Expression of the human coagulation factor VII (FVII) gene by hepatoma cells was modulated in concert with levels of glucose and insulin in the culture medium. In low glucose medium without insulin, amounts of both FVII mRNA and secreted FVII protein were coordinately increased; in the presence of glucose with insulin, both were decreased. Analysis of the FVII promoter showed that these effects could be reproduced in a reporter-gene system, and a small promoter element immediately upstream of the translation start site of the gene, which mediated these effects, was identified. Mutation of this element largely abrogated the glucose/insulin-responsive change in expression of the reporter gene. Several members of the CCAAT/Enhancer-binding protein family were found to be capable of binding the identified sequence element but not the mutated element. The expression of a FVII minigene directed by a segment of the native FVII promoter responded to co-expressed activating and inhibiting forms of CCAAT/Enhancer-binding protein β.

Numerous adaptive changes in hepatic gene expression occur in response to fluctuations in the availability of nutrients such as glucose. When glucose is abundant, it is taken up by hepatocytes and metabolized, initially to replenish glycogen stores and subsequently for synthesis of fatty acids, which are primarily converted to triglycerides for transport to and storage in adipose tissue (1). When glucose is in short supply, the pattern is reversed; biosynthetic pathways requiring glucose are inhibited, and those producing glucose from glycogen and by de novo synthesis are activated (2). The expression of many hepatic genes, particularly those encoding glucose transport proteins and enzymes that catalyze the various metabolic reactions, are induced or suppressed in concert with changes in glucose status. Because fluctuations in glucose levels are accompanied by hormonal changes, primarily reciprocal induction and suppression of pancreatic insulin and glucagon, it may be difficult to discern whether a given response is due solely to glucose metabolism, to the hormonal changes, or to a combination of both processes.

Most responsive hepatic genes are up-regulated in the presence of elevated glucose and/or insulin and down-regulated in their absence. However, a subset of responsive genes behaves in the converse manner and are down-regulated in the presence of glucose and/or insulin and up-regulated in their absence. The former group is large and includes the genes for proteins involved in glucose uptake and enzymes required for glucose metabolism and for conversion to glycogen and triglycerides, whereas the latter group is small and appears to be primarily composed of genes encoding enzymes involved in hepatic glucose synthesis and in the endoplasmic-reticulum stress response, which is triggered by glucose deprivation (reviewed in Ref. 3).

There may also be additional hepatic genes, seemingly less likely targets of glucose and/or insulin, whose expression is regulated in a similar manner. The objective of the work presented here was to evaluate the effects of glucose and insulin on transcription of the coagulation factor VII (FVII)2 gene in hepatocytes.

FVII is a vitamin K-dependent hepatic glycoprotease present in the circulation primarily as an inactive zymogen at very low concentration. Nearly all plasma FVII is in zymogen form, but ~1% has been proteolytically cleaved to the activated form, FVIIa (reviewed in Ref. 4). In solution, FVIIa demonstrates negligible activity against its substrates, the zymogens of coagulation factors X and IX (5, 6). Only when bound under the appropriate conditions to its obligate receptor, tissue factor, does FVIIa attain its full catalytic ability. Tissue factor is an integral membrane protein expressed constitutively to some degree on most cells external to the vasculature (reviewed in Ref. 7). Vascular injury exposes membrane-bound tissue factor to FVII and FVIIa from the blood, and it is capable of interaction with both. When zymogen FVII binds tissue factor, it is converted to FVIIa by trace amounts of other coagulation proteases, by the FVII-activating protease or autocatalytically (see Ref. 4 and references therein) (8, 9) and is then held in an optimal orientation relative to the cell membrane for effective interactions with factors X and IX (4). However, most cell surface tissue factor is itself in an inactive, or encrypted, form (reviewed in Ref. 10).

2 The abbreviations used are: FVII, coagulation factor VII; FVII:Ag, factor VII antigen; C/EBP, CCAAT enhancer-binding protein; LAP, liver-activating protein; LIP, liver-inhibiting protein; EMSA, electrophoretic mobility shift assay; HNF4, hepatic nuclear factor 4; AARE, amino acid response element; NSRE, nutrient-sensing response element; AS, asparagine synthase; SNAT2, sodium-coupled neutral amino acid transport; CHOP, C/EBP homologous protein; hGH, human growth hormone; CHO, Chinese hamster ovary; UTR, untranslated region.
Encrypted tissue factor binds FVII/FVIIa but does not support its catalytic activity (11). Decryption of tissue factor is associated with influx of calcium ions (12), local loss of membrane asymmetry (13), dissociation of tissue factor dimers (10), and disulfide exchange at the external Cys\(^{186} - \text{Cys}^{209}\) bond of tissue factor (14, 15). Interaction of FVIIa with decrypted tissue factor permits activation of the coagulation cascade, leading to the localized generation of thrombin at an injury site (5).

Tissue factor expression is also inducible, and tissue factor has been found within the circulation, in association with membranes of activated cells or microparticles derived from them (16, 17) as well as in an alternatively spliced, soluble form (18). It has been suggested that this “blood-borne” tissue factor participates in thrombus enlargement and perhaps in triggering of thrombosis under pathological conditions (19, 20), although this is controversial (21).

FVIIa activity is essential for coagulation by the extrinsic pathway, (22), which is involved in both hemostasis and thrombosis. Elevated plasma FVII levels are associated with polymorphisms in the promoter of the gene (23), whereas decreased levels are associated with distinct polymorphisms in the promoter, the structural gene, or both (24, 25). Whether carriage of polymorphisms influences the risk of thrombosis is presently unclear, since some studies report increased risk of myocardial infarction (26–29) or stroke (30) in carriers of FVII-elevating polymorphisms or protection from risk in carriers of FVII-lowering polymorphisms (31–33), whereas other studies do not (34, 35).

Plasma FVII levels may also be elevated in diabetes, a disease characterized by disordered glucose metabolism and increased risk of thrombotic complications (36). The mechanism through which FVII levels are modulated in diabetes is not understood. We therefore investigated whether transcription of the FVII gene was influenced by glucose and insulin.

The data presented herein indicate that deprivation of glucose and insulin, over a physiologically relevant concentration range, significantly increased expression of both FVII mRNA and FVII protein secreted by HepG2 cells, a human hepatoma cell line. The induction of FVII triggered by withdrawal of glucose and insulin could be prevented, and expression of FVII could be suppressed below basal levels, by reintroduction of insulin to deficient culture medium. These results were recapitulated using a reporter gene system, suggesting that transcriptional effects were involved. A functional region of the FVII minimal promoter capable of interaction with C/EBP isoforms was identified and found to participate in mediating the changes in reporter gene expression attributable to fluctuations in glucose and insulin levels.

**EXPERIMENTAL PROCEDURES**

**DNA Isolation, PCR, and Reporter Plasmids**—Genomic DNA was purified from leukocytes of healthy donors by standard methods (37) under a study protocol approved by the Human Studies Committee of the Brockton-West Roxbury Department of Veterans Affairs Medical Center. Segments of FVII 5’-flanking sequence (nucleotides −1012, −723, −257, and −108 to +1, with nucleotide +1 designated the first base of the initial methionine codon of FVII structural gene) were amplified by PCR with TaqDNA polymerase in a PerkinElmer Life Sciences DNA Thermal Cycler (Norwalk, CT), using primers from Integrated DNA Technologies (Coralville, IA) and subcloned into the promoterless pOGH reporter plasmid containing the human growth hormone (hGH) structural gene (Nichols Diagnostics Institute, San Juan Capistrano, CA). The plasmids are referred to as p-1012/1, p-723/1, p-257/1, and p-108/1. Additional reporter plasmids, extending from position −108 but terminating at position +134 relative to the translation initiation codon, within the first intron, and having either native sequence or sequence mutated at positions −6 to −2, identical to that of oligonucleotide TLSS-MT, described below, were also prepared by overlapping PCR methodology. These plasmids are referred to as p-108/134WT and p-108/134MT. All sequences were confirmed on a 373A DNA Sequencer (Applied Biosystems, Foster City, CA) (38) and compared with published sequences.

**Preparation of FVII Minigenes**—A segment of FVII promoter extending from position −728 to +1 was appended to a FVII cDNA segment extending from position +1 to +268 by overlapping PCR and spliced into plasmid pED-FVII using unique restriction enzyme sites present in the PCR primers and the vector to produce an expression vector with native FVII minigene, pED-FVII-MG. pED-FVII is a dicistronic mammalian expression vector carrying the dihydrofolate reductase gene as a selectable marker, in addition to 32 bp of FVII 5’ untranslated region, the complete FVII cDNA, and 1.2 kb of FVII 3’ untranslated region containing the native polyadenylation sites, under the direction of the SV40 promoter. The construction of pED-FVII has been described previously (39). During generation of the pED-FVII-MG construct, the SV40 promoter sequence was removed and replaced with the FVII promoter segment, thus placing the FVII cDNA under the direction of a segment of its native promoter. The construct was sequenced to confirm segments obtained by PCR and restriction sites used for cloning.

**Cell Culture, Treatments, and Transfections**—HepG2 cells (ATCC HB-8065) were cultured in minimal essential medium containing 5 mM glucose, supplemented with 10% certified fetal bovine serum containing 0.4 mIU/ml insulin, 4 mM/liter glutamine, 10 mM/liter pyruvate, 10 mM/liter HEPES buffer, pH 7.2, 100 units/ml penicillin G, 100 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO\(_2\). For glucose deprivation experiments, 2 × 10\(^6\) cells were plated in standard medium and allowed to attain confluence. Following a 24-h preincubation in medium with 1% FBS, media were changed to glucose-free medium with 10% dialyzed serum and further supplemented with glucose and/or recombinant human insulin as indicated. Media and sera were obtained from Invitrogen, and recombinant human insulin was from Sigma.

For transient transfection of HepG2 cells, 2 × 10\(^6\) cells were plated per dish and transfected by either the calcium phosphate method or with Lipofectamine reagent (Invitrogen) as noted in the legend to Fig. 3, with growth hormone reporter vector and pSV-β-galactosidase expression plasmid (Promega Corp.) as an internal control to monitor transfection efficiency. Media were applied to transfected cells at the end of the transfection period and maintained for 48 h. At harvest, media and lysates were assayed for hGH (Diagnostic Systems Laboratories, Webster,
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TX) and β-galactosidase (Promega). hGH values were corrected for transfection efficiency, and then reporter expression was normalized and averaged, and the S.D. values were calculated. Statistical significance of differences in expression between groups was assessed by the Student’s t test.

CHO-DUKX-B11 cells (a gift of Dr. B. Furie, Boston, MA), deficient in dihydrofolate reductase, were used for transfection with pED-FVII-MG-WT and pED-FVII. Cells were cultured in α-modified essential medium supplemented with 10% fetal bovine serum, 4 mM/liter glutamine, 10 mM/liter pyruvate, 10 mM/liter HEPES buffer, pH 7.2, 100 units/ml penicillin G, 100 µg/ml streptomycin, 10 µg/ml adenosine, 10 µg/ml deoxycytidine, and 10 µg/ml thymidine, at 37 °C, 5% CO₂. Transient transfections of 1 × 10⁶ cells with 2 µg of pED-FVII (or pED-FVII-MG) and 0.5 µg of pRSV-βgal were performed as described (39) for 16 h using Lipofectamine reagent, with or without transfection of expression vectors for C/EBPβ isoforms liver-activating protein (LAP) or liver-inhibiting protein (LIP), both kindly provided by Dr. M. Kilberg (Gainesville, FL). 48 h post-transfection, culture media and cells were harvested for assay of secreted FVII and β-galactosidase, respectively.

Preparation of Nuclear or Whole Cell Extracts—HepG2 nuclear extracts were prepared by the method of Schreiber (40). Nuclear extracts from male rat liver were obtained from Geneka Corp. (Montreal, Canada). HepG2 cells were transfected with 10 µg of expression plasmids for the rat liver-activating protein (pLAP) or liver-inhibiting protein (pLIP), isoforms of C/EBPβ, and whole cell extracts were prepared 48 h later (41). Protein concentrations of extracts were determined by Bradford assay (Bio-Rad).

FVII Antigen (FVII:Ag) Assay—FVII:Ag secreted by HepG2 cells under standard and experimental culture conditions or by CHO cells after transfection with pED-FVII-MG-WT was measured by commercial enzyme-linked immunosorbent assay of the conditioned media (American Bioproducts, Parsippany, NJ). Serum-containing medium not conditioned by cells gave responses in this assay nearly indistinguishable from diluent buffer alone, which were ~30-fold lower than those obtained for conditioned media under the standard conditions (not shown).

Real Time Reverse Transcriptase PCR—The RNeasy mini-prep protocol (Qiagen Inc., Germantown, MD) was used to prepare total RNA from HepG2 cells grown under standard or various experimental growth conditions. 20 ng of total RNA used for FVII mRNA determination and (as an internal control) for 18 S ribosomal RNA determination in multiple replicates as noted. The values were compared with those obtained for parallel reactions run with total human liver mRNA (Ambion, Inc., Austin, TX) at concentrations between 0 and 100 ng, which were used to construct standard curves for each. No amplification controls, as well as no input RNA controls, were run with each assay. The PCR primers and fluorescent probe for FVII were as follows: forward primer, 5′-GACCAG-TGGCCTCAAGTCTCC-3′; reverse primer, 5′-CCGTTTCT-CGTTTACACAGATCA-3′; probe, 5′-(6-carboxyfluorescein)-TCATCCTTGTGCCGTCACAGTTCCGT(tetramethyl-6-carboxyrhodamine). The target region produced a PCR product of the expected size with total RNA template following reverse transcription; no product was obtained with genomic DNA template or in the absence of reverse transcriptase (not shown). Primers and probe for 18 S rRNA detection were from the TaqMan ribosomal RNA control kit. All reagents for real time reverse transcriptase PCR were obtained from Applied Biosystems (Foster City, CA), and reactions were run on an ABI-7700 detector under standard conditions (30-min reverse transcriptase reaction at 48 °C, 10 min at 95 °C, and 40 cycles of PCR with 15 s at 95 °C/1 min at 60 °C).

Electrophoretic Mobility Shift Assays (EMSA)—Complementary oligonucleotides were annealed and end-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). FVII oligonucleotides (upper strand sequence) were as follows: native and mutated translation start site region, residues −20 to +14 or −20 to +25, 5′-AGCACTGCAGATTTCTATCAGCCT-CCCAGGC-3′ (TLSS-WT1) and 5′-AGCACTGCAGATgagcgCATGCTTCCCCAGGC-3′ (TLSS-MT1) or 5′-AGCAC-TGCGAGATTTCTATGTCCTCCAGCGCCTAGGACTCC-3′ (TLSS-WT2) and 5′-AGCACTGCAGATgagcgCATGCTTCCCCAGCCGCTAGGCTCC-3′ (TLSS-MT2). The mutated residues are shown in lowercase type. Incubations were done in 15 µl for 30 min at 4°C, in 50 mM HEPES, pH 7.5, 30 mM KCl, 3 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 12% glycerol, 1 µg of poly(di-l-c), with 3–5 µg of HepG2 nuclear extract or 4.4 µg of rat liver nuclear extract. Reactions were run on 5% (w/v) polyacrylamide gels in 1× TBE (90 mM Tris base, 90 mM borate, 0.5 mM EDTA) buffer and autoradiographed. For competition assays, unlabeled competitor oligonucleotides were added to reaction mixtures with the radiolabeled probe. For supershift assays, polyclonal antibodies directed against C/EBP isoforms, which recognize both the homologous human and rodent proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), were added to extracts during a 60-min preincubation at 4°C prior to the addition of oligonucleotide probe.

RESULTS

Effect of Glucose/Insulin on FVII Expression—Culture of human hepatoma (HepG2) cells for 24 h or longer in standard full growth medium produced a level of secreted FVII protein that was easily detectable by enzyme-linked immunosorbent assay. The concentration of glucose in this medium at the onset of the collection period was 5 mM, and bovine insulin was contributed by the serum to a final concentration of ~0.4 mIU/ml. HepG2 cells possess functional insulin receptors and in the presence of insulin will take up and catabolize glucose. When cells were maintained in glucose-free basal medium supplemented with dialyzed serum, the amount of FVII:Ag secreted by the cells over a 24-h time course was significantly increased. Supplementation of this glucose/insulin-deficient medium with 5 mM glucose and recombinant human insulin at 100 nM suppressed this effect (Fig. 1A). The incremental reduction in FVII:Ag level was similar at 1 and 5 mM and at 48 as well as 24 h, suggesting that the response is due to the presence or absence of insulin rather than of glucose (Fig. 1B).

Measurement of FVII mRNA levels by reverse transcription real time PCR under these different growth conditions mir-
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Biosynthesis of FVII is modulated by glucose and insulin. A, the concentrations of FVII:Ag cumulatively secreted by HepG2 cells cultured under standard growth conditions (S; 5 mM glucose, 4 IU bovine insulin), glucose/insulin limitation (−; 0 mM glucose, 0 mM insulin), or glucose/insulin supplementation (+; 5 mM glucose, 100 mM recombinant human insulin) during 24 h were determined by enzyme-linked immunosorbent assay and are graphed as percentage of FVII:Ag ± S.D. D. n = 12 per group for standard and insulin limitation conditions; n = 11 for insulin supplementation; assayed in duplicate. The amount recovered under the standard condition was considered 100%, and the other values were normalized relative to this. The increase in FVII:Ag seen when insulin/glucose were limited and the decrease seen when glucose/insulin were supplied were significant when compared either with one another or with the level seen under the standard condition (p < 0.01). B, the concentration of FVII antigen secreted by HepG2 cells cultured between 0 and 24 h and between 24 and 48 h in medium supplemented with either 1 or 5 mM glucose, and without or with 100 mM insulin, is graphed as percentage of FVII:Ag ± S.D. At 24 h, n = 6 per group; at 48 h, n = 3 for 1 mM glucose and n = 7 for 5 mM glucose per group. Here, the amount recovered during the first 24 h of culture at each glucose concentration in the absence of insulin is considered to be 100%, and the other values at that glucose level are normalized relative to this. At both 1 and 5 mM glucose, insulin treatment reduced the amount of FVII:Ag secreted by the cells, and this effect was sustained over 48 h. All differences in FVII:Ag due to insulin are significant (p < 0.01). C, the levels of FVII mRNA within HepG2 cells cultured for 24 h under standard growth conditions (n = 7), glucose/insulin limitation (n = 9), or glucose/insulin supplementation (+; n = 4) were determined by reverse transcription real time PCR and corrected by the relative levels of 18 S rRNA in each sample. Each sample was assayed in either four or six replicates for each ampiclon. The average corrected level obtained for cells under standard conditions was considered 100%, and the others are normalized to this. The increase in FVII mRNA seen when insulin/glucose were limited and the decrease seen when they were resupplied were significant when compared either with one another or with the level seen under the standard condition (p < 0.01).

Identification of a functional response element by promoter deletion and mutagenesis. A, HepG2 cells were transfected by the calcium phosphate method with 3 μg of growth hormone reporter vector either lacking promoter (OGH) or having a fragment of the human FVII gene extending from position −1012, −728, −257, or −108 to +1, the translation initiation site. 24 h post-transfection, cells were transferred to the standard growth medium (black bar) or to medium deficient in glucose/insulin (hatched bar) and subsequently assayed as described. The corrected expression from plasmid OGH with or without glucose/insulin has been subtracted from the corrected expression for each reporter plasmid under the corresponding culture condition. Data shown are from 2–6 independent experiments for each reporter. The expression of each reporter plasmid containing a fragment of FVII promoter under the standard condition was considered 100% expression for that plasmid; expression in the promoterless vector (pOGH) was used as a baseline to determine the effects seen for FVII protein levels (Fig. 1C). Thus, limitation of glucose/insulin increased expression from the endogenous FVII gene in hepatoma cells at both mRNA and protein levels, and this effect was reversed by reintroduction of these compounds.

Identification of a Glucose Response Element in the FVII Promoter—Many changes in hepatic gene expression are transcriptionally mediated, and it has been shown that the transcriptional process is primarily responsible for changes in FVII plasma levels (42, 43). To determine whether the observed effects of glucose and insulin on FVII mRNA and protein levels were due to transcriptional mechanisms, reporter gene assays were performed with vectors having various length fragments of FVII 5′-flanking sequence, placed upstream of the hGH reporter gene in promoterless plasmid pOGH. These vectors were used for transient transfection of HepG2 cells under standard or glucose/insulin depleted conditions, as shown in Fig. 2A. In this promoter deletion analysis, all vectors were responsive and had increased expression when glucose and insulin were restricted, thus recapitulating the effect seen for the endogenous FVII gene in untransfected cells. The presence of a functional promoter element residing within the minimal promoter segment was suggested by the sensitivity of even p-108/1, the smallest promoter construct tested, to glucose/insulin deprivation.

Previous characterizations of the FVII 5′-flanking region by DNase I footprinting, EMSA, and mutational analyses indicated that two regions within the minimal promoter, −40 to −70 and −84 to −108, are necessary and sufficient for liver-specific expression of the FVII gene. The former region binds hepatic nuclear factor 4 (HNF4), a transcription factor prominent in liver, and the latter binds the ubiquitously distributed transcription factor Sp1. When either the HNF4 or the Sp1 target area was disrupted by mutagenesis, binding to these
nuclear proteins was prevented, and expression of the reporter gene was greatly reduced (42–44). Subsequent analyses of the causal molecular defects in several unrelated patients with the hereditary bleeding disorder, FVII deficiency, showed that each carried a single base mutation in the FVII 5′-flanking region, confirming the importance of these promoter regions to in vivo FVII expression. These mutations are C to T at position 55 (45), T to G at position 59 (46), T to C at position 60 (47), T to G at position 61 (48), C to G at position 94 (49), and C to T at position 96 (50). In vitro analyses indicate that each mutation impairs expression of a reporter gene, whereas EMSA indicates that the four former mutations disturb interaction with HNF4 and the two latter with Sp1.

Additional footprinted areas within the minimal promoter of FVII have been also been described, although transcriptional regulators interacting with these regions (−51 to −32 and −13 to +6) are largely unknown (46). Our inspection of FVII minimal promoter sequence indicated that the sequence between positions −8 and +1 is strongly homologous to previously characterized sequence regions of the promoters of several genes, which mediate transcriptional responses to availability of nutrients, such as amino acids and/or carbohydrates (51–63). The sequences of these response elements and comparison with the −8 to +1 region of the FVII promoter are shown in Table 1. The data of Fig. 2A indicated that the sequence required for the FVII glucose/insulin response was contained within the minimal promoter, which would also encompass this putative response element at positions −8 to +1. To examine what the identified sequence mediated responses to glucose/insulin, vector p-108/134MT was generated, and a block mutation was installed between residues −6 and −2 to produce construct p-108/134MT. These longer constructs included exon 1a (residues +1 to +64) and a segment of the first intron (residues +65 to +134) of the FVII gene in addition to the minimal promoter. Further transient transfection assays were then performed.

As shown in Fig. 2B, expression of the hGH reporter gene from constructs containing native FVII sequence responded to limitation of glucose/insulin with an increase in reporter produced, and this increase could be reversed by supplementation of medium with glucose and insulin. Mutation of bases −6 through −2, however, rendered expression from the extended construct insensitive to induction by glucose/insulin deprivation without interfering with basal expression of the reporter gene. These data suggest that the identified sequence is part of a response element that modulates, but is not essential for, expression of the FVII gene.

**Nuclear Proteins, Including C/EBPs, Specifically Bind the Identified FVII Response Element**—To identify transcriptional regulators that might transduce these responses, EMSAs were done with hepatic nuclear extracts and oligonucleotides encompassing residues −20 to +14 or −20 to +25 of the FVII gene, having either native sequence (TLSS-WT1 and -WT2) or block-mutated sequence at positions −6 through −2 (TLSS-MT1 and -MT2). As shown in Fig. 3A, complexes from nuclear extracts of HepG2 cells cultured under various regimes of glucose and insulin bound to the native oligonucleotide. There was only weak binding between proteins of these same extracts and the mutated oligonucleotide, however. Also, the complexes with the mutated oligonucleotide, observed at long exposure times, had different electrophoretic mobilities in comparison with the prominent complexes observed with the native oligonucleotide and so may be composed of different nuclear proteins. Correspondingly, hepatic nuclear complexes formed with labeled native oligonucleotide were competed by excess unlabeled native oligonucleotide, indicating that the binding interactions were specific, but they were not competed by mutated oligonucleotide, indicating that the interactions were taking place through the identified sequence (Fig. 3B).

To identify nuclear proteins capable of binding to the native oligonucleotide, supershift analyses were performed. As mentioned above, sequence between −8 and +1 of the FVII promoter is strikingly similar to characterized elements in several other genes (Table 1); in accordance with the nomenclature, this region is referred to as the FVII amino acid response element, or FVII AARE. The AARE is in the asparagine synthase (AS) gene mediates up-regulation when either glucose or amino acids are limited (64), and transcription factors of the C/EBP family were shown to recognize the sequence although it is not a typical C/EBP element. Supershift analysis confirmed that members of the C/EBP family also recognize the FVII AARE. As seen in Fig. 4, antibodies recognizing particular C/EBP isoforms produced supershifted complexes with nuclear extracts prepared from HepG2 cells under standard growth conditions and with nuclear extracts from rat liver as well. Thus, CCAAT transcription factors, whether obtained from hepatoma cells or normal hepatocytes, demonstrably interact with the FVII AARE. These data indicate that C/EBPα and -δ isoforms do not bind the FVII response element, whereas the C/EBPβ, -γ, and -ε isoforms do.
Members of the C/EBP family of transcriptional regulators are abundant in liver and very important in hepatic gene expression. They interact with one another to form a large array of homo- and heterodimeric forms with diverse effects on transcription. C/EBPβ itself is present in multiple isoforms, including a full-length transcription factor, a slightly smaller isoform lacking 23 amino acid residues at the N terminus of the protein (LAP), and a truncated form lacking the transactivation domain (LIP). These isoforms arise by use of alternate translation initiation sites within the C/EBPβ mRNA (65, 66). As their names imply, LAP generally promotes transcription, whereas LIP functions as a natural dominant-negative regulator of other members of the C/EBP family. The C/EBPγ isoform, too, is a natural dominant-negative and, like LIP, could decrease C/EBP-mediated expression by making dimers defective in DNA binding or transactivation with LAP or other activating forms of C/EBP family proteins. However, only the association with C/EBPβ was further examined in this study.

First, the ability of recombinant C/EBPβ isoforms to interact with the native and mutated FVII oligonucleotides was examined. Extracts from HepG2 cells overexpressing either the full-length (LAP) or truncated (LIP) recombinant rat C/EBPβs contained prominent complexes that bound native, but not mutated, oligonucleotide, and these complexes were completely supershifted by anti-C/EBPβ antibody (Fig. 5A). Then HepG2 nuclear extracts were prepared from cells cultured under various conditions, including polyadenylation sites from the human FVII gene, was shown to bind FVII:Ag was measured; essentially, the FVII protein participated in expression of the FVII gene, an alternative approach was used. A native FVII minigene construct, consisting of 728 bp of promoter, the cDNA, and the 3' UTR, including polyadenylation sites from the human FVII gene, was transfected into CHO cells alone or with expression plasmids for LAP and/or LIP (Fig. 6). In these experiments, the normalized expression of secreted FVII:Ag was measured; essentially, the FVII protein served as the reporter gene under the direction of its own promoter. A very low but detectable level of FVII:Ag

![FIGURE 3. FVII AARE binds hepatic nuclear proteins. A, labeled unmutated TLSS-WT2 (lanes 1–4) or mutated TLSS-MT2 (lanes 5–8) oligonucleotides were incubated with 3 μg of HepG2 nuclear extract from cells maintained for 24 h at 5 mM glucose with 100 mM insulin (lanes 2 and 6), 0 mM glucose without insulin (lanes 3 and 7), or 0 mM glucose with 100 mM insulin (lanes 4 and 8). The labeled oligonucleotides alone are in lanes 1 and 5, respectively. Multiple prominent complexes formed between all three nuclear extracts and the unmutated oligonucleotide, but the mutated oligonucleotide interacted with the same extracts weakly. All lanes are from the same exposure of the one gel, but intervening lanes have been removed. B, labeled TLSS-WT1 oligonucleotide in the absence of unlabeled competitor oligonucleotide (−) or with 10-, 50-, 100-, 200-, and 400-fold excess unlabeled competitor self-oligonucleotide (lanes 3–7). As the amount of this unlabeled oligonucleotide increased, the level of labeled complexes recovered progressively decreased. However, even 200- and 400-fold unlabeled oligonucleotide with block-mutated AARE (lanes 8 and 9) did not substantially decrease binding to the labeled, unmutated oligonucleotide.](image)

![FIGURE 4. C/EBPs recognize the FVII AARE. Labeled oligonucleotide TLSS-WT1, containing the FVII AARE, was incubated with 3 μg of nuclear extract from HepG2 cells (A, left) or from rat liver (B, right), in the absence of antibody (−) or with antibody directed against multiple C/EBP isoforms (pan) or specific for a particular one, as shown above each lane. Labeled oligonucleotide alone is shown in lane 1 of each panel. The C/EBPβ, C/EBPε, and pan-specific antibodies produced supershifted complexes with both nuclear extracts (A, lanes 4, 6, and 7 versus lane 2; B, lanes 3, 6, and 7 versus lane 8). A C/EBPγ antibody produced supershifted complexes as well (B, lane 4 versus lane 8). Supershifted complexes are indicated by the brackets.](image)
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expression was observed from the minigene construct alone in CHO cells, which do not normally express FVII. In mock-transfected control cultures, no secreted protein was detectable using antibodies directed against human FVII:Ag. Co-expression of p-LAP, however, was found to increase expression from the minigene construct, whereas inclusion of p-LIP moderated the effect of p-LAP. In control experiments, the pED-FVII vector, in which the SV40 promoter directed expression of FVII cDNA and 3'-UTR, was measured. Expression of FVII:Ag in these cultures was significant but was not enhanced by co-expression of pLAP. These data indicated that manipulation of C/EBPβ levels within the cells predictably affected FVII expression from the minigene construct containing the AARE.

DISCUSSION

The data presented here indicate that expression of the FVII gene can be directly modulated by fluctuations in glucose/insulin availability. Glucose/insulin deprivation increased the levels of both FVII mRNA and secreted protein, whereas reintroduction decreased both FVII mRNA and secreted protein. These results were reproduced using a reporter gene system, suggesting that the mechanism was at least partially transcriptional. Furthermore, EMSA and mutation analysis delineated a response element in the minimal FVII promoter that was involved in mediating this transcriptional response and that strongly resembles functional elements described in other genes up-regulated by the absence of glucose and/or of amino acids. Transcriptional regulators, the C/EBPβ proteins LAP and LIP, were capable of recognizing this FVII native sequence, but not a nonfunctional mutated sequence, and of modulating expression from a FVII minigene vector construct.

These data indicate that the FVII gene must join the growing list of genes whose expression can be induced through the agency of genetic elements called “amino acid response elements” or AAREs. We use this established name, although for the AS (54, 64), CHOP (51), tribbles-related protein 3 (62), and activating transcription factor 3 (55) genes, these elements mediate induction in response to decreased glucose availability as well. Many of these elements have been shown to bind not only to members of the CCAAT family of transcription factors but also to various members of the closely related activating transcription factor/CREB family, with which CCAAT factors can cross-dimerize. Whether these transcription factors, as well as C/EBPβ isoforms, affect expression of the FVII gene is currently under study in our laboratory.

Our data suggest that the FVII AARE is not required for basal expression but instead mediates inducible expression or repression according to insulin status and perhaps in response to various types of cellular stress. The other proteins known to have functional AAREs are involved in amino acid biosynthesis or
transport (AS, SNAT2, cystine/glutamate transporter, and cationic amino acid transporter genes) or alternatively encode transcription factors induced by cellular stresses including starvation (CHOP, tribbles-related protein 3, C/EBPβ, and activating transcription factor 3 genes). The expression of a much larger group of genes is responsive to nutrient availability (reviewed in Ref. 67), but to our knowledge the existence of functional AARE elements has thus far been demonstrated only for these eight genes and, by the present work, for the FVII gene.

Sequences of the 9-bp AARE elements in these eight genes are very closely related. In Table 1, the known AAREs are arranged in the 3′–5′ orientation, for comparison with the FVII element, which extends from position −8 to +1 of the FVII minimal promoter. If the AARE of the AS gene (also referred to as the NSRE-1 element (54, 68)) is considered the prototype, only the cationic amino acid transporter element is completely identical to it. The others vary slightly (e.g. at the fourth residue from the 3′ side (activating transcription factor 3 AARE) or at both the first and fourth residues). Within the latter group of elements, the xCT forward and reverse AARE elements and the three tribbles-related protein 3 AAREs are alike, as are the CHOP AARE, C/EBPβ AARE, and SNAT2 AARE. The FVII AARE element is not completely identical to any of the others. It matches the AS AARE at 8 of 9 positions, excepting the first residue on the 3′ side, where it matches the residue found in the CHOP, C/EBPβ, and SNAT2 elements. To what extent an exact nucleotide sequence is important for AARE function is not yet clear, but the number of AAREs, their proximity to other response elements, and their location within the gene, may all be factors that provide fine control of the response and result in differential expression of genes possessing one or more AARE elements.

Most of the characterized AAREs are in the 5′-UTRs of their respective genes, but the SNAT2 AARE is within the first intron (60), whereas the cationic amino acid transporter AARE is in the first exon and the C/EBPβ AARE is in the 3′-UTR. The FVII AARE is uniquely positioned in that it is located at residues −8 to +1 of the minimal promoter, overlapping with the first base of the initiation codon of the FVII antigen. We are unaware of a similar element immediately preceding the translational start site of a gene.

Our data do not address whether the FVII AARE functions in a completely independent manner to modulate transcription. Although the AARE of the CHOP gene appears to work independently, AARE elements of several of the other genes, while necessary for up-regulation under nutrient deprivation conditions, are not themselves sufficient for robust induction and apparently cooperate with additional nearby elements, which have been found ~10 bp, or one turn of the DNA double helix, away from the AARE. For the cystine/glutamate transporter gene, the second element, spaced 9 bp from the first AARE, is a second identical AARE in the reverse orientation. These two AAREs must cooperate to mediate increased expression in amino acid deprivation, because mutation of either one reduces the amino acid responsiveness of the gene, whereas mutation of both eliminates it altogether (61). The tribbles-related protein 3 gene has three AARE sequences oriented the same way, each of which has a potentially functional, canonical C/EBP element located 6 bp upstream (62). Similarly, the single SNAT2 AARE has a potential C/EBP element 10 bp upstream (60). The AS gene contains a second element, the NSRE-2, located 11 bp downstream of the AARE, which is required for inducible expression, but the regulatory proteins it binds have not yet been identified. Also, the C/EBPβ AARE has both unfolded protein response element and NSRE-2 sites 36 and 50 bp downstream, which contribute to its effectiveness. The C/EBPβ unfolded protein response element also strongly resembles the AARE consensus (63).

It is clear that cells must be able to detect and respond appropriately to periodic fluctuations in nutrient levels in order to survive episodes of deprivation. Given this, the modulation of expression of genes such as AS, SNAT-2, cystine/glutamate transporter, and cationic amino acid transporter through AARE elements following limitation of amino acids/glucose is not unexpected, since their increased activity would mitigate the effects of nutritional deprivation. But it is not obvious why expression of the gene for FVII, a secreted protein required for the initiation of coagulation by the extrinsic pathway, should be modulated in a similar manner. Also, although members of the large b-Zip family of transcriptional regulators, which includes the C/EBPβ proteins, have been shown to bind several AAREs, it is unexpected for FVII expression to be affected by C/EBP transcription factors. Nonetheless, the ability of LAP and LIP to bind the FVII AARE are consistent with a role for C/EBPβ in glucose/insulin-responsive expression of the FVII gene.

The genes for coagulation factors are not generally thought of as targets for C/EBP transcriptional regulators, and we are aware of only three prior reports even suggesting a role for C/EBPs in their expression. First, the promoter and first exon of the factor VIII gene have multiple elements that interact with both the α and β isoforms of C/EBP; several located near the transcriptional start site were functional when assessed by in vitro assays (69). Of seven such elements, only one, called site 2, has significant homology to the AARE consensus (Table 1), but even so it differs at two positions, 5 and 8 from the 3′ side, that are invariant in all characterized AARE elements. Second, a potential binding site for C/EBPα was suggested to occur in the promoter of the human factor IX gene near the transcriptional start site and to be disrupted in some individuals with the coagulation disorder, hemophilia B Leyden, which is severe in childhood but improves with maturity (70, 71). Again, the sequence of this element differs from known AAREs at two positions, 1 and 7 from the 3′ side; the latter is invariant in characterized AAREs. And third, a potential C/EBPβ binding site was predicted to occur in the promoter of the murine FVII promoter by the MatInspector sequence recognition program (72). This predicted C/EBPβ site is upstream of the murine Sp1 and HNF4 binding sites rather than downstream from similar functional elements in the human FVII promoter, as we show here. Recombinant C/EBPβ was shown to bind to this region of the murine FVII promoter by EMSA, but a functional interaction was not demonstrated. It is interesting that the putative murine FVII C/EBPβ site matches the human FVII AARE at 6 of 9 positions but differs at positions 1, 6, and 7 from the 3′ side (Table 1).

Single nucleotide mutagenesis of individual residues of the
Insulin Down-regulates Factor VII

AARE (NSRE-1) from the AS promoter indicates that a substitution at any position is detrimental to nutrient responsiveness of that gene (54, 68). The least severe effects were observed for change at either position 1 or 6 from the 3′ side, where the response was diminished by 60%. The most severe effects were seen for change at positions 2, 4, 5, 8, or 9 from the 3′ side, where in each instance the responsiveness was reduced by 90–100%. Multiple substitutions were not evaluated. All known AARE elements match exactly at positions 5–9 according to this numbering, so it is unclear whether any other coagulation factor C/EBP elements (human coagulation factor VIII or IX, murine FVII) could behave as an AARE.

The abundance of various C/EBPs in the HepG2 cells employed in the present study, relative to adult liver, is an important consideration here. A mark of hepatoma is a change at either position 1 or 6 from the 3′ side. They further suggest that in certain pathological situations.

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