

Differential Regulation of Vitronectin in Mice and Humans *in Vitro**

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To define the cis-acting elements involved in the regulation of the murine vitronectin (Vn) gene in inflammation, the 5'-flanking region was isolated, fused to the luciferase reporter gene, and the basal and interleukin 6 (IL-6)-stimulated transcriptional activity was tested in transfection experiments using Hep3B cells. Treatment with IL-6 induced this construct by more than 20-fold, whereas the corresponding 5'-flanking region of the human Vn gene was not stimulated. Transfection studies using murine Vn constructs with serial 5'-deletions revealed that two sequences were important in the IL-6 response, and specific mutations in both sequences abolished the response. A 2-base pair mutation converted the human sequence to that of a murine IL-6 responsive element and partially conveyed IL-6 inducibility. In contrast, transforming growth factor β stimulated the human construct and the endogenous Vn gene in human Hep3B cells in a dose-dependent manner, whereas the murine construct was not responsive. The transforming growth factor β responsive region was localized to a 30-base pair fragment with little homology to the murine sequence. These studies reveal that the structural basis for the differential regulation of the human and murine Vn genes resides in the differences in promoter sequence.

Vitronectin (Vn)¹ is not only a member of a group of cell adhesion molecules that mediate adhesion through a common Arg-Gly-Asp containing sequence but also appears to regulate proteolytic enzyme cascades, including the complement, coagulation, and fibrinolytic systems (1–3). Thus, Vn may provide a unique regulatory link between cell adhesion, humoral defense mechanisms, and cell invasion (1).

Although Vn biosynthesis was originally only detected in hepatocytes (4), recent studies provide evidence that low but significant levels are also expressed in normal and diseased extra-hepatic tissues (5–7). Vitronectin biosynthesis also ap-

pears to be regulated. Transforming growth factor β (TGF β) modulates Vn gene expression in the human hepatocarcinoma cell line HepG2 (8), and rodent Vn genes are regulated by lipopolysaccharide during acute systemic inflammation (7, 9). Endotoxin induces a number of cytokines *in vivo*, including TNF α , IL-1, and IL-6 (10–12). Interestingly, Vn gene expression in the rat liver was induced within 1 h after intraperitoneal injection of purified rat IL-6 (9), suggesting that rodent Vn genes are directly responsive to IL-6. These latter observations identify Vn as an acute phase reactant.

Little is known about the Vn promoters and their regulation. The immediate 5'-flanking region of the human Vn gene has been isolated, and the 5'-end of the mRNA was determined in primer extension experiments (13). However, this fragment lacked a TATA box consensus sequence at the expected distance from the cap site, and the transcriptional activity of this fragment was not tested (13). Thus, it is unclear whether this sequence contained a functional promoter.

In view of these recent results, a detailed comparative analysis of the Vn promoter of the human and murine genes was performed. IL-6 stimulated the transcriptional activity of the murine but not of the human promoter, while TGF β specifically up-regulated the human but not the murine promoter. Evidence is presented that the two promoters contain different cis-acting elements that account for these differences in regulation.

MATERIALS AND METHODS

Isolation of the Vn 5'-Flanking Regions

Murine Vn—A genomic liver library from BALB/c mice in EMBL3 (Clontech) was screened by standard colony hybridization using the cloned Vn cDNA as a probe (4, 14). Phage DNA from positive plaques was analyzed by Southern blotting (15) after digestion with a number of restriction endonucleases. A 2.2-kilobase *Bam*HI-*Sa*II fragment was identified that hybridized only with a probe derived from the 5'-end of the Vn cDNA (bases 1–606; numbering according to Ref. 4) and subcloned into pUC18. Sequence analysis of both DNA strands was performed directly from double-stranded DNA using M13 universal- and custom-made primers by a modification of the dideoxy chain termination procedure with a Sequenase kit (U. S. Biochemical Corp.). The authenticity of the sequence was confirmed by Southern blotting using mouse genomic kidney DNA, and probes were derived from the 5'-flanking region and the 5'-end of the cDNA (not shown). The transcription start site was determined by primer extension analysis as described (15) using liver RNA isolated from BALB/c \times C57B1/6F1 mice (Scripps Clinic Rodent Breeding Colony) and the following end-labeled antisense oligonucleotide (base pairs 104–69): 5'-GGGTGCCATGGCAGGCTCTAGCCCCAGGGCCTTGGC-3'. In control experiments, liver RNA either was replaced by tRNA or no RNA was added. A number of extended fragments ranging in size from 93 to 104 bases were detected; the major cap sites appear to be two adenosines 84 and 95 nucleotides upstream of the methionine initiation codon (not shown).

Human Vn—The 5'-flanking region of the human Vn gene was PCR amplified (see below) from genomic DNA isolated from Hep3B cells or from a 9.4-kilobase *Bam*HI-*Bam*HI genomic Vn clone in pUC 19 (13). The sequence of the Hep3B-derived DNA was identical to the reported sequence (13).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X72091.

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¹ The abbreviations used are: Vn, vitronectin; IL-1, interleukin 1; IL-6, interleukin 6; TGF β , transforming growth factor β ; TNF α , tumor necrosis factor α ; PCR, polymerase chain reaction.

TABLE I
Oligonucleotides

Oligonucleotides were synthesized on a PCR-Mate EP model 391 DNA synthesizer (Applied Biosystems). Panels A and B, sequences of the PCR primer used for the human and murine Vn promoter constructs, respectively. The sequences of the 5'-PCR primer used to generate the intermediate products in the two-sided overlap PCR mutagenesis technique are indicated. The second intermediate product was obtained using a reverse and complementary PCR primer.

Vn constructs	Sequences
Human	
Upstream (5') PCR-primer	
1079 /1652	5' GAA ATG AAG CTT TGT TCT TTT ATG CTA 3'
1179 /1652	5' GAA ATG AAG CTT GCA CCC AGC CAG GGA TGT G 3'
1190 /1652	5' GAA ATG AAG CTT GGG ATG TGT CTG ATC AAA CA 3'
1212 /1652	5' GAA ATG AAG CTT CAT GGA TCA ATA GCT ATG TTTG 3'
1245 /1652	5' GAA ATG AAG CTT TGT GGC TGC TCC AGC TAC TGG 3'
1279 /1652	5' GAA ATG AAG CTT TCC AGT TCA TGT AAT CTC C 3'
1379 /1652	5' GAA ATG AAG CTT ATG AGC CTT CTT TCT GCC 3'
Downstream (3') PCR-primer	
	5' AAT TGT CAG GTA CCG AAG TCT CCG CTC TGA 3'
Site-directed mutagenesis	
	5' GGC CAG GCC CAG TGT TCT GGG AAT GTG ACC TTT GCT 3'
Murine	
Upstream (5') PCR-primer	
-528 /47	5' GAA ATG AAG CTT TGT TCT TTT ATG AG 3'
-428 /47	5' GAA ATG AAG CTT GGG GGT AGG ATG GGA CAA GC 3'
-403 /47	5' GAA ATG AAG CTT AGC CTG ATC AAA CAG TAA 3'
-399 /47	5' GAA ATG AAG CTT TGA TCA AAC AGT AAA TCG A 3'
-396 /47	5' GAA ATG AAG CTT TCA AAC AGT AAA TCG ATA ACG 3'
-393 /47	5' GAA ATG AAG CTT AAC AGT AAA TCG ATA ACG G 3'
-383 /47	5' GAA ATG AAG CTT CGA TAA CGG CTT ATG GGA ATG 3'
-378 /47	5' GAA ATG AAG CTT ACG GCT TAT GGG AAT GAG 3'
-373 /47	5' GAA ATG AAG CTT TTA TGG GAA TGA GTT GGA AGC 3'
-353 /47	5' GAA ATG AAG CTT CTA CTC CAG GCA CGA AAC 3'
-328 /47	5' GAA ATG AAG CTT CTG TTC TAG CTC ACG GAA TC 3'
-228 /47	5' GAA ATG AAG CTT GAG GGT GAC AAC ACA TGC TT 3'
-100 /47	5' GAA ATG AAG CTT CTG GGA AAT GTG ACC CT 3'
-91 /47	5' GAA ATG AAG CTT GTG ACC CTT TGC TGC AGC 3'
Downstream (3') PCR-primer	
	5' CAG GCA GGA CAG CGA GAT TCG GTA CCT GAC AATT 3'
Upstream (5') Mutagenic PCR-primer	
Mutant -100 to -92	5' GCT CTC CTG GGG CTG GGG CGT TGA GAT TGT GAC CCT TTG CTG CAG 3'
Mutant -399 to -391	5' ATG GGA CAA GCA CCT AGC CAA GAA TTC CAG TAA ATC GAT AAC GGC 3'

Reporter Gene Constructions

Specific fragments of the Vn promoters and 5'-flanking regions were amplified for 29 cycles (55 °C, 30 s; 72 °C, 1 min; 94 °C, 30 min) in 32 mM Tris-HCl (pH 8.3) containing 54 mM KCl, 4 mM MgCl₂, 0.34 mM dithiothreitol, 0.48 mM each dNTP, and 50 pmol each of upstream and downstream PCR primer (see Table I), followed by a final extension at 72 °C for 5 min. The firefly luciferase reporter gene plasmid (p19LUC; Ref. 16) was digested with the restriction endonucleases *Hind*III and *Kpn*I, gel-isolated, and ligated to the *Hind*III-*Kpn*I-digested PCR product. Mutagenesis of DNA sequences was performed by PCR using the two-sided overlap technique (17). Briefly, the intermediate products were obtained by PCR amplification as above and gel-isolated from 2% low melting point agarose gels. An equimolar amount of the two PCR products (0.1% of the DNA from the first round of amplification) was PCR amplified using 50 pmol each of upstream and downstream PCR primer and ligated into p19LUC as above. The nucleotide sequences of the relevant, fused PCR products were determined directly from double-stranded plasmid DNA using internal sequencing primers. Plasmid DNA for transfection studies was isolated either using anion exchange chromatography with a Qiagen pack 500 kit (Qiagen, Studio City, CA) or by equilibrium centrifugation in CsCl-ethidium bromide gradients (15). Similar transfection efficiencies were obtained using plasmid DNA isolated by either method.

Cell Culture and Luciferase Assays

Hep3B human hepatoma cells obtained from ATCC (HB8064) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Bio Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin (Bio Whittaker). For transfection experiments, semiconfluent cells in 6-well (10 cm² per well) tissue culture plates (Corning) were washed twice with serum-free media (Dulbecco's modified Eagle's medium/Ham's F-12) and then incubated in serum-free media. A mixture containing Lipofectin (Life Technologies, Inc.; 15 µg/well) and DNA constructs (2.5 µg/well in water) was

added to each well (50 µl/well), and the plates were incubated for 18 h. After lipofection, the cells were incubated an additional 24 h with various agonists including human recombinant IL-6 (Boehringer Mannheim; 1138600), human recombinant IL-1α (Sigma; I-6011), and TGFβ (a generous gift of Berlix Biosciences, South San Francisco, CA). As a control, cells were also treated with lipopolysaccharide (1 µg/ml, Sigma; L-2630), which failed to induce the transcriptional activity of the Vn reporter gene constructs. This rules out stimulatory effects of endotoxin contamination potentially present in the cytokine preparations. The monolayers were washed and then extracted into 0.1 M KPO₄, pH 7.8, containing 0.25% (v/v) Triton X-100 and 1 mM dithiothreitol, and then assayed for luciferase activity as described (16) by using a Monolight 2001 luminometer (Analytical Luminescence, San Diego, CA). For each experiment, at least three independent transfections were performed with every construct. The -fold induction calculated for the figures and tables represents the results from a single representative experiment. For some experiments, the Vn promoter constructs were cotransfected with the pCMVβ reporter gene plasmid (1 µg DNA/well; Clontech), a vector designed for the expression of β-galactosidase as transcribed by the cytomegalovirus promoter. β-Galactosidase activity was detected with the Galacto-Light chemiluminescent reporter assay system according to the manufacturer (Tropix Inc., Bedford, MA).

Vn Antigen Concentration

Hep3B cells were cultured as above and treated either with IL-6 (500 units/ml) or the indicated concentrations of TGFβ (see Fig. 2). The conditioned medium was harvested 24 h later, and the Vn concentration was determined by a sandwich enzyme-linked immunosorbent assay.² Briefly, a purified monoclonal antibody (clone 17.26) to Vn was coated onto microtiter plates, and after washing and blocking, samples were incubated for 1 h in the wells. Bound Vn was detected using rabbit anti-human Vn IgG followed by biotin-labeled goat anti-rabbit IgG, streptavidin alkaline phosphatase conjugate, and *p*-nitrophenyl phos-

² D. Seiffert, unpublished results.

phate. Results of duplicate wells were averaged and corrected for absorbance at 405 nm from wells not incubated with the conditioned media but otherwise treated identically. The assay was linear from 10 to 400 ng/ml using purified Vn (18) as a standard. For some experiments, cells were washed with methionine-deficient media, treated with the indicated agents (TGF β , 1 ng/ml; IL-6, 500 units/ml), and labeled for 24 h with [³⁵S]methionine (100 μ Ci/ml) in methionine-deficient media supplemented with 10% methionine-containing growth media. The conditioned media, cell lysates, and extracellular matrix were prepared as described (19). Metabolically labeled Vn was isolated by immunoprecipitation using monoclonal antibody 1224 as described (19, 20). The precipitated proteins were fractionated on 9% SDS-polyacrylamide gel electrophoresis under reducing conditions. An autoradiogram was used to localize Vn in the gel, and these regions were excised and subjected to liquid scintillation counting. The experiment was performed in duplicate wells on two different occasions, and representative results are presented in Fig. 2.

RESULTS

In the present study, the immediate 5'-flanking region of the murine Vn gene was isolated and sequenced (not shown). The major cap sites were determined by primer extension analysis and were located at two adenosine residues 84 and 95 nucleotides upstream of the methionine initiation codon (not shown). The adenosine representing the most distal transcription start site (*i.e.* 95 nucleotides upstream of the initiation codon) was designated as +1. The sequence data indicated that the murine Vn gene resembles the majority of eukaryotic genes in that a "TATA box" (position -30 to -24) is located at the conserved distance from the cap site (21).

To establish the transcriptional activity of the 5'-flanking region of the murine Vn gene, a 575-base pair fragment (-528/47) was amplified by PCR and cloned upstream of the luciferase reporter gene. The sequence of the murine 5'-flanking region also was aligned with that of the human sequence, and the corresponding human DNA fragment (1079/1652; numbering according to Ref. 13) was PCR amplified from Hep3B genomic DNA, cloned directly in front of the luciferase reporter gene, and sequenced (compare Fig. 6). The transcriptional activities of the resulting constructs were compared to the parental reporter gene construct (p19LUC; Ref. 16) after transfection into human Hep3B hepatoma cells. Cell lysates were prepared 48 h after transfection and analyzed for luciferase activity. The β -galactosidase gene, driven by the cytomegalovirus promoter, was cotransfected with the luciferase reporter gene constructs to correct for differences in DNA uptake. The promoterless reporter gene plasmid (p19LUC) produced barely detectable luciferase activity, whereas both Vn promoter constructs exhibited promoter activity approximately 300-fold above background in Hep3B cells (not shown). These results indicate that the immediate 5'-flanking regions of both genes contain a functional, active promoter. The transcriptional activity of these Vn reporter gene constructs agrees with the observation that the endogenous human Vn gene is expressed by this cell line (19).

Experiments were performed to characterize the effects of a number of cytokines and growth factors on the transcriptional activity of the Vn genes. 1 day after transfection, the cells were incubated for 24 h in the presence of the indicated agents, and cell lysates were prepared and analyzed for luciferase activity. The results were compared to those for the Rous sarcoma virus long terminal repeat promoter (Table II). The transcriptional activity of the murine Vn promoter was strongly up-regulated by IL-6, supporting the notion that the murine Vn gene is regulated as an acute phase reactant (Table II). Both the basal and the IL-6-induced transcriptional activities of the murine Vn promoter were reduced by IL-1 (Table II) and TNF α (not shown), and this down-regulation was dose-dependent with respect to the concentration of IL-1 and TNF α employed (not shown). Surprisingly, the transcriptional activity of the human

TABLE II
Relative transcriptional activity of human and murine Vn and Rous sarcoma virus (RSV) "long terminal repeat" constructs in Hep3B cells after treatment with various agents

Reporter gene constructs containing the 5'-flanking regions of the human Vn gene (1079/1652), the murine Vn gene (-528/47), or the Rous sarcoma virus long terminal repeat promoter (29) were transiently transfected into Hep3B hepatoma cells using Lipofectin. The cells were then treated with the indicated agents for 24 h, and the cell lysates were prepared and assayed for luciferase activity as described under "Materials and Methods." The -fold increase in luciferase activity following treatment is presented in comparison to untreated cells. The following agonist concentrations were used: IL-6, 500 units/ml; TGF β , 1 ng/ml; IL-1 α , 50 units/ml.

Treatment	Relative luciferase activity		
	Human Vn	Murine Vn	RSV
Control	1	1	1
TGF β	29	2	1
IL-6	2	35	1
IL-1	0.9	0.4	1
TGF β + IL-1	29	1	2
IL-6 + IL-1	4	7	2

Vn promoter was not stimulated by IL-6 (Table II). In contrast, the human Vn promoter was up-regulated by TGF β , whereas this growth factor was ineffective on the murine Vn promoter (Table II). Also, IL-1 (Table II) and TNF α (not shown) did not suppress the stimulatory effect of TGF β on the human Vn promoter. The results for the Vn promoters were compared to that for the human type 1 plasminogen activator inhibitor gene, a typical TGF β -responsive and acute phase gene (22). A DNA fragment of the human type 1 plasminogen activator inhibitor gene (-800/75) was strongly stimulated (150-fold) by TGF β (1 ng/ml) as previously reported, whereas the response to IL-6 (500 units/ml; 2-fold induction) and a combination of IL-6 and dexamethasone (500 units/ml IL-6, 10⁻⁵ M dexamethasone; 6-fold induction) was rather limited in comparison to the murine Vn gene.

The differential regulation of the Vn promoters by IL-6 and TGF β was compared in dose-response experiments (Fig. 1). The transcriptional activity of the murine Vn promoter was up-regulated by IL-6 in a dose-dependent manner, with half-maximal stimulation at approximately 250 units/ml (Fig. 1A). In contrast, IL-6 has only a minimal effect on the human Vn promoter (Fig. 1A) or the Rous sarcoma virus promoter (not shown). In contrast, TGF β stimulated the transcriptional activity of the human Vn promoter in a dose-dependent manner, with half-maximal stimulation at approximately 0.3 ng/ml (Fig. 1B). The murine Vn promoter was not stimulated by TGF β concentrations up to 10 ng/ml (Fig. 1B).

These results raised the possibility that either the human and murine endogenous Vn genes are differentially regulated or important regulatory elements (*e.g.* more distal DNA fragments, intron or exon sequences) that were not contained within the reporter gene constructs are required for the regulation. To discriminate between these two possibilities, the regulation of the endogenous human Vn gene was studied in human hepatoma cells. TGF β stimulated the accumulation of Vn in the conditioned media of Hep3B cells in a dose-dependent manner, whereas IL-6 (500 units/ml) had no effect (Fig. 2). The same was true using IL-6 concentrations up to 5,000 units/ml (not shown). Similar results were obtained when radiolabeled Vn was immunoprecipitated using a monoclonal antibody specific for human Vn (Fig. 2, *inset*). Quantification of the radioactivity of the Vn containing bands revealed that IL-6 reduced Vn biosynthesis by approximately 25%, whereas TGF β stimulated the accumulation of Vn in the conditioned medium approximately 3-fold (not shown). In contrast, we were unable to immunoprecipitate radiolabeled Vn from the extracellular ma-

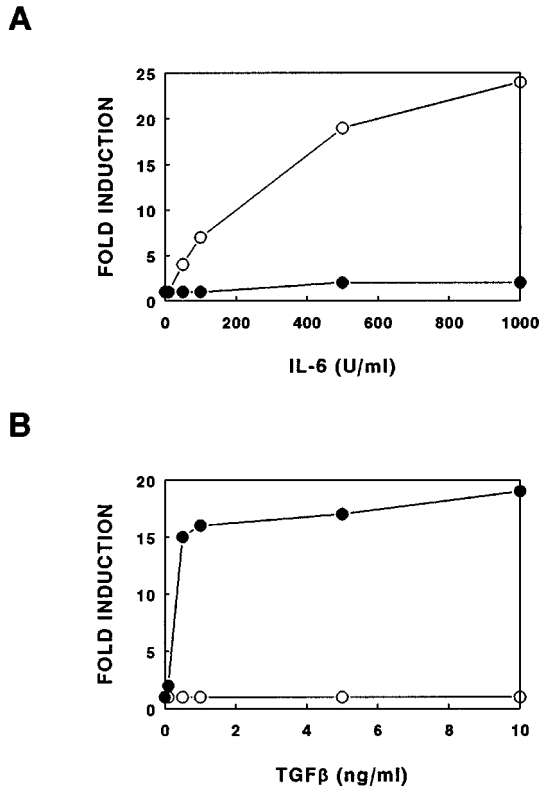


FIG. 1. Regulation of the Vn promoters by IL-6 and TGF β . Constructs containing either the murine Vn (-528/47, open circles) or the human Vn (1079/1652, closed circles) promoters were transfected into Hep3B cells. The cells were treated with the indicated concentration of IL-6 (panel A) or TGF β (panel B) for 24 h and assayed for luciferase activity (see "Materials and Methods"). The -fold induction is plotted on the y axis (linear scale) against the concentration of cytokine or growth factor on the x axis (linear scale).

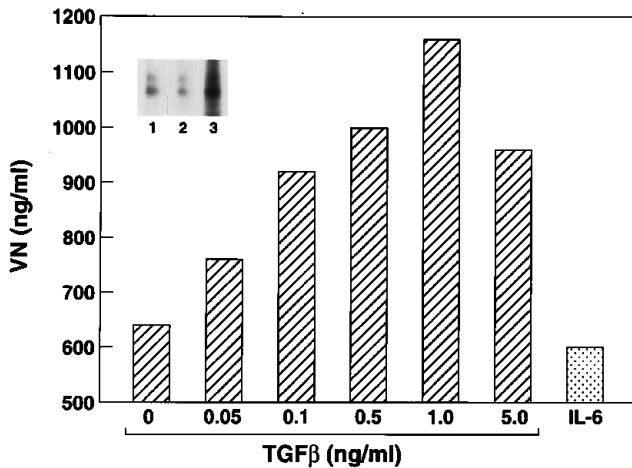


FIG. 2. Regulation of the endogenous Vn gene in Hep3B cells by IL-6 and TGF β . Confluent Hep3B cells were serum-starved for 24 h and incubated with the indicated concentration of TGF β or IL-6 (500 units/ml) for an additional 24 h. The accumulation of Vn in the conditioned medium was determined by a sandwich enzyme-linked immunosorbent assay (see "Materials and Methods"). Inset, Hep3B cells were serum-starved for 24 h and metabolically labeled with [35 S]methionine for an additional 24 h in serum-free medium (lane 1) or in medium containing either IL-6 (500 units/ml, lane 2) or TGF β (1 ng/ml, lane 3). The radiolabeled Vn was collected by immunoprecipitation using monoclonal antibody 1244 raised against human Vn and protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

trix (not shown), indicating that the majority of the synthesized Vn accumulated in the conditioned medium. The metabolic activity of the TGF β -stimulated wells was determined by tri-

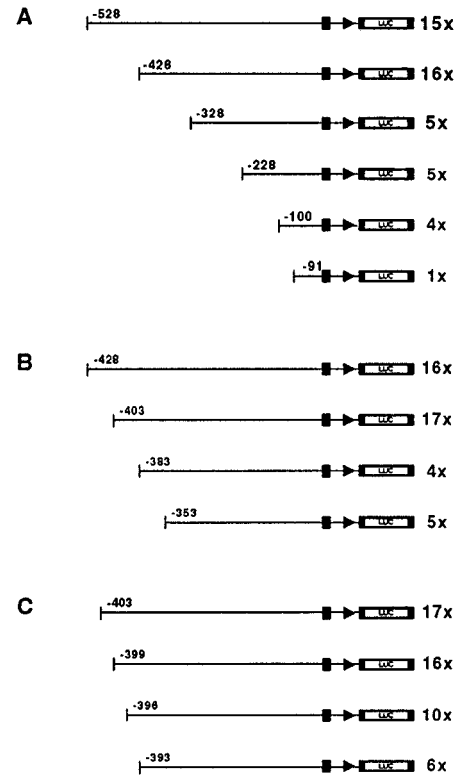


FIG. 3. Deletional analysis of IL-6 responsive elements as shown by stimulation of the murine Vn promoter by IL-6. Panel A, a series of progressive 5'-deletions were prepared between -528 and -91. Panel B, A 5'-deletion series between -428 and -353 was prepared. Panel C, A more detailed analysis of the active region (-403 to -393) is shown. The resulting constructs were transfected into Hep3B cells and assayed for luciferase activity following treatment with 500 units/ml IL-6 (see "Materials and Methods"). The 5' boundaries are indicated to the left of each construct. The -fold increase in luciferase activity following IL-6 treatment is presented to the right of each construct. The solid box represents the "TATA" box at position -24. The arrow indicates the location and direction of transcriptional initiation at position 1.

chloroacetic acid precipitation of radiolabeled proteins and was not significantly (*i.e.* less than 30% variation) different from that of untreated or IL-6-treated cells (not shown). It should be noted that TGF β , but not IL-6, stimulated the accumulation of Vn in the conditioned medium of human HepG2 cells approximately 2-fold, consistent with previous reports (8). These results clearly indicate that the endogenous human Vn gene and the human promoter construct studied are regulated in a similar fashion.

Experiments were performed to specifically localize the region(s) in the Vn promoters that mediate inducibility in Hep3B cells. In the first set of experiments, Hep3B cells were transfected with a series of overlapping 5'-deletion constructs of the murine Vn promoter and then incubated for 24 h in the presence of 500 units/ml IL-6. Cell lysates were prepared and analyzed for increased luciferase activity (Fig. 3). Initial studies implicated two areas in the regulation by IL-6 (Fig. 3, panel A), a distal element located between -428 and -328, and a proximal element between -100 and -91. The latter sequence contains a C/EBP consensus sequence (see also Fig. 7, top panel). To more precisely define the distal IL-6 responsive sequence, several progressive 5'-deletions were made through this region (Fig. 3, panels B and C). When sequences between -403 and -383 were deleted, the level of IL-6 induction fell from 16-fold to 4-fold. The remaining level of induction was still constant in deletions to -353 (Fig. 3, panel B). To further define this distal IL-6-responsive element, the sequence be-

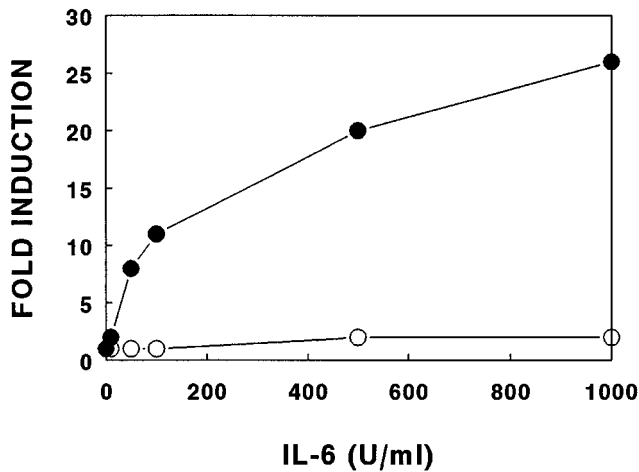


FIG. 4. **Mutational analysis of the IL-6 responsive elements in the murine Vn promoter.** The regions encompassing the proximal (–100/–92) and distal (–399/–391) IL-6 responsive elements were scrambled by site-directed mutagenesis in the intact Vn promoter (–528/47) and assayed for luciferase activity following treatment with the indicated concentration of IL-6. The -fold induction is plotted on the y axis (linear scale) against the concentration of IL-6 on the x axis (linear scale). *Closed circles*, wild-type murine Vn promoter; *open circles*, mutations in both the proximal and distal IL-6 responsive elements.

tween –403 and –383 was scanned by deleting 3–4 bases at a time (Fig. 3, *panel C*). Constructs containing 5'-deletions to –399 retained full IL-6 inducibility, whereas further deletion to –393 progressively reduced the -fold induction to 6-fold (Fig. 3, *panel C*). These results suggest that the distal IL-6 regulatory sequence is located between –399 and –393. To confirm these results in the context of the intact promoter (–528/47), both regulatory sequences were scrambled by site-directed mutagenesis. IL-6 dose-response experiments revealed that the IL-6 inducibility of the murine Vn promoter was drastically reduced (Fig. 4). These results indicate that at least two different sequences, located between –399/–393 and –100/–92, are involved in the regulation of the murine Vn gene by IL-6.

In the second set of experiments, a series of 5'-overlapping deletion constructs of the human Vn promoter were transfected into Hep3B cells and then incubated for 24 h with 1 ng/ml TGF β . These initial studies implicated the region between 1179 and 1279 in the regulation by TGF β (Fig. 5, *panel A*). The responsive sequence was further defined by several progressive 5'-deletions. An initial 2-fold loss in inducibility was observed by deletion to 1190, and further deletion to 1212 abolished the majority of the TGF β response (Fig. 5, *panel B*). Inspection of the sequence revealed no evidence for any reported TGF β responsive elements. The results are summarized in Fig. 6.

Alignment of the murine Vn promoter sequence (–528/47) with that of the corresponding human gene (1079/1652) revealed that the overall sequence identity is 65%. Notably, the upstream IL-6 responsive sequence was also present in the human Vn promoter, whereas the downstream sequence contained two mismatches in the center of the recognition sequence (Fig. 7, *top panel*). Site-directed mutagenesis was employed to convert the human downstream sequence into the murine sequence. The resulting reporter gene construct was transiently transfected into Hep3B cells, and the luciferase activity was compared to the wild-type human and murine promoters after treatment with IL-6 (Fig. 7). The mutant human Vn promoter was stimulated by IL-6 in a dose-dependent manner, indicating the importance of the downstream IL-6 responsive element in a heterologous promoter.

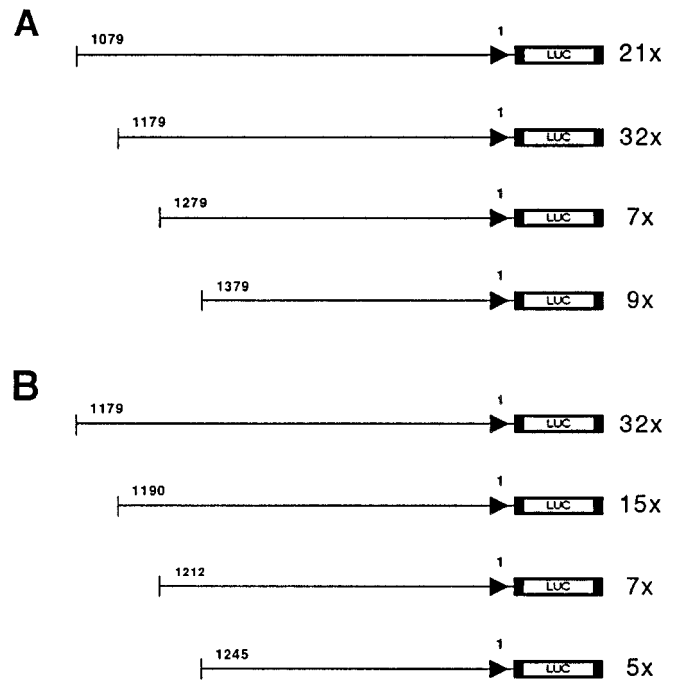


FIG. 5. **Deletional analysis of the Vn promoter and 5'-flanking region as shown by stimulation of the human Vn promoter by TGF β .** *Panel A*, a series of progressive 5'-deletions were prepared between 1079 and 1379. *Panel B*, a more detailed analysis of the region between 1179 and 1245 is depicted. The resulting constructs were transfected into Hep3B cells and then assayed for luciferase activity following treatment with 1 ng/ml TGF β (see "Materials and Methods"). The 5' boundaries are indicated to the left of each construct. The -fold increase in luciferase activity following TGF β treatment is presented to the right of each construct. The arrow at position 1 indicates the location and direction of transcription initiation.

murine Vn



human Vn



FIG. 6. **Schematic alignment of the murine and human Vn promoters and 5'-flanking regions.** The solid boxes in the upper panel (murine Vn) represent the two IL-6 responsive elements, whereas the solid box in the lower panel (human Vn) represents the TGF β responsive element. The arrows indicate the direction of transcriptional initiation. The 5' and 3' boundaries of the full-length promoter constructs are indicated. The numbering of the murine construct is based on the experimentally determined transcriptional start sites, and the adenosine representing the most distal transcription start site was designated as +1. The numbering of the human construct is based on the published sequence (13).

DISCUSSION

Although the human and murine Vn proteins are functionally indistinguishable (4), we provide evidence that the two 5'-flanking regions fused to a reporter gene are regulated quite differently. Specifically, IL-6 stimulated the murine construct in a dose-dependent manner but was ineffective when the human construct was studied. In contrast, the human but not

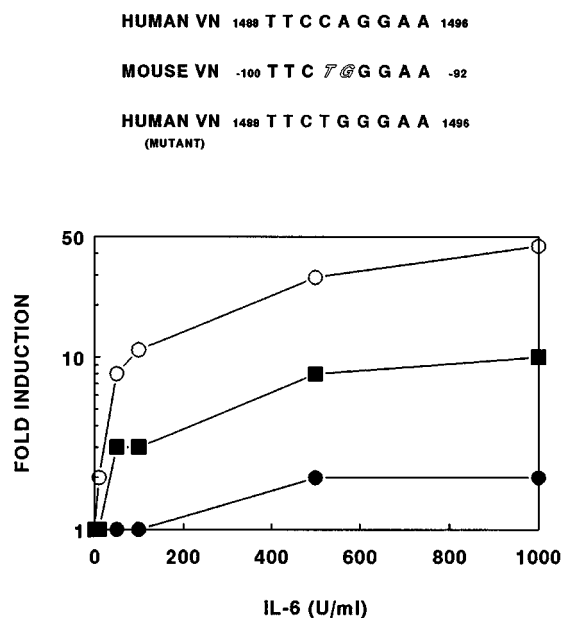


FIG. 7. **Introduction of IL-6 responsiveness to the human Vn promoter.** Two adjacent single-point mutations (position 1491 C to T, 1492 A to G) were introduced into the human Vn promoter by site-directed mutagenesis (see "Materials and Methods"). The resulting construct was transfected into Hep3B cells and then assayed for luciferase activity following treatment with the indicated concentration of IL-6 (filled squares). The -fold induction was compared to the wild-type mouse (open circles) and wild-type human Vn promoters (closed circles), respectively. The -fold induction is plotted on the y axis (logarithmic scale) against the concentration of IL-6 on the x axis (linear scale).

the murine construct was strongly stimulated by TGF β . A number of observations support the notion that the endogenous genes and the reporter gene constructs are regulated in a similar fashion. First, TGF β stimulated the accumulation of Vn in the conditioned medium of Hep3B cells in a dose-dependent manner (Fig. 2), consistent with recent reports on HepG2 cells, another hepatocarcinoma cell line (8). It should be noted that in the latter study, the steady-state level of Vn mRNA increased upon stimulation with TGF β (8), indicating that the stimulation of Vn biosynthesis by TGF β is, at least in part, on the transcriptional level. This observation is consistent with preliminary results that TGF β but not IL-6 increases the steady-state level of Vn mRNA in Hep3B cells approximately 2-fold (not shown). In addition, IL-6 failed to elicit stimulation of the endogenous Vn gene in Hep3B cells (Fig. 2), consistent with the lack of effect on the reporter gene construct (Fig. 1). Thus, the endogenous human Vn and the human Vn promoter reporter gene construct appear to be regulated in a similar fashion. Second, the endogenous murine and rat Vn genes are stimulated in acute inflammation *in vivo* (7, 9) and purified IL-6-induced rat hepatic Vn gene expression within 1 h after injection, suggesting a direct effect on the transcriptional activity of rodent Vn genes (9). In agreement with these observations, the murine Vn promoter fused to the luciferase reporter gene construct was strongly induced by IL-6 (Table II and Fig. 1). These results indicate that with respect to IL-6 induction, the murine reporter gene construct and the endogenous gene are regulated in a similar fashion. We attempted to confirm these results using a number of established murine hepatoma cell lines. Unfortunately, none of the cells studied expressed Vn. Information regarding the regulation of the endogenous murine Vn gene by TGF β is not available. However, the identification of a TGF β responsive sequence in the human Vn promoter construct and the lack of the corresponding sequence in the murine Vn promoter (see below) would predict that the endogenous

murine Vn gene is not stimulated by TGF β .

Acute phase genes fall into two categories. Type 1 acute phase genes require IL-1 and IL-6 for maximal stimulation, whereas induction of type 2 acute phase genes requires only IL-6 (23). The observation that maximal stimulation of the murine Vn gene is achieved by IL-6 alone (Table II), whereas a combination of IL-1 and IL-6 (Table II) or TNF α and IL-6 (not shown) actually leads to a down-regulation of the IL-6 induction, suggests that Vn is a type II acute phase protein.

The molecular basis for the differential regulation of the human and murine Vn genes was elucidated. The studies summarized in Figs. 3 and 4 demonstrate that the response of the murine Vn promoter to IL-6 is contained within two separate regions. This conclusion is based on both 5'-deletional analysis of these regions and scrambling of the respective sequences by site-directed mutagenesis in the context of the whole promoter. The proximal sequence at -100 to -92 is identical to the IL-6 responsive transcription control element present in several acute phase genes, including fibrinogen and C-reactive protein (consensus sequence (T/A)T(C/G)TGGGA(A/T), Ref. 24). Thus, it is likely that NF IL-6 binds to this sequence, leading to an up-regulation of Vn gene expression. The second sequence (-399/-391) shares only 3 bases with this consensus sequence, raising the possibility that transcription factor(s) distinct from NF IL-6 may bind to this site. Clearly, additional studies are required to identify the nature of the transcription factor(s) that interact with each site. The importance of the proximal sequence was confirmed with a heterologous promoter (*i.e.* the human Vn promoter) that lacked IL-6 responsiveness.

The TGF β responsive sequence was localized to a 33-base pair stretch between 1179 and 1212, and this sequence appears to be unrelated to previously described TGF β responsive elements. The human and murine 5'-untranslated sequences were aligned, and while the overall identity was 65%, the sequence between 1179 and 1212 was only 45% conserved, and no continuous stretch with more than 3-base pair matches was detected. This comparison thus strongly suggests that the TGF β responsive element is not contained within the murine Vn promoter.

The physiological significance of the regulation of the Vn genes remains to be elucidated. The homeostatic response of the body to trauma or infection consists of an orderly and orchestrated series of events that results in the halting of the process of injury, the protection of the rest of the organism against further injury, and the initiation of repair processes aimed at returning the body to normal function. One of the striking changes that occurs during these events is the dramatic alteration in the plasma concentration of a series of proteins, collectively called acute phase proteins (reviewed in Refs. 24-27). Many of the acute phase proteins act as antiproteases, opsonins, or blood-clotting and wound-healing factors, which may protect against generalized and local tissue destruction associated with inflammation (28). Vn shares several functional similarities with classical acute phase proteins, including regulatory functions in the complement, blood coagulation, and fibrinolytic systems (reviewed in Refs. 1 and 2). The physiological significance of the up-regulation of rodent Vns should probably be viewed in the context of these functions.

Interestingly, the human Vn gene appears to be regulated quite differently. Species differences with respect to acute phase regulation have been reported for a number of acute phase proteins, the most prevalent example being C reactive protein, the classical human, but not murine acute phase protein (24). However, we are unaware of any example that the corresponding mammalian genes are either regulated by a cytokine (*i.e.* IL-6) or growth factor (*i.e.* TGF β). The physiolog-

ical significance of the regulation of human Vn by TGF β remains to be elucidated. Recent reports indicate that the Vn gene is expressed at significant levels at extrahepatic sites (7). This observation raises the possibility that the up-regulation of the Vn gene by TGF β could be of importance for stimulating Vn biosynthesis in localized areas of inflammation and tissue damage.

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