The role of the 5'-flanking region in the cell-specific transcription of the human von Willebrand factor gene

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Transcriptional regulation of the human von Willebrand factor (vWF) gene was investigated in calf pulmonary artery endothelial (CPAE), HeLa, COS 7 and Hep G2 cells. Various lengths of flanking sequences extending up to 2123 bp 5' of the transcription initiation site and containing 19 bp of the first exon, were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene and these constructs were assayed in transient transfection assays. Sequences up to 89 bp upstream of the cap site showed transcriptional activity in all cell types. Sequences between -147 and -419 bp markedly reduced CAT activity in CPAE cells and abolished it in other cell lines. A domain from -592 to -810 bp generated low levels of expression only in CPAE cells. This transcriptional activity was repressed with constructs containing 1041 to 1240 bp upstream of the cap site. Endothelial cell-specific

transcription was restored by a construct that contained 1286 bp upstream of the cap site. The additional 46 bp upstream of the negative regulatory domain were within the 5' end of an inverse human Alu-family DNA repeat. RNAase-protection assays confirmed the correct transcriptional initiation. The sequence between -89 and -420 contained at least one negative regulatory element able to repress the CAT gene expression controlled by the heterologous thymidine kinase promoter in all cell types. A construct that included the sequence from -89 to -1286 bp increased the transcriptional activity directed by the thymidine kinase promoter only in CPAE cells. These results indicate that negative and positive elements in the 5'-flanking region interact to regulate vWF gene expression.

INTRODUCTION

von Willebrand factor (vWF), a multimeric adhesive glycoprotein, binds platelets to the subendothelium at high shear rates [1]. vWF also serves as a carrier protein for coagulation factor VIII in plasma and stabilizes its activity [2]. Abnormalities of vWF result in von Willebrand disease, the most frequent inherited disorder of haemostasis in man with over 20 subtypes identified [3].

vWF undergoes complex post-transcriptional processing and assembly within endothelial cells. The mRNA is translated into a large approx. 309000 Da precursor protein followed by glycosylation, dimerization, sulphation, multimerization and cleavage of a prosequence [4–6]. vWF is stored in Weibel–Palade bodies within endothelial cells and in platelet α -granules and released in response to a wide variety of secretagogues *in vitro* including thrombin [7,8], phorbol esters [9], calcium ionophore A23187 [10], fibrin [11] and *in vivo* analogues of vasopressin [12].

The only known cells to synthesize vWF are endothelial cells and megakaryocytes [13,14]. Furthermore, endothelial cells show differential expression of vWF based on the location of the cell in the vascular system, and mRNA studies suggested that this heterogeneity in the expression of vWF was at the level of transcription [15]. The plasma levels of vWF are increased in pregnancy and diabetes, and decreased in hypothyroidism and lymphoproliferative disorders [16–19]. The levels of vWF have been found to be modulated *in vitro* by 4- β -phorbol 12-myristate 13-acetate, 17- β -oestradiol and dexamethasone [9,16,20]. Nevertheless, because the vWF molecule undergoes very complex processing and assembly, it has been difficult to demonstrate at what level the cell is regulating the expression of the protein.

The tissue-specific and hormonal-mediated pattern of vWF gene expression is presumably controlled by the interaction between regulating factors and specific DNA elements within or adjacent to the gene. Characterization of the transcriptional regulatory region of the gene would help identify the DNA sequences involved in the control of vWF expression. The human vWF gene sequence, 2138 bp upstream of the transcription initiation site, has been reported [21–24]. This region contains an atypical TATA box, a CCAAT box downstream of the TATA box, a poly dG-dT sequence and two Alu repeats in opposite orientation.

In order to identify factors regulating vWF gene expression we analysed DNA sequences extending up to 2123 bp 5' of the cap site for their ability to direct expression of bacterial chloramphenicol acetyltransferase (CAT) reporter gene in endothelial cells and cells that do not express vWF. Transient expression experiments demonstrated an ubiquitous promoter which includes the putative TATA box. An upstream negative regulatory domain repressed the CAT gene expression driven by the vWF promoter as well as by a heterologous promoter. Transcriptional activity was restored only in endothelial cells by a complex mechanism involving upstream positive regulatory elements.

MATERIALS AND METHODS

Materials

Restriction enzymes, other nucleic-acid-modifying enzymes and fetal-calf serum (FCS) were purchased from Boehringer-Mannheim (Meylan, France) and RNAase-free DNAase I from

Abbreviations used: vWF, von Willebrand factor; MAb, monoclonal antibody; CPAE, calf pulmonary artery endothelial; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco's modified essential medium; α -MEM, alpha-minimum essential medium; FCS, fetal-calf serum; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; ONPG, o-nitrophenol β -D-galactoside; HeLa cells, human epitheloid carcinoma cells; SV 40, simian virus 40; Hep G2, human hepatocellular carcinoma.

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Promega (Madison, WI, U.S.A.). Acetyl-CoA, *o*-nitrophenol β -D-galactoside (ONPG) and alpha-minimum essential medium (α -MEM) were obtained from Sigma (St. Louis, MO, U.S.A.) and D-threo-[dichloroacetyl-1- 14 C]chloramphenicol (50 mCi/mmol) and [α - 32 P]UTP (800 Ci/mmol) from Amersham International (Amersham, Bucks., U.K.). The Sequenase kit was from United States Biochemical Corp. (Cleveland, OH, U.S.A.). Plasmids pBS M13⁺ and M13⁻, and the *in vitro* transcription kit were purchased from Stratagene Inc. (La Jolla, CA, U.S.A.). Plasmid pCH110 was obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Dulbecco's modified essential medium (DMEM) was purchased from Gibco (Life Technologies SARL, Cergy Pontoise, France) and Ultrosor from IBF (Villeneuve-la-Garenne, France). Centricon 100 microconcentrators were obtained from Amicon (Danvers, MA, U.S.A.).

Plasmid constructions

The 5'-flanking sequences of the vWF gene were subcloned upstream of the CAT gene into the promoterless plasmids pSB1 or pGCAT-A [25,26]. Plasmid pBE6 has been described previously [21] and contains 2123 bp at the 5' end and 1480 bp at the 3' end of the transcription initiation site of the vWF gene. Plasmid pBE6 was linearized with *Sac*I 1480 bp downstream of the transcription initiation site. Exonuclease *Bal*31 was used to digest the 3' end of the linearized plasmid to remove a potential open reading frame with an inframe stop codon in the non-translated region 43 bp downstream of the cap site [21]. The *Bal*31-digested plasmids were cut with *Xba*I, 2123 bp upstream of the cap site, and fragments of the expected size were subcloned into the *Xba*I and *Sma*I sites of the multiple cloning site of plasmid pBS M13⁺. A plasmid pBvW -2123/+19 with an insert spanning from -2123 bp to +19 bp relative to the cap site [21] was selected and used for the 5' deletions. DNA fragments with different lengths at the 5' end were excised from pBvW -2123/+19 after cleavage at *Xba*I, *Sma*I, *Bam*HI and *Hind*III restriction sites within the 5'-flanking region and the *Eco*RI site in the polylinker 3' of the insert. The four resulting fragments spanned 2123, 1240, 420 and 89 bp upstream of the cap site and included 19 bp of the first exon. These DNA fragments were converted into blunt ends with Klenow DNA polymerase and inserted into the filled in *Hind*III site of pSB1 to generate four different pvWCAT constructions. Two additional plasmids were constructed. One contained a fragment spanning from -89 to +19 bp in the opposite orientation upstream of the CAT gene (pvWCAT +19/-89), and the other included the longest 5'-flanking fragment deleted of 108 bp of the 3' end (pvWCAT -2123/-89).

Another series of constructs was generated by progressive deletions of the 5'-flanking sequence of the vWF gene. The plasmid pBvW -2123/+19 was linearized at the *Xba*I site at nucleotide -2123 and digested with *Bal*31 [27]. The digested genomic fragments were separated from the vector by *Sac*I in the polylinker 3' of the insert. Fragments of various lengths, containing 19 bp of the first exon and 147 to 1286 bp upstream of the transcription initiation site, were inserted between the *Sma*I and *Sac*I sites of the multiple cloning site of plasmid pGCAT-A to generate pGvWCAT constructions.

Fragments of the 5'-flanking sequence of the vWF gene were also inserted in front of the Herpes simplex virus thymidine kinase (tk) gene promoter of plasmid pBLCAT2 [28]. Inserts were isolated either from the plasmid pBvW -2123/+19 by digestions at restriction sites inside the vWF gene sequence, or from pGvWCAT constructions by digesting 5' in the multiple cloning site and 3' at one site inside the vWF gene sequence. Three

fragments spanning from -89 to -420, -89 to -1240 or -89 to -1286 bp and one segment from -1199 to -1363 bp were filled in with Klenow enzyme and ligated in two orientations to the blunt-ended *Bam*HI site of pBLCAT2 to make the pvWtkCAT constructs. The orientation and boundaries of all the constructs were confirmed by restriction analysis and sequencing [29].

Cell cultures and transfections

Calf pulmonary artery endothelial (CPAE) cell [30], human epitheloid carcinoma cells (HeLa) and simian virus 40 (SV 40)-transformed monkey kidney cells (COS 7) were from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Human hepatocellular carcinoma (Hep G2) cells were kindly given by C. Bodard (INSERM U. 33, Le Kremlin-Bicêtre, France). Human endothelial cells were isolated from umbilical vein (HUVEC). Cells were cultured in α -MEM supplemented with FCS at a concentration of 20 % (v/v) for endothelial cells and 10 % (v/v) for HeLa and COS 7 cells. Hep G2 cells were cultured in DMEM and 10 % (v/v) FCS. All media contained penicillin (100 units/ml) and streptomycin (100 μ g/ml).

Transfections were performed by the calcium phosphate-DNA co-precipitation method [27]. CPAE (2×10^6) cells and HeLa, COS 7 and Hep G2 (1×10^6) cells were plated on 10-cm diam. Petri dishes in 10 ml of complete medium at 5 % CO₂. The medium was removed 24 h later and replaced with the same type of medium for HeLa, COS 7 and Hep G2 cells, whereas CPAE cells received 10 ml of DMEM plus 10 % (v/v) FCS. Cells were incubated with the fresh medium for 3 h before transfection. The calcium phosphate-DNA precipitates were prepared in Hepes-buffered saline [27] and optimal conditions were found when the pH value of the buffer was 7.13 for HeLa, COS 7 and Hep G2 cells and 7.2 for CPAE cells. Cells were transfected with 15 μ g of either pvWCAT, pGvWCAT or pvWtkCAT constructs. CPAE, COS 7 and Hep G2 cells were co-transfected with the pCH110 vector, which contains a functional LacZ gene downstream of

Figure 1 SDS/agarose gel electrophoresis of vWF secreted from CPAE cells, HUVEC and Hep G2 cells into serum-free media

The gels were reacted with a mixture of 125 I-labelled polyclonal and monoclonal antibodies raised against human vWF. Lane 1, CPAE cells; lane 2, HUVEC; lane 3, Hep G2 cells.

Figure 2 Transient expression of CAT gene constructs with different lengths of the 5'-flanking region of the vWF gene

A cloned fragment containing 2123 bp of the 5'-flanking region of the vWF gene and 19 bp of the first exon was deleted from the 5' end by restriction-enzyme digestion at sites within the flanking region, excised from the plasmid and subcloned into pSB1 plasmid upstream of the CAT gene. Deleted constructs (15 µg) were transfected into CPAE, COS 7, HeLa and Hep G2 cells. CPAE, COS 7 and Hep G2 cells were co-transfected with the SV 40 β -galactosidase plasmid pCH110 (5 µg) to estimate the transfection efficiency. (a) The vWF gene 5'-flanking sequence is schematically shown in the top line with the position of Alu repeats and TAATTA sequence indicated. The black vertical rectangle indicates the 19 bp of the first exon cloned immediately upstream of the CAT gene. The restriction sites in the 5'-flanking DNA that were used to generate the vWF-CAT deletions were indicated by the capital letters. The position of the insert upstream of the CAT gene is indicated on the left. The insert +19/-89 is cloned in the reverse orientation. The broken line indicates that the first 89 bp upstream and 19 bp downstream of the cap site were deleted. The pSVECAT plasmid that contains the SV 40 early promoter and enhancer upstream of the CAT gene is indicated by the hatched bar. CAT activity is expressed as the percentage conversion of [14 C]chloramphenicol per 400 units of β -galactosidase activity for CPAE, COS 7 and Hep G2 cells and per 700 µg of protein for HeLa cells. In assays of cells transfected with pSVECAT it was necessary to dilute the cell lysates approx. 10-fold before analysis to be in the linear range of the curve for the measurement of CAT activity. The values are the mean CAT activity $\pm$ S.E.M. for four to seven experiments. Abbreviations: X, *Xba*I; S, *Sma*I; B, *Bam*HI; H, *Hind*III. (b) Representative results of the corresponding CAT assays in CPAE and HeLa cells. The deletion endpoints relative to the cap site are given for each construction. Abbreviations: CM, [14 C]chloramphenicol; 1-Ac, [14 C]1-monoacetylated chloramphenicol; 3-Ac, [14 C]3-monoacetylated chloramphenicol.

the strong SV 40 early promoter [31]. The β -galactosidase produced in the cells by the LacZ gene was assayed to monitor transfection efficiency. The β -galactosidase activity appeared to be inhibited in extracts from HeLa cells and therefore HeLa cells were only transfected with CAT gene constructs.

CAT and β -galactosidase assays

Medium was removed 20 h after transfection and the cells were washed and incubated in fresh medium for a further 48 h. The cells were then washed with 12 mM Na_2HPO_4 /1.5 mM KH_2PO_4 /0.14 M NaCl/3 mM KCl (pH 7.4) (PBS), scraped in 4 ml of PBS, pelleted and resuspended in 200 µl of 250 mM sucrose/10 mM Tris/HCl (pH 7.4)/10 mM EDTA. After five cycles of freezing and thawing the samples were centrifuged for 5 min at 15000 g at 4 °C. Before measuring CAT activity, 20 µl samples of the supernatants were withdrawn to determine the β -galactosidase activity to act as a control for transfection efficiency. The β -galactosidase activity was measured by a spectrophotometric assay in the presence of 100 nM ONPG [31]. One

unit of β -galactosidase activity was arbitrarily defined as 100 times the absorbance change at 420 nm of the enzymically cleaved ONPG extrapolated to 1 h of enzymic reaction. The remainder of the cell extract was heated to 65 °C to inactivate any endogenous inhibitor [32]. Cell extracts corresponding to 400 units of β -galactosidase or 700 µg of protein from HeLa cells were adjusted to a final concentration of 250 mM Tris/HCl (pH 8), 5 mM EDTA, 1.3 mM acetyl-CoA and 0.2 µCi of D-threo-[dichloroacetyl- 14 C]chloramphenicol in 300 µl and incubated for 3 h at 37 °C. The chloramphenicol and its acetylated forms were extracted with ethylacetate and analysed by t.l.c. [33]. The percentage conversions of chloramphenicol into its acetylated derivatives were calculated by counting of the radioactive spots after autoradiography.

vWF antigen analysis

Confluent cell cultures (10 cm diam. Petri dishes) were extensively rinsed with PBS and incubated for 48 h in 12 ml of serum-free medium containing 1.0% Ultrosor. Media were withdrawn and

concentrated 20-fold using a Centricon 100 microconcentrator. The amount of cells in a culture was 3.2×10^6 for CPAE cells, 3.7×10^6 for HUVEC and 3×10^6 for Hep G2 cells. Aliquots (2 μ l) of the concentrated media were diluted 10-fold in 10 mM Tris/HCl, pH 8.8, containing 2% (w/v) SDS and vWF multimer analysis was performed by 0.1% SDS/1.4% (w/v) agarose gel electrophoresis [34]. After electrophoresis the gels were fixed, washed and reacted with a mixture of 125 I-labelled polyclonal antibody and monoclonal antibody (MAb) 9 raised against human vWF as described [34]. MAb 9 recognizes an epitope on bovine vWF [35].

RNAase mapping

A fragment from the pGvWCAT -222/+19 construct, containing 222 bp of the vWF gene 5'-flanking region, 19 bp of the first exon and a 18 bp linker sequence was excised from the plasmid at the *Bam*HI cloning site upstream of the insert and at the *Eco*RI site 250 bp inside the CAT gene. The fragment was subcloned into the *Bam*HI and *Eco*RI sites of pBS M13⁺ (pBS M13 -222/CAT). The plasmid was linearized at the *Bam*HI site and antisense RNA transcripts were made from the T7 promoter of pBS M13 -222/CAT using the RNA *in vitro* transcription kit and 80 μ Ci of [α - 32 P]UTP by following the suggested procedures. After transcription *in vitro*, plasmid DNA was digested with 5 units of DNAase I for 15 min and transcripts of the proper size were purified from a 4.5% (w/v) acrylamide-7 M urea gel.

Total cellular RNA was isolated using a guanidinium isothiocyanate procedure described by Chomczynski and Sacchi [36]. RNA (50 μ g) was treated with 5 units of DNAase I in the presence of 20 units RNAasin for 20 min followed by phenol-chloroform extraction. RNAase mapping was done essentially as described in Sambrook et al. [27]. The RNA was dissolved in 30 μ l of 80% (v/v) formamide/40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl and labelled antisense transcript (7.5×10^5 c.p.m.) in a volume of 1 μ l was added. The RNA was heated for 10 min at 85 °C and hybridized overnight at 45 °C. Unprotected RNA was digested for 30 min at 37 °C in 300 μ l of 10 mM Tris/HCl (pH 7.4)/5 mM EDTA/0.3 M NaCl containing 50 μ g/ml RNAase A and 1500 units/ml RNAase T1. After digestion the solution was phenol- and phenol-chloroform-extracted and ethanol-precipitated. The protected RNA was dissolved in 8 μ l of 95% (v/v) formamide/10 mM EDTA/0.05% Bromophenol Blue/0.05% xylene cyanol and applied to a 6% (w/v) acrylamide-7 M urea sequencing gel. The sequence of single-stranded M13mp18 DNA using the -40 primer provided in the Sequenase kit was used as molecular-mass markers.

RESULTS

vWF antigen analysis

The CPAE cells used in this study synthesize and secrete vWF (Figure 1). vWF secreted from the CPAE cells and HUVEC (Figure 1) showed the same multimeric pattern, including the presence of high-molecular mass forms found in plasma vWF. The intensities for human and bovine vWF antigen shown by the multimeric analysis (Figure 1) were different due to the mixture of antibodies against human vWF, which do not completely cross-react with the bovine protein. The same relative relationship of vWF antigen was found in the human and bovine plasma samples (results not shown), although concentrations of vWF in these plasma samples are in the same range [37]. A similar analysis of the Hep G2 cell line did not show any vWF antigen (Figure 1).

Transient transfections with vectors containing different lengths of the vWF-gene 5'-flanking sequence

Sequences of different lengths containing 19 bp of the first exon and 89 to 2123 bp upstream of the transcription initiation site of the vWF gene were inserted in front of the CAT gene in pSB1 [25]. CPAE, HeLa, COS 7 and Hep G2 cells were transfected with these pvWCAT constructs (Figure 2). CPAE, COS 7 and Hep G2 cells were also co-transfected with the β -galactosidase expression vector pCH110 to control for transfection efficiency. The analysis of β -galactosidase activity is impaired in extracts of HeLa cells, making it difficult to calculate transfection efficiency. Figure 2 demonstrates that 700 μ g of protein from cell extracts of HeLa cells transfected with pSVECAT, in which the SV 40 early promoter is inserted upstream of the CAT gene, showed approx. the same amount of CAT activity as the volume of extracts from other cells containing 400 β -galactosidase units. Therefore, CAT activity for HeLa cell extracts was based on the activity present in 700 μ g of protein.

The CAT activities with the different plasmids are shown in Figure 2 along with representative CAT assays. Separate cultures of cells were also transfected with the promoterless plasmid pSB1 to monitor the background level of CAT expression. In addition, plasmid pSVECAT was used in order to compare the expression level of an ubiquitous strong promoter and enhancer [38]. Results clearly show that the construct -89/+19 is expressed in these

Figure 3 Effect of progressive deletions of the 5'-flanking region of the vWF gene on CAT gene expression in CPAE cells

A plasmid containing 2123 bp of the vWF-gene flanking sequence and 19 bp of the first exon was linearized at the 5' end of the insert and progressive deletions were generated with the exonuclease *Bal*31. The inserts were subcloned into the vector pGCAT-A. The deletion end points relative to the cap site are given for each construct. CAT activities were calculated as in Figure 2. Results were then normalized between experiments by expressing CAT activity relative to the activity obtained with the -1240/+19 constructs. Hatched bars represent the relative CAT activity determined with the series of constructions expressed in the vector pGCAT-A. Open bars represent the relative CAT activity observed with fragments cloned into the vector pSB1 shown in Figure 2. The values represent the mean $\pm$ S.E.M. for four to seven plates.

Figure 4 RNAase mapping of transcriptional initiation in CPAE cells transfected with the pGvWCAT constructs

(a) RNA was isolated from a 10-cm-diam. culture dish of CPAE cells after transfection with three of the pGvWCAT constructs (Figure 3) and the plasmid pSVGCAT-A with the SV 40 early promoter/enhancer [26]. RNA (50 μ g) was hybridized with 32 P-labelled 525-nucleotide antisense RNA probe as described in the Materials and methods section. Protected fragments were analysed on a 6% (w/v) acrylamide–7 M urea sequencing gel. The A and C ladder produced by dideoxy sequencing of the single-stranded M13mp18 DNA sequence, using the –40 primer provided in the Sequenase kit, is shown on the left. Lane 1, untransfected CPAE cells; lane 2, pSVGCAT-A; lanes 3–5, pGvWCAT constructs –810/+19, –1240/+19, –1286/+19; lane 6, tRNA. The protected fragment from CPAE cells transfected with pSVGCAT-A is smaller than that shown by cells transfected with pGvWCAT constructs (arrow) because the probe hybridizes only to the CAT gene fragment on the transcript. Parallel culture dishes demonstrated that the transfection efficiency of cells transfected with the pGvWCAT vectors were similar. (b) Diagram showing the position of the probe and the protected fragment with respect to the hybrid vWF–CAT transcript. Solid bar, the vWF gene fragment included in the probe containing 222 bp upstream and 19 bp downstream of the vWF transcription initiation site (+1); open bar, linker fragment; and hatched bar, the segment of the CAT gene included in the probe. Abbreviations: nt, nucleotide; B, *Bam*H1; E, *Eco*R1.

four cell lines at a level corresponding to an average of 15 times more activity than that observed upon transfection with pSB1. This transcriptional activity is lost when the cells are transfected with a plasmid in which the insert –89/+19 is cloned in the reverse orientation (+19/–89, Figure 2).

Increasing the length of the 5'-flanking sequence of 420 bp and to 1240 bp upstream of the transcription initiation site inhibited the CAT gene expression in all four cell lines. Transfection of the endothelial cells with the longest construct –2123/+19 restored the CAT activity to approx. the same level as with the –89/+19 construct (Figure 2). In contrast the HeLa, COS 7 and Hep G2 cells transfected with this same construct –2123/+19 did not exhibit any significant CAT activity. Deletion of 108 bp from the 3' end of the –2123/+19 construct demonstrated no CAT activity in the endothelial cells, as shown upon transfection with the –2123/–89 construct. These results demonstrated that both positive and negative regulatory elements are present within 2123 bp of the 5'-flanking sequence of the vWF gene.

Transfections with progressive deletions of the vWF gene-flanking sequence

To study these DNA elements in more detail progressive deletions

Figure 5 The effect of vWF gene regulatory domains on the transcriptional activity of the tk promoter

Blunt-ended fragments from the vWF gene 5'-flanking sequence were ligated in the sense or antisense direction into the filled in *Bam*HI site of pBLCAT 2 upstream of the tk promoter. Calcium phosphate-mediated transfections were performed in CPAE or COS 7 cells with 15 μ g of the pvWtkCAT plasmids. Cells were co-transfected with the β -galactosidase pCH110 (2.5 μ g) expression vector to control for transfection efficiency. Cell extracts were prepared 68 h after transfection and aliquots normalized for transfection efficiency were used for the determination of CAT activity. (a) CAT gene expression from the pvWtkCAT plasmids construct with a vWF-gene fragment –420/–89 in sense or antisense (–89/–420) direction upstream of the tk promoter. pBLCAT 2 is the CAT gene expression vector with only the tk promoter and pBLCAT 3 is the promoterless plasmid. A representative of four experiments is shown. (b) CAT activity from the pvWtkCAT vectors with vWF gene fragments –1286/–89 and –1240/–89 in the sense direction. A representative experiment with two separate clones (1,2) containing the same fragment is given. Transfections were repeated twice for each clone. Abbreviations: CM, [14 C]chloramphenicol; 1-Ac, [14 C]1-monoacetylated chloramphenicol; 3-Ac, [14 C]3-monoacetylated chloramphenicol.

of the 5'-flanking region were made as described in the Materials and methods section, and the fragments were inserted into a different vector, pGCAT-A, taking advantage of the multiple cloning site upstream of the CAT gene in this plasmid [26]. These pGvWCAT constructions were used to transfect CPAE, HeLa and Hep G2 cells. The CAT activity measured in CPAE cells is indicated in Figure 3. Results from the transfections with the pvWCAT vectors (Figure 2) were added for comparison. In order to normalize between experiments, CAT activity was expressed relative to the activity obtained with the –1240/+19 constructs.

Analysis of CAT activity in CPAE cells transfected with the pGvWCAT constructions revealed several upstream positive regulatory domains separated by areas that repressed activity (Figure 3). CAT synthesis decreased in CPAE cells transfected with constructs further upstream of –89/+19 but the inhibition of transcriptional activity appeared to be mollified by another potential positive regulatory domain between 592 and 810 bp

Figure 6 Comparison of CAT gene expression in CPAE, HeLa and COS 7 cells transfected with pvWtkCAT constructs

A diagram of the 5'-flanking sequence of the vWF gene and the location of some internal elements are shown in the top line on the left. The expression vectors containing various lengths of 5'-flanking sequences in the two orientations used for transfection assays are given under the diagram. The hatched box indicates the location of the tk promoter and the bent arrow refers to the transcription initiation site for the reporter CAT gene. The CAT gene vectors (15 µg) were transfected into CPAE, HeLa or COS 7 cells and CAT activity compared after 68 h. CPAE and COS 7 cells were co-transfected with pCH110 (2.5 µg) as an internal control for transfection efficiency. Aliquots of cellular extracts containing 50 β-galactosidase units for CPAE and COS 7 cells, or 20 µg of protein of HeLa cells, were assayed for CAT activity. The CAT activity of the transfected vector pBLCAT 2 with the only tk promoter was set at 100%. The CAT activities associated with the other vectors expressed relative to the activity of pBLCAT 2 are given on the right. pBLCAT 3 refers to the plasmid without a tk promoter. The results are the mean ± S.E.M. of four experiments.

upstream of the cap site. The constructs -1041/+19, -1199/+19 and -1240/+19 demonstrated striking inhibition of transcriptional activity in CPAE cells. Constructs upstream of -1240 restored transcription in CPAE cells. In contrast, cell lysates from Hep G2 and HeLa cells transfected with the same series of plasmids exhibited no detectable CAT synthesis, indicating that the upstream positive control elements were specific for CPAE cells (results not shown).

RNAase mapping

RNAase mapping was performed on total cellular RNA from CPAE cells transfected with selective pGvWCAT constructs to determine whether RNA transcription involved the same initiation site as the vWF gene (Figure 4). Protected RNA fragments from cells transfected with pGvWCAT constructs exhibited the expected size of approx. 292 bp when compared with DNA molecular-mass markers, demonstrating that transcription was initiating at the correct cap site. In addition, protected fragments were clearly evident with the -1286/+19 construct, while longer autoradiography times were needed to demonstrate transcripts with the -810/+19 and -1240/+19 constructs correlating to the relative CAT activity.

Influence of the vWF regulatory domains on the expression of the tk gene promoter

To analyse the effect of vWF gene regulatory domains on the expression of an ubiquitous heterologous promoter, four fragments were cloned in two orientations upstream of the tk gene promoter in the pBLCAT2 vector [28]. Representative CAT determinations with the pvWtkCAT vectors are given in Figure 5 and associated CAT activities relative to pBLCAT2 expression are listed in Figure 6. The construct -420/-89, which includes the negative regulatory domain directly upstream of the vWF basal promoter, decreased the CAT expression from the tk

promoter in all cell lines by more than 50%. Increasing the length of the fragment to -1240 bp restored baseline CAT activity. Enlarging the fragment by 46 additional bp to -1286 markedly enhanced, in CPAE cells, the transcriptional activity under control of the tk gene promoter and CAT gene expression increased almost 3-fold. This result corroborated the fact that the construct containing 1286 bp of flanking region also increased in a cell-specific manner the CAT activity directed by the vWF promoter (Figures 3 and 4). The enhancement, however, involved complex interactions because the fragment -1363/-1199, which contains 136 bp of the Alu sequence including the 46 bp segment, did not increase CAT expression driven by the tk gene promoter. In fact, when the -1363/-1199 construct was in the sense direction CAT activity in CPAE cells slightly diminished and in the antisense direction transcriptional activity fell by 50%. Modulation of tk gene promoter function by the -420/-89 and -1286/-89 constructs was orientation dependent which is observed frequently with the tk gene promoter [39].

DISCUSSION

The CPAE cells synthesized and secreted significant levels of vWF and were used as a model to study the endothelial-cell-specific expression of the vWF gene. Transient transfection assays with constructs containing progressive deletions of the vWF gene 5'-flanking sequences demonstrated both positive- and negative-regulatory regions in CPAE, COS 7, HeLa and Hep G2 cells. A proximal element was located within the first 89 bp upstream of the cap site and showed functional promoter activity in all cell types. Sequences within these 89 bp were necessary for transcription, as deletion of the entire 89 bp abolished CAT activity. The proper orientation was also important, as a construct with the fragment in the reverse direction was transcriptionally inactive. This area appeared to be a constitutive promoter element since it was required for activity

in all cells and includes a TAATTA sequence at position -32 and a putative CCAAT box at -18.

Transcriptional activity decreased in all cell types with constructs containing sequences further upstream of these proximal elements. CPAE cells appeared to maintain a basal level of CAT activity cumulating in a potential specific activating domain from 592 to 810 bp upstream of the cap site. Plasmids containing 1041 to 1240 bp of flanking sequence suppressed CAT activity in CPAE cells. Therefore, two areas in the 5'-flanking region of the vWF gene repressed the transcriptional activity directed by the proximal element. Negative regulation would be important to inactivate the vWF gene in non-expressing cells because the proximal element was functional in all cell types analysed. Constructs containing sequences immediately upstream of -1240 bp restored transcriptional activity in a cell-specific manner only in CPAE cells and at a level greater than or equal to that observed with the proximal element.

To confirm that transcription started at the expected site we analysed cellular RNA after transfection of CPAE cells with selected constructs by RNAase mapping. Transcripts of the predicted size were clearly detectable in cells transfected with constructs that showed CAT activity, demonstrating that the inserted sequences were indeed activating transcription from the vWF initiation site.

The element(s) within the region -89 to -420 bp in the flanking region that negatively modulated the activity of the vWF gene promoter also repressed the activity of the heterologous tk transcriptional unit in all cell types. This negative regulation was more apparent when the fragment was in the sense orientation. Although the transcriptional activity of the vWF promoter (-89/+19) was suppressed by a construct containing 1240 bp of flanking sequence, the insert -89 to -1240 had no effect on the activity of the tk gene promoter. Negative regulatory elements may have differential effects depending on the transcriptional strength of the promoter element [40].

The construct containing 1286 bp of flanking region enhanced the activity of the tk promoter only in CPAE cells. The same length of flanking sequence also increased in a cell-specific manner the CAT activity directed by the vWF promoter. Constructs containing these 1286 bp of flanking sequence are only 46 bp longer than the downstream constructs that suppressed CAT activity of the vWF promoter. These 46 bp are in the 5' end of an inverse human Alu element. The expression of the -1286/+19 construct only in CPAE cells correlates with the cell-specific expression of vWF that suggests the presence of cis-acting regulatory elements within the bovine vWF gene, which may be analogous to the human sequence. The positive effect not only depended on the 46 bp fragment but appeared to involve sequences downstream because the insert -1199 to -1363, which includes the fragment, did not increase the CAT activity directed by the tk gene promoter. Interestingly, when the -1199 to -1363 insert was in the reverse orientation an approx. 50% decrease in transcriptional activity occurred in all cell types. This effect could be due to the presence of a consensus T-antigen-binding site (-1222 to -1210 bp in the vWF gene flanking sequence) in Alu repeats orientated 5' → 3' that has been shown to repress CAT activity driven by the SV 40 promoter/*c-myc* enhancer in HeLa cells [41].

The analysis of the 5' region of the vWF gene suggests a complex interaction of *trans*-acting factors, with upstream sequences involved in both positive and negative regulation of vWF gene transcription. A recent abstract suggested that positive elements in the non-translated region may also play a role in the cell-specific expression of vWF in bovine aortic endothelial cells

[42]. It remains to be determined whether the positive regions upstream that we have described here act synergistically with the putative elements 3' of the transcription initiation site.

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REFERENCES

- Baumgartner, H. R., Tschopp, T. B. and Meyer, D. (1980) *Br. J. Haematol.* **44**, 127-139
- Weiss, H. J., Sussman, I. I. and Hoyer, L. W. (1977) *J. Clin. Invest.* **60**, 390-404
- Baruch, D., Bahnak, B. R., Girma, J. P. and Meyer, D. (1989) *Platelet Disorders*, (Caen, J. P., ed.), vol. 2, 627-672, Baillière Tindall-WB, London
- Lynch, D. C., Williams, R., Zimmerman, T. S., Kirby, E. P. and Livingston, D. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2738-2742
- Wagner, D. D. and Marder, V. J. (1984) *J. Cell Biol.* **99**, 2123-2130
- Fay, P. J., Kawai, Y., Wagner, D. D., Ginsburg, D., Bonthron, D., Ohlsson-Wilhelm, B. M., Chavin, S. I., Abraham, G. N., Handin, R. I., Orkin, S. H., Montgomery, R. R. and Marder, V. J. (1986) *Science* **232**, 995-998
- Levine, J. D., Harlan, J. M., Harker, L. A., Joseph, M. L. and Counts, R. B. (1982) *Blood* **60**, 531-534
- De Groot, P. G., Gonsalves, M. D., Loesberg, C., Van Buul-Wortelboer, M. F., Van Aken, W. G. and Van Mourik, J. (1984) *J. Biol. Chem.* **259**, 13329-13333
- Reinders, J. H., Vervoor, R. C., Verweij, C. L., Van Mourik, J. A. and De Groot, P. G. (1987) *J. Cell. Physiol.* **133**, 79-87
- Loesberg, C., Gonsalves, M. D., Zandbergen, J., Willems, C., Van Aken, W. G., Stel, H. V., Van Mourik, J. A. and De Groot, P. G. (1983) *Biochim. Biophys. Acta* **763**, 160-168
- Ribes, J. A., Feng, N., Wagner, D. D. and Francis, C. W. (1989) *J. Clin. Invest.* **84**, 435-442
- Mannucci, P. M., Aberg, M., Nilssen, I. M. and Robertson, B. (1975) *Br. J. Haematol.* **30**, 81-92
- Jaffe, E. A., Hoyer, L. W. and Nachman, R. L. (1973) *J. Clin. Invest.* **52**, 2754-2764
- Nachman, R. L., Levine, R. and Jaffe, E. A. (1977) *J. Clin. Invest.* **60**, 914-921
- Bahnak, B. R., Wu, Q. Y., Coulombel, L., Assouline, Z., Kerbiriou-Nabias, D., Pietu, G., Drouet, L., Caen, J. P. and Meyer, D. (1989) *J. Cell. Physiol.* **138**, 305-310
- Harrison, R. L. and McKee, P. A. (1984) *Blood* **63**, 657-665
- Dalton, R. G., Dewar, M. S., Savidge, G. F., Kernoff, P. B. A., Matthews, K. B. and Greaves, M. (1987) *Lancet* **1**, 1007-1009
- Ostermann, H. and Van De Loo, J. (1986) *Haemostasis* **16**, 386-416
- Mannucci, P. M., Lombardi, R., Bader, R., Horellou, M. H., Finazzi, G., Besana, C., Conard, J. and Samama, M. (1984) *Blood* **64**, 614-621
- Piovela, F., Giddings, J. P., Almasio, P., Ricetti, M. M. and Thomas, J. (1983) *Thromb. Res. (Suppl. 4)*, 69-73
- Assouline, Z., Kerbiriou-Nabias, D., Pietu, G., Thomas-Maison, N., Bahnak, B. R. and Meyer, D. (1988) *Biochem. Biophys. Res. Commun.* **153**, 1159-1166
- Collins, C. J., Underdahl, J. P., Levene, R. B., Ravera, C. P., Morin, M. J., Dombalagian, M. J., Ricca, G., Livingston, D. M. and Lynch, D. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4393-4397
- Bonthron, D. and Orkin, S. H. (1988) *Eur. J. Biochem.* **171**, 51-57
- Mancuso, D. J., Tuley, E., Westfield, L. A., Worrall, N. K., Shelton-Inloes, B. B., Sorace, J. M., Alevy, Y. G. and Sadler, J. E. (1989) *J. Biol. Chem.* **264**, 19514-19527
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) *Cell* **39**, 653-662
- Frebourg, T. and Brison, O. (1988) *Gene* **65**, 315-318
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Luckow, B. and Schutz, G. (1987) *Nucleic Acids Res.* **5**, 5490
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467
- Del Vecchio, P. J. and Smith, J. R. (1981) *J. Cell. Physiol.* **108**, 337-345
- Hall, C. V., Jacob, P. E., Ringold, G. M. and Lee, F. (1983) *J. Mol. Appl. Genet.* **2**, 101-109
- Crabb, D. W. and Dixon, J. E. (1987) *Anal. Biochem.* **163**, 88-92
- Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044-1051
- Nishino, M., Girma, J. P., Rothschild, C., Fressinaud, E. and Meyer, D. (1989) *Blood* **74**, 1591-1599

-
- 35 Bakhshi, M. R. and Kirby, E. P. (1990) *Thromb. Haemostasis* **63**, 517–523
- 36 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 37 Binnie, C., Wagner, R. H., Cooper, H. and Hermans, J. (1985) *Thrombosis Res.* **40**, 523–532
- 38 Herbomel, P. and De Crombrughe, B. (1983) *Cold Spring Harbor Conferences on Cell Proliferation* 10, Teratocarcinoma Stem Cells, pp. 285–294, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 39 Rousseau, S., Renaud, S. and Ruiz-Carillo, A. (1989) *Nucleic Acids Res.* **17**, 7495–7511
- 40 Baniahmad, A., Muller, M., Steiner, C. H. and Renkawitz, R. (1987) *EMBO J.* **6**, 2297–2303
- 41 Tomilin, N. V., Iguchi-Arigo, S. M. M. and Ariga, H. (1990) *FEBS Lett.* **263**, 69–72
- 42 Jahroudi, N., Hague, Z. and Lynch, D. C. (1991) *Blood* **78**, Suppl-178a
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