treatment (Figure 4A). This is in contrast with the increased FVII mRNA expression at 24 h after ribavirin treatment (Figure 1B). There were no significant changes in ELL3 and FVII mRNA expression in untreated HepG2 cells. This ELL3 up-regulation was also induced by MPA treatment, and restored by guanosine

Table 1 Microarray screening for whole-gene expression in HepG2 and HuH7 cells treated with ribavirin and confirmed by qRT-PCR

Analysis of mRNA expression by microarray and qRT-PCR. Microarray data show selected genes whose expression was changed more than 2-fold in cell-to-cell comparisons (cells treated with ribavirin compared with without ribavirin). For reverse transcription–PCR (primers are listed in Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490231add.htm), values were normalized to GusB mRNA and are represented relative to that of untreated HepG2 cells. $*P < 0.05$ compared with untreated cells.

Screen	Treatment	ELL2	ELL3	Elongin A
Microarray	Ribavirin	2.32	3.99	2.57
qRT-PCR	Ribavirin	1.79 ± 0.46	7.86 ± 1.83*	1.62 ± 0.46
qRT-PCR	MPA	0.78 ± 0.14	4.40 ± 0.19*	0.66 ± 0.05

supplementation at both mRNA and protein levels (Figures 4B and 4C).

To verify the function of ELL3 in the up-regulation of *F7* expression by ribavirin, we performed RNAi experiments on ELL3. We transfected HepG2 cells with ELL3-specific and NS siRNA and analysed ELL3 expression levels by Western blotting and qRT-PCR. We observed that ribavirin-induced ELL3 protein up-regulation was abolished in the ELL3 siRNA-transfected cells (Figure 4D). We also observed diminished expression of ELL3 mRNA levels in the ELL3 siRNA-transfected cells (Figure 4E). Next, we investigated the effects of ELL3 knockdown on *F7* expression by qRT-PCR, and observed an attenuated ribavirin-induced up-regulation of FVII mRNA expression in ELL3 siRNA-transfected HepG2 cells, whereas there was no significant difference in FVII mRNA levels between the NS siRNA and ELL3 siRNA-transfected HepG2 cells (Figure 4F).

Ribavirin treatment resulted in modulation of Pol II CTD phosphorylation and enhanced the recruitment of Pol II and elongation factors in HepG2 cells

As shown in Figures 3 and 4, increased FVII mRNA expression was likely to be dependent on transcription elongation associated with P-TEFb and to some extent with ELL3. We performed ChIP analyses to evaluate the recruitment of Pol II to the *F7* gene, the status of serine phosphorylation of the Pol II CTD, and the P-TEFb and/or ELL3 contribution to transcription elongation efficacy by Pol II on *F7* gene expression (Figures 5A and 5B). Indeed, ribavirin treatment increased Pol II occupancy and the level of phosphorylation of CTD Ser^2. Ribavirin treatment did not increase the levels of CTD $pSer^5$, but decreased slightly at some points of *F7* gene context. Ribavirin treatment also increased the recruitment of both CDK9 (P-TEFb) and ELL3, which displayed a similar distribution pattern. In addition, we observed ribavirin treatment-enhanced AFF4 recruitment to the FVII, which was occupied by CDK9 and ELL3.

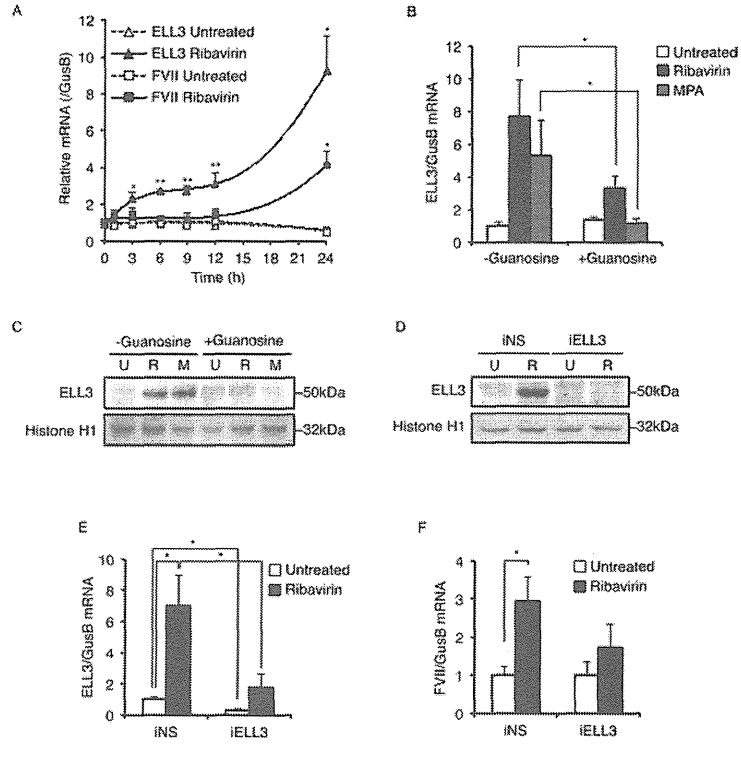
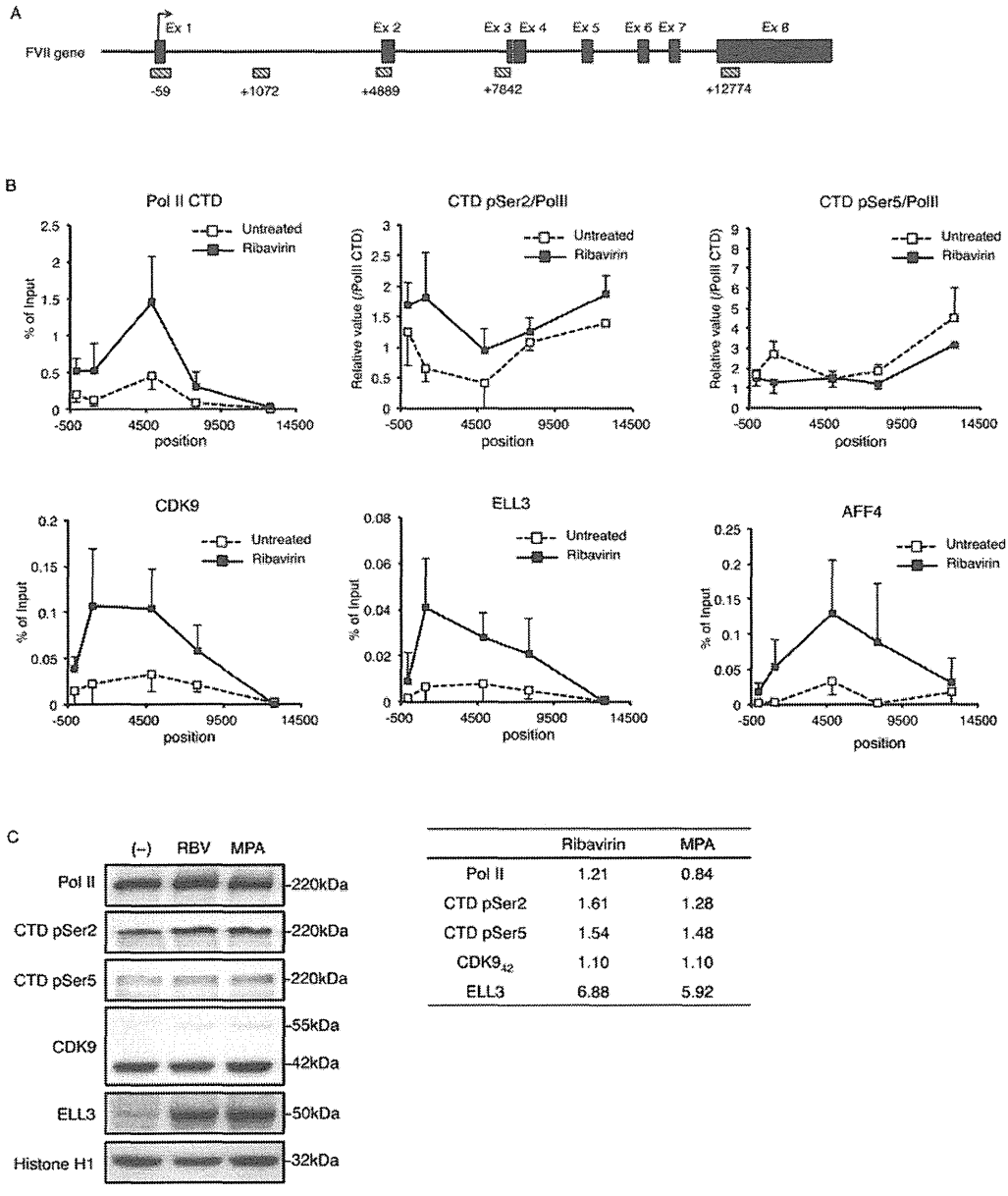


Figure 4 Time-course analysis for ELL3 expression induced by ribavirin treatment and knockdown of ELL3 by RNAi

(**A**) The expression levels of ELL3 and FVII mRNA in HepG2 cells treated with ribavirin at various time points were determined by qRT-PCR. (**B**) IMPDH inhibitors up-regulated ELL3 expression and supplementation of guanosine retraced the ELL3 up-regulation. (**C**) ELL3 protein induction by IMPDH inhibitors was cancelled by the addition of guanosine. U, untreated; R, ribavirin-treated; M, MPA-treated. (**D**) The effects of ELL3 RNAi in HepG2 cells were analysed by Western blotting. The ELL3-specific siRNA (iELL3) or NS siRNA (iNS) was transfected into HepG2 cells at a final concentration of 10 nM and treated with ribavirin (100 μg/ml) for 24 h. The nuclear extracts were isolated from the cells and subjected to Western blotting. Histone H1 was used as a loading control. U, untreated; R, ribavirin-treated. (**E**) Quantification of ELL3 mRNA in HepG2 cells transfected with iNS or iELL3 (10 nM). After transfection, cells were treated with or without ribavirin (100 μg/ml) and total RNA was isolated and subjected to qRT-PCR. The mRNA levels were expressed as relative values to that of untreated iNS-transfected cells. (**F**) FVII mRNA levels were examined in siRNA-transfected HepG2 cells treated with or without ribavirin. The expression levels were normalized to GusB mRNA, and shown as relative values to that of untreated cells. $*P < 0.05$ compared with untreated cells or iNS-transfected cells; $**P < 0.01$ compared with untreated cells.



	Ribavirin	MPA
Pol II	1.21	0.84
CTD pSer2	1.61	1.28
CTD pSer5	1.54	1.48
$CDK9_{42}$	1.10	1.10
ELL3	6.88	5.92

Figure 5 Recruitment of Pol II and transcription elongation factors induced by ribavirin treatment

(**A**) The genomic locus of human *F7* (Gene ID: 2155; sequence derived from GenBank®) with the primer positions we used in the present study. Numbers (transcription start site as + 1) indicated the 5′ end as the forward primer. The primers are described in Supplementary Table S2 at http://www.biochemj.org/bj/449/bj4490231add.htm. The arrow indicates transcription start site of the *F7* gene. The relative quantities of $pSer^2$ and $pSer^5$ were normalized to Pol II levels. Ex, exon. (**B**) ChIP–qRT-PCR analyses to assess the occupancy of Pol II, $pSer^2$ of CTD, $pSer^5$ of CTD, CDK9, ELL3 and AFF4 on the *F7* using specific antibodies. The density was determined by quantitative PCR and is represented as a percentage of input DNA, with values subtracted from that of normal IgG immunoprecipitation. The *x*-axis indicates the position of quantitative PCR amplification in *F7*. Values are the mean ± S.D. for at least five independent experiments. (**C**) Western blot analyses for nuclear extracts from HepG2 cells treated with IMPDH inhibitors (ribavirin, MPA) using specific antibodies against Pol II CTD, CTD $pSer^2$, CTD $pSer^5$, CDK9, ELL3 and histone H1. Histone H1 was used as the loading control. Molecular masses are indicated in kDa to the right of the blots.

We assessed the status of pSer residues of Pol II CTD and expression levels of elongation factors in HepG2 cells treated with IMPDH inhibitors by Western blotting (Figure 5C). No change was observed in the total amount of Pol II CTD in both the IMPDH inhibitor-treated and -untreated cells, whereas ribavirin or MPA treatment increased the level of CTD $pSer^2$. The levels of CTD $pSer^5$ were slightly increased. We also checked the expression levels of CDK9 and ELL3 and found that ribavirin or MPA treatment clearly increased the ELL3 protein levels. By contrast, there was no significant change in the expression level of the 42-kDa CDK9 protein, which is a predominant form of CDK9.

DISCUSSION

The major finding of the present study is that intracellular GTP depletion induced by IMPDH inhibitors modulated Factor VII

gene transcription, mainly in the elongation phase rather than transcription initiation.

Ribavirin, a synthetic nucleoside analogue, is used in combination with pegylated IFN (interferon)-α as the standard treatment for patients with chronic hepatitis C. A previous study demonstrated that ribavirin treatment increased FVII activity in HCV-infected haemophilia patients as well as increased FVII mRNA expression in both normal human hepatocytes and a human hepatocarcinoma cell line (HepG2) *in vitro* [8]. In the present study, we investigated further the molecular mechanisms of ribavirin on FVII mRNA up-regulation in HepG2 cells.

We observed that ribavirin treatment increased FVII mRNA expression in HepG2 cells in a dose- and time-dependent manner. Because the ribavirin metabolite RMP inhibits IMPDH, we tested other IMPDH inhibitors (MPA and 6-MP) and found that they also up-regulated FVII mRNA in HepG2 cells. Interestingly, it was reported that ribavirin treatment induced the expression of particular ISGs (IFN-stimulated genes) by activating transcription [13,14], whereas MPA induced the expression of ISGs by promoter activation [13,15]. Thus there may be a close relationship between intracellular GTP depletion caused by sequential inhibition of IMPDH and modulation of various gene expressions. Indeed, guanine nucleoside repletion restored ribavirin-induced FVII mRNA up-regulation.

In general, control of gene expression in eukaryotic cells involves regulatory events at multiple transcriptional and post-transcriptional stages. Transcriptional regulation by Pol II is a multifaceted process, requiring the combined action of a large number of factors for the steadfast synthesis of full-length mRNA [16–20]. Transcription by Pol II is divided into four stages: initiation, promoter clearance, elongation and termination. For many years, transcription initiation was considered as the rate-limiting step to the whole transcription process; however, a large number of studies have demonstrated that the elongation stage of transcription, regulated by a number of factors, is essential for productive transcription [17,18,21]. Recently, the elongation phase of transcription has gained increasing importance as numerous positive and negative elongation factors have been identified [18]. We analysed which step of transcription was critical for ribavirin-induced FVII mRNA up-regulation. We found that ribavirin treatment did not affect the FVII mRNA stability. In the luciferase analysis, we observed an approximate 1.5-fold increase in *F7* promoter activity by ribavirin treatment, but also found a similar increase of 'ribavirin-insensitive' *PROS1* promoter activity, suggesting that we might overestimate the effects of ribavirin in our luciferase assay system. By contrast, ribavirin treatment sufficiently increased nascent FVII mRNA synthesis in HepG2 cells, as analysed by the nuclear run-on and nascent mRNA capturing assay, which was abolished by inhibiting CDK9 using DRB. These findings indicate that ribavirin-induced *F7* up-regulation is mainly induced by acceleration of transcription elongation, which is associated with P-TEFb, rather than by promoter activation. The marginally enhanced transcription initiation could contribute to some extent in the ribavirin-induced *F7* up-regulation, but the enhanced transcription by elongation factors would perform a critical contribution in the up-regulation.

Previous evidence has indicated that elongation is tightly linked to post-transcriptional events such as mRNA capping, splicing, polyadenylation and nuclear export [22–24]. The CTD of the largest subunit of Pol II plays a critical role in the integration of these events. The CTD comprises multiple heptad repeats (YSPTSPS motifs, 52 in mammals) each containing two main phosphor-acceptor sites, Ser^2 and Ser^5. Several CTD kinases have been identified, with CDK7 and CDK9 being the most prominent among them. CDK7 is part of general transcription factor TFIIH and catalyses Ser^5 phosphorylation [25]. CDK9 is the catalytic subunit of the P-TEFb, with site specificity to preferentially phosphorylate Ser^2 or Ser^5 depending on the experimental paradigm used [26–30]. CDK9 kinase activity of P-TEFb phosphorylates Ser^2 of the CTD and signals the release of the stalled Pol II into productive transcription elongation [31,32].

In the present study, we found that IMPDH inhibitors increased nascent FVII mRNA expression in HepG2 cells, possibly by acceleration of transcription elongation. The increase in FVII mRNA expression was inhibited by DRB treatment, indicating that CDK9 is critical for up-regulation of *F7* expression by acceleration of transcription elongation. Consistently, ChIP assays revealed that recruitment of Pol II and CDK9, and phosphorylation of Ser^2 of the Pol II CTD were reinforced by ribavirin treatment. To investigate further the elongation steps necessary for increased FVII mRNA expression, we searched ribavirin-responsive genes by cDNA microarray analysis and found that transcription elongation factor ELL3 expression was significantly up-regulated in HepG2 cells treated with ribavirin, and this ELL3 up-regulation was induced in response to intracellular GTP depletion. In addition, we found that ribavirin-induced an increase in ELL3 mRNA expression before an increase in FVII mRNA expression in HepG2 cells, suggesting that induced ELL3 may contribute to FVII mRNA up-regulation.

ELL3, a member of the Pol II elongation factors, is an approximately 400-amino-acid protein with high homology for the C-terminal sequences of ELL and ELL2 [33]. To test the effects of ELL3 on *F7* transcription, we performed an RNAi analysis of ELL3 and observed that diminished ELL3 expression by ELL3-specific siRNA and that ribavirin-induced FVII mRNA up-regulation was attenuated by ELL3 knockdown in HepG2 cells. A ChIP assay indicated that ELL3 was bound to *F7* in a similar distribution pattern to that of CDK9, suggesting that CDK9 and ELL3 may work together in the transcription elongation phase of ribavirin-induced *F7* gene up-regulation.

A previous study reported that ELL proteins are a component of the SEC (super elongation complex) that contains P-TEFb [34]. AFF (AF4/FMR2) proteins are also activators of transcription elongation and interacts CDK9 and ELL [35]. It was concluded that AFF4 was a component of the transcription elongation complex containing P-TEFb and ELL and that AFF4 was required for ELL–P-TEFb complex stability. We found that CDK9, ELL3 and AFF4 were recruited to the *F7* gene, as shown by the ChIP assay. A study by Shilatifard and co-workers suggested that these elongation factors form the SEC in the elongation stage [36]. Consistent with previous findings, our findings suggested that ELL3 is involved in SEC with P-TEFb and AFF4 for *F7* transcription elongation. We proposed that ELL3 has an important role in ribavirin-induced FVII mRNA up-regulation, even though it may not be essential. It is possible that other molecules could contribute to increased FVII mRNA expression, or the function of ELL3 may be compensable in ribavirin-induced *F7* transcription elongation. Our proposed model for the up-regulation of *F7* gene transcription is shown in Figure 6.

We wonder whether *F7* gene expression was specifically modulated by intracellular GTP depletion. In fact, we observed that some other genes of blood coagulation factors were also fairly up-regulated by ribavirin exposure, and now we are investigating the molecular basis of their gene expressions.

In summary, we found that ribavirin increased FVII mRNA expression by acceleration of its transcription elongation in HepG2 cells and that CDK9 kinase activity of P-TEFb has a crucial role in *F7* transcription elongation. Although further study is required to determine why intracellular GTP depletion

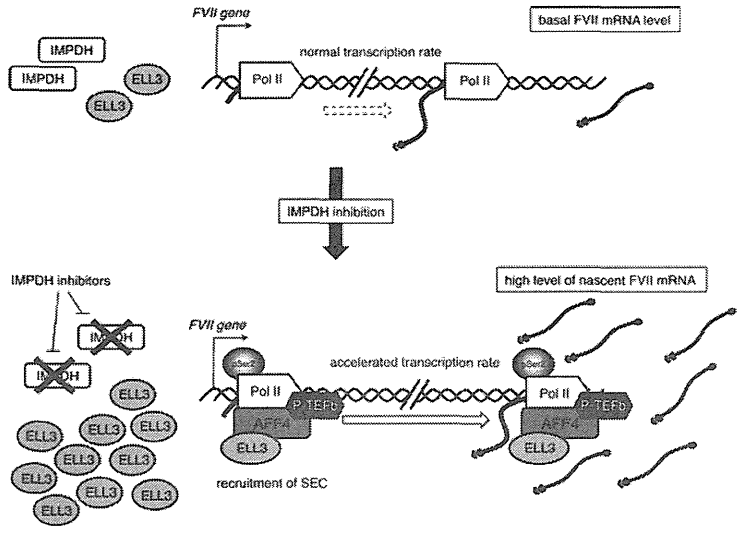


Figure 6 A proposed model for the acceleration of *F7* transcription elongation by ribavirin treatment

IMPDH inhibitors induce the transcription elongation on F7. Intracellular GTP depletion accelerates newly synthesized FVII mRNA by recruiting the SEC, consisting of P-TEFb, AFF4 and ELL3. For a detailed explanation, see the Discussion.

causes accelerated *F7* transcription elongation, our findings have contributed to elucidate the precise mechanisms of ribavirin-induced *F7* up-regulation. Finally, the present study highlights the possible development of novel pharmaceutical therapies for haemophilia.

AUTHOR CONTRIBUTION

Atsuo Suzuki designed and performed the experiments, and drafted the paper. Yuhri Miyawaki, Eriko Okuyama, Moe Murata, Yumi Ando, Io Kato and Yuki Takagi analysed and interpreted the data and contributed to the analytical methodology. Akira Takagi and Takashi Murate designed the experiments and analysed the data. Hidehiko Saito supervised the project and edited the paper before submission. Tetsuhito Kojima designed the project, analysed data and wrote the paper. All authors were involved in a critical reading of the paper before submission.

ACKNOWLEDGEMENTS

We thank Dr Nobuaki Suzuki, Dr Shinji Kunishima, Dr Akira Katsumi and Dr Tadashi Matsushita for their helpful discussions.

FUNDING

A.S. is a research fellow of the Japan Society for the Promotion of Science (JSPS). This study was supported in part by grants-in-aid for JSPS Fellows [grant number 23-5159] (to A.S.)] and by Scientific Research (C) [grant number 22590524 (to T.K.)] from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and for Research on Measures for Intractable Diseases to T.K. from the Japanese Ministry of Health, Labour and Welfare.

REFERENCES

1 Bajaj, S. P., Rapaport, S. I. and Brown, S. F. (1981) Isolation and characterization of human Factor VII. Activation of Factor VII by Factor Xa. J. Biol. Chem. **256**, 253–259
2 Fair, D. S. (1983) Quantitation of Factor VII in the plasma of normal and warfarin-treated individuals by radioimmunoassay. Blood **62**, 784–791
3 Rao, L. V. and Rapaport, S. I. (1988) Activation of Factor VII bound to tissue factor: a key early step in the tissue factor pathway of blood coagulation. Proc. Natl. Acad. Sci U.S.A. **85**, 6687–6691
4 Nemerson, Y. (1988) Tissue factor and hemostasis. Blood **71**, 1–8
5 Ingerslev, J. and Sørensen, B. (2011) Parallel use of by-passing agents in haemophilia with inhibitors: a critical review. Br. J. Haematol. **155**, 256–262
6 Oyesiku, J. O. (2011) Home treatment of haemophilia patients with inhibitors. Haemophilia **17**, 173–178
7 Brinkhous, K. M., Hedner, U., Garris, J. B., Diness, V. and Read, M. S. (1989) Effect of recombinant Factor VIIa on the hemostatic defect in dogs with hemophilia A, hemophilia B, and von Willebrand disease. Proc. Natl. Acad. Sci. U.S.A. **86**, 1382–1386
8 Yamamoto, K., Honda, T., Matsushita, T., Kojima, T. and Takamatsu, J. (2006) Anti-HCV agent, ribavirin, elevates the activity of clotting Factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin. J. Thromb. Haemostasis **4**, 469–470
9 Feld, J. J. and Hoofnagle, J. H. (2005) Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature **436**, 967–972
10 Suzuki, A., Sanda, N., Miyawaki, Y., Fujimori, Y., Yamada, T., Takagi, A., Murate, T., Saito, H. and Kojima, T. (2010) Down-regulation of *PROS1* gene expression by 17β-estradiol via estrogen receptor α (ERα)–Sp1 interaction recruiting receptor-interacting protein 140 and the corepressor–HDAC3 complex. J. Biol. Chem. **285**, 13444–13453
11 Yamada, M., Horiguchi, K., Umezawa, R., Hashimoto, K., Satoh, T., Ozawa, A., Shibusawa, N., Monden, T., Okada, S., Shimizu, H. and Mori, M. (2010) Troglitazone, a ligand of peroxisome proliferator-activated receptor-γ, stabilizes *NUCB2* (Nesfatin) mRNA by activating the ERK1/2 pathway: isolation and characterization of the human *NUCB2* gene. Endocrinology **151**, 2494–2503
12 Peng, J., Zhu, Y., Milton, J. T. and Price, D. H. (1998) Identification of multiple cyclin subunits of human P-TEFb. Genes Dev. **12**, 755–762
13 Thomas, E., Feld, J. J., Li, Q., Hu, Z., Fried, M. W. and Liang, T. J. (2011) Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. Hepatology **53**, 32–41
14 Pan, Q., Tilanus, H. W., Janssen, H. L. and van der Laan, L. J. (2011) Ribavirin enhances interferon-stimulated gene transcription by activation of the interferon-stimulated response element. Hepatology **53**, 1400–1401
15 Pan, Q., de Ruiter, P. E., Metselaar, H. J., Kwekkeboom, J., de Jonge, J., Tilanus, H. W., Janssen, H. L. and van der Laan, L. J. (2012) Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C virus infection *in vitro* and *in vivo*. Hepatology **55**, 1673–1683
16 Workman, J. L. and Kingston, R. E. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. **67**, 545–579
17 Shilatifard, A., Conaway, R. C. and Conaway, J. W. (2003) The RNA polymerase II elongation complex. Annu. Rev. Biochem. **72**, 693–715
18 Sims, R. J., Belotserkovskaya, R. and Reinberg, D. (2004) Elongation by RNA polymerase II: the short and long of it. Genes Dev. **18**, 2437–2468
19 Brès, V., Yoh, S. M. and Jones, K. A. (2008) The multi-tasking P-TEFb complex. Curr. Opin. Cell Biol. **20**, 334–340
20 Boettiger, A. N. and Levine, M. (2009) Synchronous and stochastic patterns of gene activation in the *Drosophila* embryo. Science **325**, 471–473
21 Levine, M. (2011) Paused RNA polymerase II as a developmental checkpoint. Cell **145**, 502–511

22 Hirose, Y. and Manley, J. L. (2000) RNA polymerase II and the integration of nuclear events. Genes Dev. **14**, 1415–1429
23 Proudfoot, N. J., Furger, A. and Dye, M. J. (2002) Integrating mRNA processing with transcription. Cell **108**, 501–512
24 Bentley, D. L. (2005) Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr. Opin. Cell Biol. **17**, 251–256
25 Trigon, S., Serizawa, H., Conaway, J. W., Conaway, R. C., Jackson, S. P. and Morange, M. (1998) Characterization of the residues phosphorylated *in vitro* by different C-terminal domain kinases. J. Biol. Chem. **273**, 6769–6775
26 Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E. and Jones, K. A. (2000) CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat–P-TEFb complex to TAR RNA. Mol. Cell. Biol. **20**, 6958–6969
27 Ramanathan, Y., Rajpara, S. M., Reza, S. M., Lees, E., Shuman, S., Mathews, M. B. and Pe'ery, T. (2001) Three RNA polymerase II carboxyl-terminal domain kinases display distinct substrate preferences. J. Biol. Chem. **276**, 10913–10920
28 Shim, E. Y., Walker, A. K., Shi, Y. and Blackwell, T. K. (2002) CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the *C. elegans* embryo. Genes Dev. **16**, 2135–2146
29 Ahn, S. H., Kim, M. and Buratowski, S. (2004) Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3′ end processing. Mol. Cell **13**, 67–76
30 Ni, Z., Schwartz, B. E., Werner, J., Suarez, J. R. and Lis, J. T. (2004) Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol. Cell **13**, 55–65
31 Jones, K. A. and Peterlin, B. M. (1994) Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. **63**, 717–743
32 Fuda, N. J., Ardehali, M. B. and Lis, J. T. (2009) Defining mechanisms that regulate RNA polymerase II transcription *in vivo*. Nature **461**, 186–192
33 Miller, T., Williams, K., Johnstone, R. W. and Shilatifard, A. (2000) Identification, cloning, expression, and biochemical characterization of the testis-specific RNA polymerase II elongation factor ELL3. J. Biol. Chem. **275**, 32052–32056
34 Lin, C., Smith, E. R., Takahashi, H., Lai, K. C., Martin-Brown, S., Florens, L., Washburn, M. P., Conaway, J. W., Conaway, R. C. and Shilatifard, A. (2010) AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. Mol. Cell **37**, 429–437
35 Esposito, G., Cevenini, A., Cuomo, A., de Falco, F., Sabbatino, D., Pane, F., Ruoppolo, M. and Salvatore, F. (2011) Protein network study of human AF4 reveals its central role in RNA Pol II-mediated transcription and in phosphorylation-dependent regulatory mechanisms. Biochem. J. **438**, 121–131
36 Lin, C., Garrett, A. S., De Kumar, B., Smith, E. R., Gogol, M., Seidel, C., Krumlauf, R. and Shilatifard, A. (2011) Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC). Genes Dev. **25**, 1486–1498

Received 14 August 2012/25 September 2012; accepted 11 October 2012
Published as BJ Immediate Publication 11 October 2012, doi:10.1042/BJ20121286

Biochem. J. (2013) **449**, 231–239 (Printed in Great Britain) doi:10.1042/BJ20121286

online data

SUPPLEMENTARY ONLINE DATA

Ribavirin-induced intracellular GTP depletion activates transcription elongation in coagulation Factor VII gene expression

Atsuo SUZUKI*, Yuhri MIYAWAKI*, Eriko OKUYAMA*, Moe MURATA*, Yumi ANDO*, Io KATO*, Yuki TAKAGI*, Akira TAKAGI*, Takashi MURATE*, Hidehiko SAITO† and Tetsuhito KOJIMA*[1]

*Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya 461-8673, Japan, and †National Hospital Organization-Nagoya Medical Center, Nagoya 460-0001, Japan

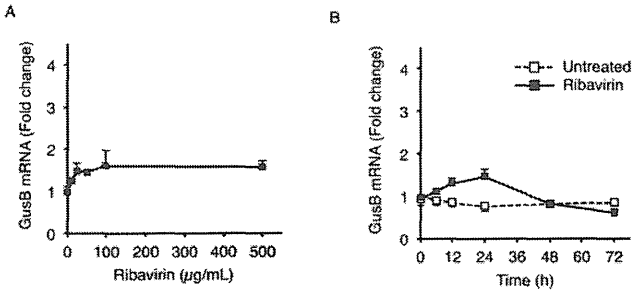


Figure S1 Quantification of *GusB* mRNA used for reference in ribavirin-treated HepG2 cells

(**A**) GusB mRNA levels in HepG2 cells treated for 24 h with ribavirin (0, 10, 25, 50, 100 and 500 μg/ml) were determined by qRT-PCR. (**B**) Time-course analysis of GusB mRNA levels under ribavirin treatment in HepG2 cells. The cells were treated with 100 μg/ml ribavirin for 6, 12, 24, 48 or 72 h and at each time point, total RNA was isolated as described in the Experimental section of the main text. The levels of GusB mRNA are shown as the relative raw values to that of untreated cells.

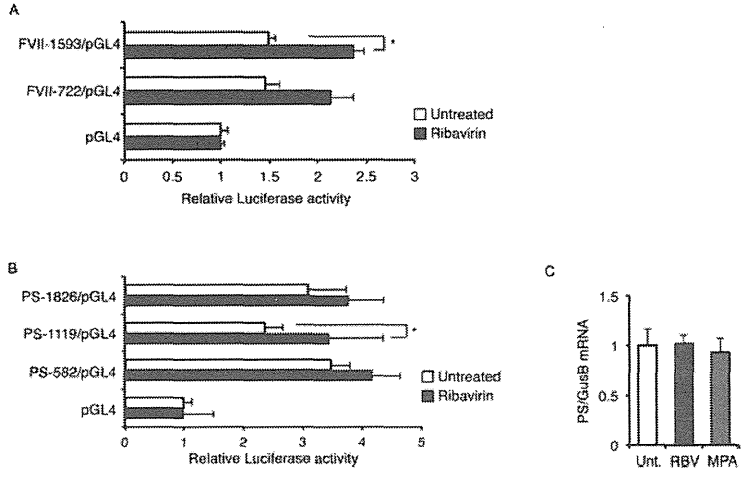


Figure S2 Ribavirin affected ribavirin-insensitive *PROS1* promoter activity as well as *F7* promoter

(**A**) Luciferase activity of HepG2 cells transiently transfected with luciferase-reporter vector containing the *F7* promoter region, FVII-1593/pGL4 or its 5′-truncated construct FVII-722/pGL4 with or without ribavirin was measured. (**B**) Luciferase activity in HepG2 cells transiently transfected with luciferase-reporter vector containing the *PROS1* gene promoter region, PS-1826/pGL4 or its 5′-truncated construct PS-1119/pGL4 or PS-582/pGL4 with or without ribavirin was measured. The luciferase activity levels of the respective constructs were expressed as relative values to that of the pGL4 empty vector. (**C**) *PROS1* (PS) mRNA levels were determined by qRT-PCR in HepG2 cells treated with or without ribavirin (100 μg/ml) and MPA (100 μM). Unt., untreated.

[1] To whom correspondence should be addressed (email kojima@met.nagoya-u.ac.jp).

Table S1 Primers used in qRT-PCR

Target	Forward (5′→3′)	Reverse (5′→3′)	Amplicon (bp)
F7	AAGGACGCGGAGAGGACGAAG	CAGGGAGGCAGAAGCAGATATAGG	130
GUSB	GCGTGGAGCAAGACAGTGGGC	GGTCCCAGTCCCATTCGCCA	122
HPRT1	CATGGACTGATTATGGACAGGAC	GTAATCCAGCAGGTCAGCAAAG	129
ELL2	CAAGCTAAATAACTCCAGTCCAA	GGAGACGATAGCGATATATTTTATC	121
ELL3	GAAGATATACCAGACTACCTCCTGC	CGGTATTCAGCATAATCTGTCTC	101
TCEB3	ATTACAGTAGACATTCTTGCGGAG	CAGCATTTCGTTCCACAGG	136

Table S2 Primers used in quantitative PCR for ChIP

Position numbers (transcription start site as +1) indicate the 5′ end of the forward primers.

Position	Forward (5′→3′)	Reverse (5′→3′)	Amplicon (bp)
−59	CTGTGTCCTCCCCTCCCCCATC	AAAACCTCCCCGGACGCACCT	193
+1072	CAGGAGGAGAAACACGGGAC	GGAGGGGAAGGAGGTGATGT	116
+4889	CATTTCCCAGTCTTCGTAACCC	CTCCTCCTTGCACTCCCTCTC	120
+7842	CCCACCCCGCCAGACCCAG	GCACCTTCACCAGAGCACCCAACG	87
+12774	CCCCTCTGCCTGCCCGAA	GGGGCACGTTGAGGACCATGA	133

Received 14 August 2012/25 September 2012; accepted 11 October 2012
Published as BJ Immediate Publication 11 October 2012, doi:10.1042/BJ20121286

Minimizing the Inhibitory Effect of Neutralizing Antibody for Efficient Gene Expression in the Liver With Adeno-associated Virus 8 Vectors

Jun Mimuro[1], Hiroaki Mizukami[2], Shuji Hishikawa[3], Tomokazu Ikemoto[4], Akira Ishiwata[1], Asuka Sakata[1], Tsukasa Ohmori[1], Seiji Madoiwa[1], Fumiko Ono[5], Keiya Ozawa[2] and Yoichi Sakata[1]

[1]Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi-ken, Japan; [2]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Tochigi-ken, Japan; [3]Division of Medical Skill Training, Center for Development of Advanced Medical Technology, Jichi Medical University, School of Medicine, Tochigi-ken, Japan; [4]Division of Cardiovascular Medicine, Department of Medicine, Jichi Medical University, School of Medicine, Tochigi-ken, Japan; [5]The Corporation for Production and Research of Laboratory Primates, Tsukuba, Japan

Neutralizing antibodies (NAbs) against adeno-associated viruses (AAVs) are known to interfere with AAV vector-mediated gene transfer by intravascular delivery. Evading the inhibitory effects of antibodies against AAV vectors is necessary for efficient transfer of therapeutic genes clinically. For this purpose, we tested the efficacy of saline flushing in order to avoid contact of vectors with NAbs present in blood. Direct injection of the AAV8 vector carrying the *factor IX* (*FIX*) gene into the portal vein of macaques using saline flushing achieved transgene-derived FIX expression (4.7 ± 2.10–10.1 ± 5.45% of normal human FIX concentration) in the presence of NAbs. Expression was as efficient as that (5.43 ± 2.59–12.68 ± 4.83%) in macaques lacking NAbs. We next tested the efficacy of saline flushing using less invasive balloon catheter-guided injection. This approach also resulted in efficient expression of transgene-derived FIX (2.5 ± 1.06–9.0 ± 2.37%) in the presence of NAbs (14–56× dilutions). NAbs at this range of titers reduced the efficiency of transduction in the macaque liver by 100-fold when the same vector was injected into mesenteric veins without balloon catheters. Our results suggest that portal vein-directed vector delivery strategies with flushing to remove blood are efficacious for minimizing the inhibitory effect of anti-AAV antibodies.

Received 21 August 2012; accepted 14 November 2012; advance online publication 18 December 2012. doi:10.1038/mt.2012.258

INTRODUCTION

Gene and cell therapies are expected to be the next generation of therapies for a variety of inherited diseases. Hemophilia is thought to be an ideal target disease for these approaches as it is caused by a genetic abnormality in the factor VIII gene for hemophilia A, or the factor IX (FIX) gene for hemophilia B.[1–7] The current strategy of hemophilia gene therapy involves inducing expression of the normal coagulation factor gene or transplanting cells expressing the respective coagulation factor. The liver is normally the primary target of gene transfer for coagulation factors since the majority of these coagulation factors are synthesized in the liver with appropriate post-translation modifications before secretion into the circulatory system.

Substantial effort has been applied to express coagulation factor genes using various vector types. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for therapeutic gene transfer *in vivo* because they reside in the episome and rarely integrate into genomes. However, retrovirus vectors including lentivirus vectors require integration into the host cell genome.[6,7] In addition, AAV vectors can transfer genes to nondividing cells and allow long-term expression of transgenes in these cells.

Clinical trials for hemophilia gene therapy have recently been conducted using various types of vectors.[4–11] These trials were designed based upon data obtained from mouse models of hemophilia and hemophiliac dogs and proved to be more efficient in these models than for humans. Species differences between humans and these other animal models might partially account for the results observed. Therefore, gene transfer studies in non-\ human primates may well predict the efficacy of gene transfer in humans. Indeed, *FIX* gene transfer studies using a new type of vector have been conducted in rhesus macaques.[12,13] The results from these studies provided the basis for recent hemophilia B gene therapy clinical trials employing an AAV8 vector.[13–16] Gene transfer in mice using AAV vectors results in excellent transduction efficiency. This is especially so for AAV8 vector-mediated gene transfer in the mouse liver;[12–14,17] however, the efficacy of AAV8 vectors is modest in macaques.[13]

There are also difficulties associated with *FIX* gene expression when using AAV8 vectors in nonhuman primates. Growing evidence suggests that the presence of neutralizing antibodies (NAbs) against AAV8, due to previous natural infection by wild-type AAV, significantly inhibits transduction in the macaque liver. It is likely that antibodies against one serotype of AAV cross-react with other AAV serotypes.[18] A hemophilia B gene therapy

J.M., H.M., S.H., and T.I. contributed equally to this work.

Correspondence: *Jun Mimuro, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke 329-0498, Japan. E-mail: mimuro-j@jichi.ac.jp*

Table 1 Expression of macaque T262A in nonhuman primates with AAV8-HCRHAAT-macFIXT262A

Macaque number	Age	Vector dose (vg/kg)	Route of vector injection	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#14	5.7	1×10^{12}	Mesenteric vein	0.02 ± 0.019	0.1	56×
#17	5.8	1×10^{13}	Mesenteric vein	0.13 ± 0.081	0.4	56×
#24	6.6	1×10^{12}	Mesenteric vein	0.09 ± 0.048	0.5	14×
#28	7.8	5×10^{12}	Saphenous vein	12.68 ± 4.83	38.2	Negative
#30	2.9	5×10^{12}	Saphenous vein	5.43 ± 2.59	48.2	Negative
#31	2.9	5×10^{12}	Saphenous vein	7.64 ± 2.32	49.6	Negative

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region.
The concentration of macaque FIX T262A is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 neutralizing antibody (NAb) titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

clinical study using an AAV8 vector was successfully conducted in hemophilia B patients negative for pre-existing antibodies against AAV8.[15] Because of the high prevalence of AAV infection in humans,[18] evading NAbs against this virus is an important hurdle to overcome before AAV8 vectors can be routinely and effectively employed for therapies.

The aim of our study was to develop an administration method of AAV8 vectors that assisted in minimizing the inhibitory effect of NAbs against AAV in macaques that were already seropositive for AAV8 antibodies.

RESULTS

The AAV8 vector carrying the macaque *FIX T262A* gene located downstream of the liver-specific chimeric promoter consisted of an enhancer element of hepatic control region (HCR) of the *ApoE/C-I* gene and the 5′ flanking region of the *α1-antitrypsin* (HAAT) gene (AAV8-HCRHAAT-macFIXT262A). This vector was used to express mutant macaque FIX containing a single amino acid substitution of Thr to Ala at the position 262 (macaque FIX T262A) in the following experiments. Macaque FIX T262A but not wild-type macaque FIX could be bound to human FIX-specific monoclonal antibody 3A6, thereby macaque FIX T262A expressed in macaques with AAV8-HCRHAAT-macFIXT262A could be precisely quantified by an enzyme immunoassay with 3A6.[17] The amino acid sequence of macaque FIX is highly homologous to the human FIX amino acid sequence. Twelve amino acid residues of human FIX are different at corresponding positions of macaque FIX, while only one amino acid of macaque FIX T262A is different from wild-type macaque FIX. Expression of macFIX T262A in a macaque would mimic a situation where normal human FIX is expressed in a hemophilia B patient with a missense mutation in the *FIX* gene.

Results corresponding to the expression of macaque FIX T262A following injection of AAV8HCRHAATmacFIXT262A can be seen in **Table 1**. When AAV8HCRHAATmacFIXT262A (5×10^{12} vector genome copies (vg)/kg) was injected into the saphenous veins of three AAV8 NAb-negative macaques (#28, #30, #31), expression of macFIX T262A in the therapeutic range (>5% of normal FIX concentration) was achieved. However, injection of the same vector (1×10^{12}–1×10^{13} vg/kg) into the mesenteric vein branches of AAV8 NAb-positive macaques (#14, #17, #24; inhibitory titers: 14–56×) resulted in subtherapeutic levels (<0.2%) of macFIX T262A expression. The amount of vector DNA in the liver of AAV8 NAb-positive macaques was ~1% of that seen in AAV8 NAb-negative macaques (**Table 1**). These data suggest that low titers of NAbs against AAV8 significantly inhibit transduction even when the vector is injected into the mesenteric vein branches. In addition, only short period of time may be required for NAbs in the blood to neutralize the AAV8 vector since the blood of the mesenteric vein rapidly goes to the liver through the portal vein after gathering with the blood from other viscera.

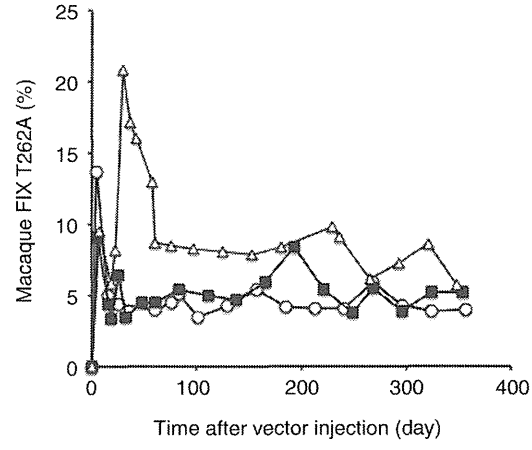


Figure 1 Expression of FIX T262A in macaques after direct vector injection into portal veins. Macaques ($n = 3$) were subjected to direct injection of AAV8 vector into the portal vein. Concentrations of FIX T262A in macaque plasma samples (macaque #26, open triangles; #27, open circles; #29, closed squares) were measured by ELISA. AAV, adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Evading AAV8 NAbs could be achieved by ensuring the AAV8 vector and NAbs do not come into physical contact with each other in the blood. Blood enters the liver from the hepatic artery and portal vein. The hepatic artery accounts for ~20–30% of blood flow, while the portal vein supplies the remaining blood flow to hepatocytes.[19,20] Blood from the portal vein and hepatic artery are eventually mixed in the sinusoids of the liver; however, the blood from the portal vein mainly supplies hepatocytes. Therefore, direct injection of AAV8 vectors into the portal vein branch was

Table 2 Expression of macaque T262A in nonhuman primates with direct, and balloon catheter-guided vector (AAV8-HCRHAAT-macFIXT262A) injection into the portal vein

Macaque ID	Age	Vector dose (vg/kg)	Injection method to portal vein branch	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#26	10.1	5×10^{12}	Direct	4.7 ± 2.10	77.9	28×
#27	7.4	5×10^{12}	Direct	10.1 ± 5.45	28.5	14×
#29	11.0	5×10^{12}	Direct	5.3 ± 1.40	64.3	14×
#37	7.5	5×10^{12}	Catheter-guided	9.0 ± 2.37	61.1	14×
#38	10.7	5×10^{12}	Catheter-guided	3.2 ± 1.21	13	56×
#42	7.7	5×10^{12}	Catheter-guided	2.5 ± 1.06	15.3	14×

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region; Nab, neutralizing antibody.
FIX T262A concentration is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 NAb titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

investigated to determine whether saline flushing to remove blood from the portal vein just before injection of the vector would diminish the inhibitory effects of anti-AAV8 NAbs. Three macaques (#26, #27, #29; inhibitory titers: 14–28×) were directly injected with vector (5×10^{12} vg/kg) into the left portal vein after flushing saline to remove blood (**Supplementary Table S1**). Expression of transgene-derived FIX (macaque FIX T262A) increased to therapeutic levels with the AAV8 vector carrying the macaque *FIX T262A* gene and persisted for greater than 1 year in the three macaques (**Figure 1**). Average FIX and vector genome levels in macaque liver tissues are presented in **Table 2**. Compared with the results of vector injection to the mesenteric vein of NAb-positive macaques #14, #17, and #24 (**Table 1**), the levels of macaque FIX T262A in the circulation of the macaques #26, #27, and #29 that received vector injection directly to the left portal vein with flushing to remove blood, were increased to therapeutic levels with significant amounts of vector genome detected (**Table 2**).

Blood chemistry analysis and liver biopsies were conducted following administration of the vector to determine whether there were any adverse effects induced by the injection. Moderate increases in liver enzymes, such as transaminases, were observed just after injection of the vector (**Supplementary Figure S1**). However, no significant pathological changes were seen in liver biopsy samples taken on days 14, 28 or 48 (data not shown). We did not observe an increase in the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive hepatocytes in the liver biopsy specimens (data not shown).

The direct injection of the AAV8 vector into the left portal vein branch with saline flushing to remove blood from the portal vein just before injection of the vector was effective to minimize the inhibitory effects of anti-AAV8 NAbs. Therefore, we explored the possibility of utilizing a balloon catheter to perform the vector injection into the portal vein branch with saline flushing to remove blood, taking the concern about the safety of the procedures into consideration. Using a microballoon catheter, we injected the vector into the left portal vein of three anti-AAV8 antibody-positive macaques (#37, # 38, #42; inhibitory titers: 14–56×) (**Table 2, Supplementary Table S2**). Fluorography in macaque #37 representing angiography of the portal vein branch is shown in **Figure 2** and **Supplementary Video S1**. Increase of FIX T262A to therapeutic levels was achieved in the three macaques (#37, # 38, #42),

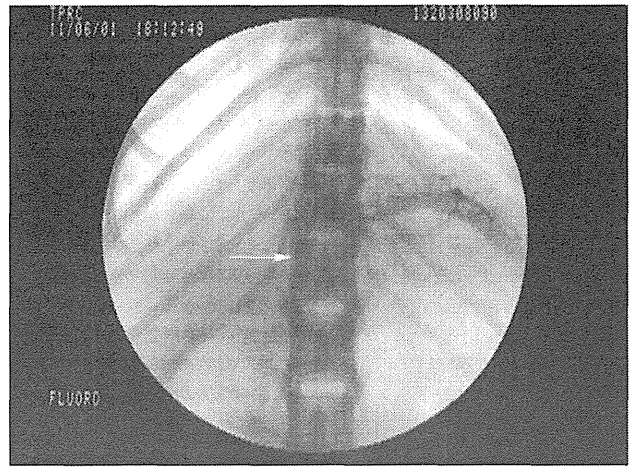


Figure 2 Fluorography in macaque #37. A balloon catheter was inserted into the portal vein of macaque #37 and contrast medium injected before vector administration. The left portal vein branches can be visualized. The arrow (white) indicates the tip of the catheter. See **Supplementary Video S1** which also recorded inflation of the balloon before the vector administration and deflation of the balloon after the administration.

and macaque FIX T262A expression in the circulation persisted (**Figure 3**, **Table 2**). The two portal vein vector delivery methods were successful in expressing macaque FIX T262A with the AAV8 vector in NAb-positive macaques (**Table 2**). The data suggest that the gene transfer efficiency using the catheter-guided vector injection method is similar to that of the direct vector injection into the portal vein branch with flushing.

Blood chemistry analysis and liver biopsies were conducted following injection of the vector. Increases in the levels of liver enzymes just after injection of the vector were not observed, suggesting that the ischemic effect of the temporary occlusion of the left portal vein branch was minimum compared with that of the direct vector injection procedure. Moderate increases in transaminases were observed following the vector injection, but did not persist (**Supplementary Figure S1**). Although the cause of the changes in the liver enzymes was not elucidated, no animals showed pathological changes, including histology of liver biopsy samples (data not shown).

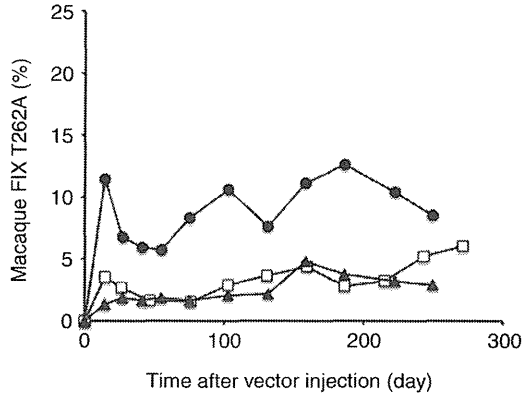


Figure 3 Expression of FIX T262A in macaques following balloon catheter-guided vector injection into portal veins. Three macaques (n = 3) were subjected to balloon catheter-guided vector injection into the portal vein. Concentrations of FIX T262A in macaque plasma samples (#37, closed circles; #38, open squares; #42, closed triangles) were measured by ELISA. ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Table 3 Vector injection rate

Route of injection (macaque number)	Elapsed time (seconds)	Rate of injection (vg/kg/second)
Mesenteric vein (#14, #17, #24)	5	2×10^{11}–2×10^{12}
Saphenous vein (#28, #30, #31)	5	1×10^{12}
Portal vein (direct) (#26, #27, #29)	8–10	5×10^{11}
Portal vein (catheter) (#37, #38, #42)	15–22	2.3×10^{11}–3.3×10^{11}

Abbreviation: vg, vector genome.

Vector injection rates of the four different vector injection procedures are listed in Table 3 for comparison. The vector injection rates of the portal vein-directed strategies were similar to those of bolus vector injection into the saphenous vein and the mesenteric vein. Thus, the effect of vector injection speed on the transduction efficiency of the vector was thought to be minimal.

DISCUSSION

There are many features that make recombinant AAV vectors attractive for transferring therapeutic genes into target organs, and many vectors have been tried for the treatment of various diseases.[6,7,11,15,21–23] However, lines of evidence suggest that NAbs against AAV interfere with AAV vector-mediated gene transfer by intravascular vector delivery.[7,23–26] A clinical gene therapy trial for hemophilia B using a self-complementary AAV8 vector carrying the FIX gene has been conducted and reported to be successful.[15] However, even the self-complementary AAV8 vector failed to express FIX in a subject with a relatively high anti-AAV8 antibody titer compared with other subjects with no or lower antibody titers.[15]

According to the previous reports on the prevalence of NAbs against various AAV serotypes in normal subjects, the seropositivity against AAV8 is 15–30%, which is lower than that against AAV2 (50–60%), although the technical details of the NAb assay is different.[27,28] These reports have also demonstrated that the antibody titer against AAV8 is generally lower than for AAV2. Our data suggest that a low titer of NAbs against AAV8 can interfere with transduction even if the vector is injected into the mesenteric vein. Therefore, the use of another serotype vector such as AAV5 vector could be the next approach for this type of gene therapy because of the divergence in capsid sequence of AAV5 from other AAV serotypes.[13,16] Although the prevalence of NAbs against AAV5 is much lower than those against AAV1 and AAV2, and the prevalence of NAbs against AAV5 is comparable to or even lower than that against AAV8 in humans,[25,27] it is possible that subjects of gene therapy may contain cross-reactive NAbs against various AAV serotypes.

Another approach for evading NAbs against AAV could be the use of chemically or genetically modified AAV variants. Such variants could include AAV vector mutants with amino acid substitutions, or chimeric AAV vectors made by serotype shuffling.[23] Approaches that enable evasion of NAb inhibitory effects are necessary if researchers and clinicians wish to effectively apply AAV vectors for gene therapy because of NAb cross-reactivity.

An alternative approach for overcoming the inhibitory effect of NAb against AAVs is to develop a vector injection method. In the current study, two portal vein vector delivery strategies were employed that ensured that the AAV8 vector and NAbs do not come into physical contact with each other in the blood. These strategies were investigated using macaques whether the strategies could efficiently transduce hepatocytes with the AAV8 vector in the presence of NAbs. The first approach was the direct injection of AAV8 vectors into the portal vein branch after flushing with saline to remove blood. This strategy proved to be successful for the vector expressing FIX T262A in anti-AAV8 antibody-positive macaques. Since there are safety concerns about the direct vector injection method, injection of the vector into the portal vein using a balloon catheter was investigated. The catheter-guided vector injection may be less invasive than the direct vector injection into the portal vein branch because exfoliation of hepatic hilum is not required. In addition, fine surgical skills, such as manipulation of the hepatic hilum and suturing the venotomy site of portal vein after the direct vector injection without causing stenosis, are required for the direct vector injection method into the left portal vein. Obviously, catheterization from the mesenteric vein branch is required for the balloon catheter-guided vector injection method but insertion of a catheter into the portal vein from a branch of the mesenteric vein is not difficult for a cardiologist and a radiologist familiar with angiography. In addition, suturing the venotomy site of the mesenteric vein branch is easier and safer than suturing the venotomy site of portal vein, and the ischemic effect of this procedure was expected to be less than that of the direct vector injection into the portal vein branch. Taken together, these studies suggested that both the direct vector injection into the left portal vein and the balloon catheter-guided vector injection into the left portal vein were similarly effective for hepatocyte transduction with the AAV8 vector in the presence of low titer NAbs but the balloon catheter-guided vector injection method into the left portal vein was thought to be safer than the direct vector injection into the left portal vein.

Considering that the antibody titer against AAV8 was generally lower than that against AAV2 and that NAbs at low titers could interfere with the AAV8 vector-mediated gene transfer to the liver significantly, we selected macaques with low NAb titers

for the portal vein vector delivery strategies. However, the impact of the presence of high titer NAbs on the efficacy of these methods was not studied. Thus, the extent of AAV8 NAb titer, for that this approach is effective, needs to be investigated in the future.

In conclusion, we have provided the basis for an alternative approach for gene transfer to the liver that minimizes the deleterious effects of anti-AAV NAbs. Our result might expand the potential of the AAV vector-mediated gene delivery for medical application.

MATERIALS AND METHODS

AAV vector production. Construction of pAAV2-HCRHAAT-macFIX T262A and production of AAV8 carrying the macaque FIX T262A gene (AAV8HCRHAATmacFIXT262A) has been previously described.[17] Briefly, DNA fragments harboring the *macFIXT262A* gene located downstream of the chimeric promoter consisted of an enhancer element of HCR of the human ApoE/C-I gene and the 5′ flanking region of the human HAAT gene (HCRHAAT promoter), and the SV40 polyadenylation signal sequence flanked by AAV2 inverted terminal repeats in pAAV2-HCRHAAT-macFIX T262A. The genes were packaged by triple plasmid transfection of human embryonic kidney 293 cells (Avigen, San Diego, CA) to generate AAV8-HCRHAAT-macFIXT262A, with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA), as previously described.[17] The chimeric packaging plasmid for AAV8 capsid pseudotyping[29] was constructed by inserting the synthetic AAV8 *Cap* gene (Takara Bio, Otsu, Shiga, Japan) downstream of the AAV2 Rep gene of pHelp19. For virus vector purification, the DNase (Benzonase; Merck Japan, Tokyo, Japan)-treated viral particles containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) supplemented with 25 mmol/l EDTA at 21 °C, as previously described.[17] Titration of recombinant AAV vectors was carried out by quantitative PCR using a real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan).[17] AAV8HCRHAATmacFIXT262A was previously shown to express macaque FIXT262A in mice efficiently.[17] Human FIX could be expressed in macaques and detected, however, macaques developed antibody against human FIX under certain experimental conditions. Only one amino acid residue at position 262 was humanized in macaque FIX T262A for detection with the human FIX-specific monoclonal antibody.

Animals. Cynomolgus macaques were bred and maintained at the Tsukuba Primate Research Center (Ibaraki, Japan). The animal experiments using macaques were performed at the Tsukuba Primate Research Center according to the guidelines of the Institutional Animal Care and Concern Committees at Jichi Medical University and the Tsukuba Primate Research Center. The use of macaques in animal experiments was approved by the Animal Care and Concern Committees. All surgical procedures were carried out under anesthesia, with vital signs and electrocardiogram monitoring conducted in accordance with the stipulated guidelines. Male macaques with low NAb titers (<56×) were used in this study.

Vector injection from peripheral and mesenteric vein. Injection of AAV8 vector to a saphenous vein (peripheral vein) was performed under intramuscular anesthesia. Injection of the AAV8 vector into a terminal branch of the superior mesenteric vein was carried out with laparotomy under anesthesia with isoflurane and electrocardiogram monitoring.

Direct portal vein vector injection with saline flushing. Direct injection of the vector solution into the left portal vein was carried out after induction of general anesthesia with isoflurane and sterilization. A right subcostal incision (5 cm) was made through the skin and the subcutaneous tissue. The abdominal cavity was explored and the soft tissue of hepatic hilum was exfoliated surgically, then the main portal vein and its right and left branches were exposed. The main portal vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo, Tokyo, Japan), which was advanced into the left portal vein branch. The left and right portal vein branches were then clamped with vascular forceps. After flushing the left portal vein with saline, the vector solution was injected, and then a second saline solution, for flushing, was injected. Volumes of solutions used in the experiments were determined by taking a standard liver volume, a hepatic vascular bed volume, and effects of solutions on the systemic circulation into consideration.[19,30] A standard liver volume of a macaque was estimated with the formula (standard liver volume = 706.2 × body surface area + 2.4)[31] and the vascular bed volume of the liver was estimated to 25–30% of the standard liver volume.[19] A hepatic vascular bed volume can increase to 60% of the liver volume upon infusion of solutions and this may function as a reservoir and reduce the effects of the solutions on the systemic circulation.[20,32,33] The forceps were then removed immediately and the venotomy site was closed with an 8-0 prolene suture.

Catheter-guided vector injection to the portal vein with saline flushing. Balloon catheter-guided injection of the vector into the left portal vein of AAV8 antibody-positive macaques was carried out after the induction of general anesthesia. A 5-cm right paramedian incision was made through the skin and subcutaneous tissue. The abdominal cavity was carefully entered, with a part of the ileum identified and brought out through the incision. A peripheral branch of the superior mesenteric vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo). A temporary occlusion microcatheter (liguman 3.3F; Fuji System, Tokyo, Japan) was advanced into the left portal vein using a guide-wire (run through 0.014 (0.36 mm); Terumo) under a fluoroscope. The positions of the catheter and the balloon were confirmed by imaging with contrast medium. Blood flow in the left portal vein was occluded with a silicone balloon catheter and 40 ml of saline, followed by the AAV8 vector solution, and another 20 ml of saline was injected sequentially through the microcatheter. Volumes of solutions used in the experiments were determined as above with taking the result of the experiment of direct vector injection to the left portal vein branch into consideration. Following deflation of the balloon, the microcatheter was withdrawn and the peripheral venotomy ligated. The abdominal wall was then closed in layers.

Analysis of macaque FIX T262A expression in macaques. Macaque FIX T262A was bound to 3A6, a human FIX-specific monoclonal antibody for analyses. An enzyme-linked immunosorbent assay (ELISA) for the detection of macaque FIX T262A was carried out using 3A6, as previously described.[17,34]

NAb assay. An assay for the detection of anti-AAV8 NAbs was performed as previously reported, with some modifications.[35,36] Briefly, 5×10^4 2V6.11 cells/well were seeded in the wells of 96-well culture plates. Ponasterone A was added to the culture media the day before transduction to induce expression of the E4 gene. On the day of transduction, 10 µl of serum (undiluted, or subject to serial twofold dilutions) was incubated with the vector (AAV8-CMV-LacZ, 5×10^7 vg/10 µl) at 37 °C for 1 hour, and this mixture was added to a culture well. Sucrose was added to the culture medium such that the final concentration was 125 mmol/l. The culture medium was removed after a 48-hour incubation, and β-galactosidase activity quantified with a β-Gal assay kit (Invitrogen, Carlsbad, CA). Briefly, o-nitrophenyl-β-D-galactopyranoside was added to cell lysates, incubated for 30 minutes, and color change quantified with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA). If β-galactosidase activity was inhibited with a test sample that contained more than 50% of control fetal bovine serum, it was judged as positive for neutralizing capacity. The inhibitory titer of the serum sample was expressed as the highest final dilution in the culture medium that showed inhibitory activity.

Quantitation of AAV8 vector DNA in macaque tissue. Quantitation of AAV8 vector DNA in macaque tissues was performed using quantitative

PCR assays using a StepOnePlus instrument (Applied Biosystems Japan). DNA was isolated from macaque tissues using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) and subjected to PCR using primers 5′-GAT AAC TGG GGT GAC CTT GG-3′ and 5′-GCC TGG TGA TTC TGC CAT GA-3′, and Cybergreen reagent (Applied Biosystems Japan).

SUPPLEMENTARY MATERIAL

Figure S1. Changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in macaques.
Table S1. Direct vector injection into the portal vein of macaques.
Table S2. Balloon catheter-guided vector injection into macaques.
Video S1.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249, and 21790920) and the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Health, Labor, and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labor, and Welfare. This work was done in Shimotsuke, Tochigi and Tsukuba, Ibaraki, Japan. The authors declared no conflict of interest.

REFERENCES

1. Mannucci, PM and Tuddenham, EG (2001). The hemophilias–from royal genes to gene therapy. *N Engl J Med* **344**: 1773–1779.
2. Pasi, KJ (2001). Gene therapy for haemophilia. *Br J Haematol* **115**: 744–757.
3. VandenDriessche, T, Collen, D and Chuah, MK (2003). Gene therapy for the hemophilias. *J Thromb Haemost* **1**: 1550–1558.
4. Chuah, MK, Collen, D and Vandendriessche, T (2004). Preclinical and clinical gene therapy for haemophilia. *Haemophilia* **10** (suppl. 4): 119–125.
5. Chuah, MK, Collen, D and VandenDriessche, T (2004). Clinical gene transfer studies for hemophilia A. *Semin Thromb Hemost* **30**: 249–256.
6. Hasbrouck, NC and High, KA (2008). AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects. *Gene Ther* **15**: 870–875.
7. Mingozzi, F and High, KA (2011). Immune responses to AAV in clinical trials. *Curr Gene Ther* **11**: 321–330.
8. Kay, MA, Manno, CS, Ragni, MV, Larson, PJ, Couto, LB, McClelland, A *et al.* (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* **24**: 257–261.
9. Jiang, H, Pierce, GF, Ozelo, MC, de Paula, EV, Vargas, JA, Smith, P *et al.* (2006). Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. *Mol Ther* **14**: 452–455.
10. Manno, CS, Chew, AJ, Hutchison, S, Larson, PJ, Herzog, RW, Arruda, VR *et al.* (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* **101**: 2963–2972.
11. Manno, CS, Pierce, GF, Arruda, VR, Glader, B, Ragni, M, Rasko, JJ *et al.* (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**: 342–347.
12. Nathwani, AC, Davidoff, AM, Hanawa, H, Hu, Y, Hoffer, FA, Nikanorov, A *et al.* (2002). Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* **100**: 1662–1669.
13. Nathwani, AC, Gray, JT, Ng, CY, Zhou, J, Spence, Y, Waddington, SN *et al.* (2006). Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107**: 2653–2661.
14. Nathwani, AC, Gray, JT, McIntosh, J, Ng, CY, Zhou, J, Spence, Y *et al.* (2007). Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* **109**: 1414–1421.
15. Nathwani, AC, Tuddenham, EG, Rangarajan, S, Rosales, C, McIntosh, J, Linch, DC *et al.* (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**: 2357–2365.
16. Nathwani, AC, Rosales, C, McIntosh, J, Rastegarlari, G, Nathwani, D, Raj, D *et al.* (2011). Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* **19**: 876–885.
17. Ishiwata, A, Mimuro, J, Mizukami, H, Kashiwakura, Y, Yasumoto, A, Sakata, A *et al.* (2010). Mutant macaque factor IX T262A: a tool for hemophilia B gene therapy studies in macaques. *Thromb Res* **125**: 533–537.
18. Calcedo, R, Vandenberghe, LH, Gao, G, Lin, J and Wilson, JM (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* **199**: 381–390.
19. Lautt, WW and Greenway, CV (1987). Conceptual review of the hepatic vascular bed. *Hepatology* **7**: 952–963.
20. Saxena R, Zucker SD and Crawford JM (2003). Anatomy and physilogy of the liver. In: Zakim D and Boyer TD (eds). *Hepatology: A Textbook of Liver Disease*, 3rd edn. Saunders: Philadelphia. pp. 3–30.
21. High, KA (2007). Update on progress and hurdles in novel genetic therapies for hemophilia. *Hematology Am Soc Hematol Educ Program*: 466–472.
22. Sands, MS (2011). AAV-mediated liver-directed gene therapy. *Methods Mol Biol* **807**: 141–157.
23. Bartel, M, Schaffer, D and Büning, H (2011). Enhancing the Clinical Potential of AAV Vectors by Capsid Engineering to Evade Pre-Existing Immunity. *Front Microbiol* **2**: 204.
24. Hurlbut, GD, Ziegler, RJ, Nietupski, JB, Foley, JW, Woodworth, LA, Meyers, E *et al.* (2010). Preexisting immunity and low expression in primates highlight translational challenges for liver-directed AAV8-mediated gene therapy. *Mol Ther* **18**: 1983–1994.
25. Li C, Narkbunnam, N, Samulski, RJ, Asokan, A, Hu, G, Jacobson, LJ *et al.* (2012). Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. *Gene Ther* **19**: 288–294.
26. Chandler, RJ and Venditti, CP (2011). A barrel of monkeys: scAAV8 gene therapy for hemophilia in nonhuman primates. *Mol Ther* **19**: 826–827.
27. Boutin, S, Monteilhet, V, Veron, P, Leborgne, C, Benveniste, O, Montus, MF *et al.* (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* **21**: 704–712.
28. Calcedo, R, Morizono, H, Wang, L, McCarter, R, He, J, Jones, D *et al.* (2011). Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin Vaccine Immunol* **18**: 1586–1588.
29. Gao, GP, Alvira, MR, Wang, L, Calcedo, R, Johnston, J and Wilson, JM (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* **99**: 11854–11859.
30. Muir, AL, Flenley, DC, Kirby, BJ, Sudlow, MF, Guyatt, AR and Brash, HM (1975). Cardiorespiratory effects of rapid saline infusion in normal man. *J Appl Physiol* **38**: 786–775.
31. Urata, K, Kawasaki, S, Matsunami, H, Hashikura, Y, Ikegami, T, Ishizone, S *et al.* (1995). Calculation of child and adult standard liver volume for liver transplantation. *Hepatology* **21**: 1317–1321.
32. Greenway, CV and Lister, GE (1974). Capacitance effects and blood reservoir function in the splanchnic vascular bed during non-hypotensive haemorrhage and blood volume expansion in anaesthetized cats. *J Physiol (Lond)* **237**: 279–294.
33. Lautt, WW and Greenway, CV (1976). Hepatic venous compliance and role of liver as a blood reservoir. *Am J Physiol* **231**: 292–295.
34. Mimuro, J, Mizukami, H, Ono, F, Madoiwa, S, Terao, K, Yoshioka, A *et al.* (2004). Specific detection of human coagulation factor IX in cynomolgus macaques. *J Thromb Haemost* **2**: 275–280.
35. Moskalenko, M, Chen, L, van Roey, M, Donahue, BA, Snyder, RO, McArthur, JG *et al.* (2000). Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *J Virol* **74**: 1761–1766.
36. Mohammadi, ES, Ketner, EA, Johns, DC and Ketner, G (2004). Expression of the adenovirus E4 34k oncoprotein inhibits repair of double strand breaks in the cellular genome of a 293-based inducible cell line. *Nucleic Acids Res* **32**: 2652–2659.

OPEN ACCESS Freely available online

PLOS ONE

Porcine Model of Hemophilia A

Yuji Kashiwakura[1☯], Jun Mimuro[1*☯], Akira Onishi[2☯], Masaki Iwamoto[2,3☯], Seiji Madoiwa[1], Daiichiro Fuchimoto[2], Shunichi Suzuki[2], Misae Suzuki[2], Shoichiro Sembon[2], Akira Ishiwata[1], Atsushi Yasumoto[1], Asuka Sakata[1], Tsukasa Ohmori[1], Michiko Hashimoto[3], Satoko Yazaki[3], Yoichi Sakata[1]

1 *Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi-ken, Japan,* **2** *Transgenic Animal Research* Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki-ken, Japan, **3** Advanced Technology Development Team, Prime Tech Ltd., Tsuchiura, Ibaraki-ken, Japan

Abstract

Hemophilia A is a common X chromosome-linked genetic bleeding disorder caused by abnormalities in the coagulation factor VIII gene (*F8*). Hemophilia A patients suffer from a bleeding diathesis, such as life-threatening bleeding in the brain and harmful bleeding in joints and muscles. Because it could potentially be cured by gene therapy, subhuman animal models have been sought. Current mouse hemophilia A models generated by gene targeting of the *F8* have difficulties to extrapolate human disease due to differences in the coagulation and immune systems between mice and humans. Here, we generated a porcine model of hemophilia A by nuclear transfer cloning from *F8*-targeted fibroblasts. The hemophilia A pigs showed a severe bleeding tendency upon birth, similar to human severe hemophiliacs, but in contrast to hemophilia A mice which rarely bleed under standard breed conditions. Infusion of human factor VIII was effective in stopping bleeding and reducing the bleeding frequency of a hemophilia A piglet but was blocked by the inhibitor against human factor VIII. These data suggest that the hemophilia A pig is a severe hemophilia A animal model for studying not only hemophilia A gene therapy but also the next generation recombinant coagulation factors, such as recombinant factor VIII variants with a slower clearance rate.

Citation: Kashiwakura Y, Mimuro J, Onishi A, Iwamoto M, Madoiwa S, et al. (2012) Porcine Model of Hemophilia A. PLoS ONE 7(11): e49450. doi:10.1371/journal.pone.0049450

Editor: Christopher B. Doering, Emory University School of Medicine, United States of America

Received April 10, 2012; **Accepted** October 9, 2012; **Published** November 28, 2012

Funding: This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249 and 21790920) and the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Health, Labour and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: MI, MH, and SY are employees of Prim Tech Ltd., the developer of Piezo micromanipulators for nuclear transfer, and are collaborative with National Institute of Agrobiological Sciences and Jichi Meidical University for basic research. Other authors declare no conflict of interest. This does not alter the authors adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: mimuro-j@jichi.ac.jp

☯ These authors contributed equally to this work.

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities in the coagulation factor VIII (FVIII) gene (*F8*). The genetic abnormalities result in FVIII deficiency, which in turn creates a bleeding diathesis, such as life-threatening bleeding in the brain and harmful bleeding in joints and muscles. The morbidity of hemophilia A is one in 5,000 male live births [1]. The current standard therapy for hemophilia A is intravenous injection of recombinant FVIII or monoclonal antibody-purified FVIII from plasma. Prophylactic administration of FVIII is effective in preventing harmful bleeding; however, hemophilia A patients are still not free from the risks of life-threatening intracranial and other harmful bleeding [1] [2]. In addition, severe hemophilia A patients develop antibody against FVIII (inhibitor) upon infusion of FVIII frequently [1].

Gene therapy, that enables sustained elevation of coagulation factor levels, will provide the next-generation therapy for hemophilia [1,3,4]. In fact, gene and cell therapy for hemophilia clinical trials were conducted. Compared with clinical trials of the gene therapy for hemophilia B [5,6], gene and cell therapies for hemophilia A have had limited successes [7,8]. Upcoming therapeutic alternatives for hemophilia A are FVIII variants with a slower clearance rate. Therapeutic factors, such as recombinant activated factor VII and plasma-derived activated prothrombin complex, are used for the treatment of hemophilia A patients with inhibitors, and the second generation therapeutic factors for hemophilia A patients with inhibitors are also currently under development. For studying next-generation therapeutics, good animal models are required. Hemophilia A mice generated by targeted ablation of mouse *F8* [9] have been the mainstay for assessment of hemophilia A gene therapy and evaluation of FVIII variants. However, there are significant species differences between mice and humans. For example, the half-life of human FVIII in the mouse circulation is very short, making it difficult to analyze the efficacy of human FVIII-expressing vectors for gene therapy or novel FVIII variants. As alternatives, there are natural hemophilia A dogs and hemophilia A sheep. Hemophilia A dogs have been used for gene therapy studies [10,11,12]. Hemophilia A sheep would be an alternative model [13]. There may be interspecies differences, such as body size, physiology, disease

progression and chromosome structure homology, between these animal models and humans.

Pigs are excellent biomedical models of human diseases [14,15]. The porcine blood coagulation system is very similar to that in humans, because of the high homology between the coagulation factor amino acid sequences [16,17,18]. In addition, porcine FVIII has been used to treat hemophilia A patients with FVIII inhibitors [19,20,21]. Therefore, the hemophilia A pig could be a good animal model to study the next-generation therapeutics for hemophilia A. Moreover, a miniature pig strain exists, and thus, cloned pigs could be downsized to an adequate size, approximately 20–30 kg in weight. For these reasons, we decided to generate hemophilia A pigs by cloning technology.

Results

Firstly, we constructed a *F8* targeting vector (Figure 1**A**) and targeted *F8* in male porcine embryonic fibroblasts (PEF) with the *F8*-targeting vector as shown in Figure 1. The DNA fragment amplified from the non-transfected PEF DNA migrated at 6.5 kb on agarose gel electrophoresis, whereas two DNA fragments, migrating at 6.5 kb and 8.3 kb, were amplified from PEF colony 134. The 8.3 kb DNA was not amplified from genomic DNA of PEF colonies 135–137. The 8.3 kb fragment PCR-amplified from PEF colony 134 was cleaved into a 2.4-kb fragment and a 5.9-kb fragment by *Stu* I, whereas the PCR-amplified 6.5-kb DNA fragment was not susceptible to *Stu* I digestion. This supports that the PCR-amplified 8.3-kb fragment is derived from the *F8*-targeted genome because a *Stu* I recognition sequence present in the neo-resistant gene but not in the PCR amplified DNA fragment from the wild-type *F8*. The expected DNA fragments were amplified by PCR with Neo primers from genomic DNA from PEF colony 134, but not from wild-type genomic DNA (WT). PCR analysis of genomic DNA with three primer sets revealed a recombination event in *F8* of a colony, 134 (PEF-134). PEF-134 nuclei were then injected into enucleated oocytes. After an electrical pulse, the oocytes were transplanted into the oviduct of a female pig [22,23]. Transplantation of nucleus-transferred oocytes to the oviducts of female pigs was repeated four times. Three months later, a fetus was obtained by induced abortion. Dermal fibroblasts from this PEF-134-derived fetus (134-fetus) were isolated and cultured, and genomic DNA was isolated for analysis by PCR and by Southern blotting (Figure 2). The PCR amplified wild-type (WT) *F8* exon 14–18 fragment migrated at 6.5 kb, whereas the 8.3-kb targeted DNA fragment was amplified solely from 134-fetus fibroblast DNA. PCR-amplified DNA fragments using an *F8* exonic primer and a Neo primer were obtained only from 134-fetus DNA. The PCRs demonstrated insertion of the neomycin-resistance gene in *F8* (Figure 2**A**). Southern blotting showed that the 5′ probe hybridized with the 8.1 kb DNA fragment of Sac I-digested wild-type DNA while the 5′ probe hybridized with 9.9 kb DNA fragment of Sac I-digested 134-fetus DNA. Southern blotting with the 3′ probe confirmed recombination in the *F8* gene in the 134-fetus genome because a Sph I recognition sequence and a Xba I recognition sequence located in the 3′ end of the Neo resistant gene of the targeted allele (Figure 2**B**). Therefore, five transfers of fetal fibroblast nuclei to oocytes followed by oocyte transplantation were performed. Four females became pregnant and each produced a full-term delivery.

Four live offspring were obtained and PCR analysis and Southern blotting were carried out. As shown in Figure 3**A**, the 8.3 kb DNA fragments were PCR amplified from piglets DNA as same as that of 134-fetus (Figure 2). Similarly, Southern blotting of Sac I-treated and *Sac* I and *Stu* I-treated DNA of the piglets with

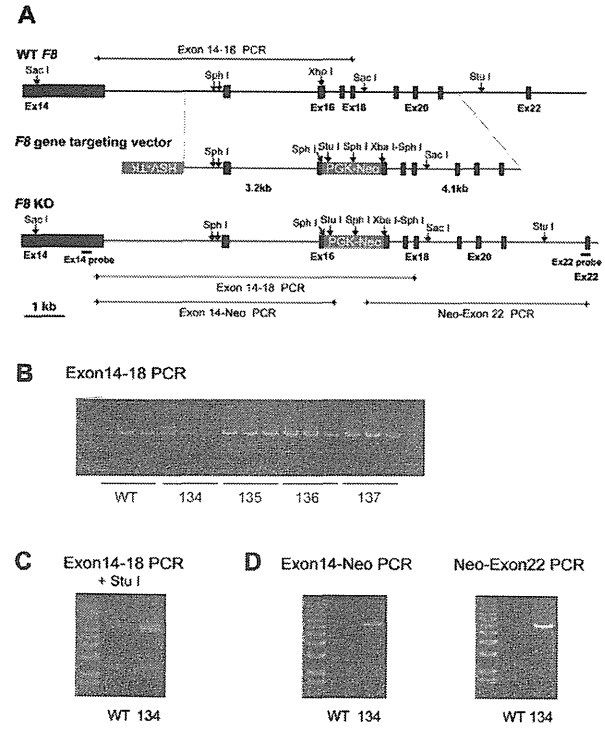


Figure 1. *F8* targeting of porcine fetal fibroblasts (PEF). (**A**) Schematic diagram of part of porcine *F8*, the positions of the restriction endonuclease sites, the *F8* targeting vector structure, and the targeted *F8* (*F8* KO) allele are shown. The neomycin-resistance gene (PGK-neo) was inserted in the exon 16 DNA fragment with deletion of a part of exon 16 and was flanked by two *F8* DNA fragments (5′ arm: 3.2 kb; 3′ arm: 4.1 kb) in *F8* targeting vector. The positions of PCR primers (arrowheads), expected amplified DNA fragments (bars), and restriction endonuclease sites used for the Southern blot analysis are indicated in the schema for *F8* KO. (**B**) *F8* exon 14–18 PCR on genomic DNA from non-transfected PEF (WT), PEF colony 134 (134), and three other PEF colonies (135–137) was shown. (**C**) The *F8* exon 14–18 PCR products were treated with *Stu* I and analyzed by agarose gel electrophoresis. (**D**) PCR analyses with two sets of primer pairs for exon 14 and the neomycin resistance gene and for the neomycin resistance gene and exon 22 were shown.
doi:10.1371/journal.pone.0049450.g001

the 5′ probe confirmed the recombination of F8 of piglets and showed that each piglet had a single copy of the targeted *F8* (Figure 3, **A** & **B**). RT-PCR analysis revealed that FVIII mRNA was not detected in the liver of piglet #3 (Figure 3**C**). Analysis of the blood of piglets #3 and #4 confirmed that the FVIII level was severely decreased to less than 1%, using an activated partial thromboplastin time (APTT)-based coagulation assay for human FVIII (Table 1). Other coagulation factors were moderately decreased (Table 1). The levels of albumin and cholinesterase of these piglet blood were also measured as the references to study whether the decreased level of coagulation factors II, V, VII, IX, and X were specific or not. The albumin levels of piglet #3 and #4 were decreased significantly compared with the wild type piglets. However, the cholinesterase activities of piglets #3 and #4 were not decreased. The data suggested that synthesis of some proteins in the liver of the cloned piglets was altered. The precise mechanism of the moderately decreased levels of coagulation factors II, V, VII, IX, and X, and albumin was not elucidated in this study.