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Identification of Regulatory Sequences and Protein-Binding Sites in the Liver-Type Promoter of a Gene Encoding 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase

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The liver-type and muscle-type isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase are encoded by one gene that uses two alternative promoters. We have identified *cis*-acting sequences and protein-binding sites on the liver-type promoter. Transfection assays with deleted promoters showed that maximal promoter activity is contained within 360 bp upstream of the cap site. DNase I footprinting experiments with liver and spleen nuclear extracts and with purified proteins revealed several protein-binding sites in this region. These included four binding sites for nuclear factor I, one site that contains an octamer consensus but showed a liver-specific footprint pattern, two liver-specific protein-binding sites, and one poly(dG)-containing binding site. Transfection of cells of hepatic origin suggested that all these sites except one are involved in transcriptional regulation. The region between −360 and −2663 contained an element that functioned as a silencer in a nonhepatic cell line. We conclude that in liver transcription from the liver-type promoter of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene is controlled by ubiquitous and tissue-specific factors and involves activating and derepressing mechanisms.

Fructose-2,6-bisphosphate is the most potent stimulator of 6-phosphofructo-1-kinase, a key enzyme of glycolysis. The synthesis and degradation of fructose-2,6-bisphosphate are catalyzed, respectively, by 6-phosphofructo-2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2). In liver these two activities are borne by separate domains of a homodimeric protein which is therefore one of the rare bifunctional enzymes. Several PFK-2/FBPase-2 isozymes whose expression differs depending on the tissue have been described (for a review, see reference 12). The molecular structure of the isozymes is unknown, except for the liver (L) and muscle (M) isozymes. We have indeed cloned a 55-kb rat gene, containing 15 exons, that encodes the L and M isozymes by alternative use of two different promoters, referred to as L and M promoters. The L and M mRNAs share the same 13 exons. They have an additional exon which is specific for either the M isozyme (first exon of the gene) or the L isozyme (second exon of the gene). Thus, the L promoter region lies between these two exons (7). The concentration of L mRNA is highest in liver, but this mRNA is detectable in other tissues (5). Its concentration in liver is regulated by hormones and is increased by insulin in diabetic rats (3) and by triiodothyronine (T_3) in hypothyroid rats (31). Transcription of the liver mRNA is also increased by glucocorticoids in adrenalectomized rats (16). The PFK-2/FBPase-2 L promoter is therefore an interesting model for studying the tissue-specific and hormonal regulation of transcription. We have delineated here the 5′-flanking sequences that influence promoter activity by transfecting hepatoma cells and isolated normal hepatocytes as well as pituitary tumor cells with L promoter mutants linked to a reporter gene. The factors that bind to these sequences have been identified by in vitro DNase I footprinting and band-shift assays.

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MATERIALS AND METHODS

Cell cultures. ATIII-SV40 cells derive from a mouse liver tumor expressing a transgene, the simian virus 40 T antigen under the control of the liver-specific human antithrombin III gene promoter. The cells were grown for 50 to 60 passages in continuous monolayer culture in Ham's F12 medium supplemented with 5% fetal calf serum (FCS), 1 μM dexamethasone, 1 μM T_3, and 10 nM insulin. Twenty-four hours before transfection, 1.5×10^6 ATIII-SV40 cells per 60-mm dish were plated in Dulbecco's modified Eagle's medium (DMEM) plus 5% FCS without addition of hormones. Rat pituitary tumor GC cells were grown as monolayers in Ham's F12 medium supplemented with 10% FCS. Rat hepatoma Fa32 cells and human cervix carcinoma HeLa cells were grown as monolayers in DMEM supplemented. respectively, with 10 and 5% FCS.

Primary cultures of hepatocytes isolated from rats fasted for 72 h were prepared as previously described (8) with the following modifications. After liver perfusion, the cells were collected in L-15 medium plus 0.2% bovine serum albumin (BSA) and 10 nM insulin. After two centrifugations, the hepatocytes were resuspended in DMEM containing 10 nM insulin, 1 μM T_3, and 10% FCS and plated on 60-mm dishes at 3.2×10^6 cells per dish. After 4 h, the medium was replaced by DMEM plus 1 μM T_3, 0.1% BSA, and 1 μM dexamethasone, and the adhering cells were incubated further for 18 h. The medium was then replaced by DMEM plus 0.1% BSA. After 24 h of this hormone-free culture, the cells were transfected.

Transfection and determination of reporter gene activity. Transfection of ATIII-SV40 cells and isolated hepatocytes was performed by the calcium phosphate-DNA coprecipitation method (32). ATIII-SV40 cells were incubated for 16 h with a coprecipitate of calcium phosphate-DNA (10 μg of test plasmid plus 1 μg of pRSVβgal as an internal control) in DMEM plus 5% FCS, washed with phosphate-buffered saline (PBS), and incubated for 24 h in DMEM plus 5% FCS

before being harvested. Isolated hepatocytes were incubated for 16 h in DMEM plus 10% FCS with the coprecipitate of calcium phosphate-DNA (10 μg of test plasmid plus 1 μg of pRSVβgal), washed with PBS, and incubated further for 24 h in DMEM plus 0.1% BSA before being harvested. GC cells, collected during exponential growth phase, were electroporated (25×10^6 cells per pulse) at 1,800 μF and 260 V (SEDD Cellject electroporator; Eurogentec, Liège, Belgium) in the presence of 20 μg of test plasmid and 1 μg of pRSVβgal in Ham's F12 medium without serum. After the electric shock, the cells were transferred to 100-mm dishes in Ham's F12 medium plus 10% FCS.

To quantify luciferase activity (test plasmid), each dish was washed with ice-cold PBS and the cells were collected in 200 μl (60-mm dish) or 500 μl (100-mm dish) of lysis buffer (19). After centrifugation ($1,600 \times g$ for 5 min), 50 μl of cell extract was mixed with 350 μl of 25 mM glycylglycine (pH 7.8)–15 mM $MgSO_4$–5 mM ATP. Duplicate tubes were placed in a Lumac biocounter M2000, the reaction was initiated by injecting 100 μl of 1 mM luciferin, and the luminescence units (Lumac light units) were recorded during the first 10 s. Background values, obtained with extracts of nontransfected cells, were about 3 U. Maximal values obtained with test plasmids were 200 to 300 U in ATIII-SV40 cells, 30 to 50 U in isolated hepatocyes, and 1,000 to 1,200 U in GC cells. β-Galactosidase activity (control plasmid) was determined on 50 μl of cell extract according to the method of Sambrook et al. (26), and in each series of transfection experiments the measurements were taken when all the reactions had reached simultaneously an optical density value at 420 nm in the range of linearity. Within such series, the activities of the control plasmid varied by up to sixfold with ATIII-SV40 cells and by up to threefold in isolated hepatocytes, in keeping with the literature (11). L promoter activity was therefore expressed as the ratio of luciferase to β-galactosidase activity in each cell extract.

DNA-binding assays. Nuclear extracts from rat liver and spleen were prepared by the method of Gorski et al. (9), except that the final dialysis was performed in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6)–100 mM KCl–10 mM $MgCl_2$–1.5 mM dithiothreitol–0.2 mM EDTA–15% glycerol. Final protein concentration ranged from 3 to 6 mg/ml. Whole extracts from GC, HeLa, and Fa32 cells were as previously described by Manley (15).

For DNase I footprinting, 5 to 40 μg of nuclear protein was incubated for 20 min at 0°C in a final volume of 50 μl containing 25 mM HEPES (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 0.75 mM dithiothreitol, 0.1 mM EDTA, 7.5% glycerol, 1 μg of poly(dI-dC), 2% polyvinyl alcohol, and 1 to 5 ng of radioactive DNA probe ^{32}P labeled as described in the legends to the figures. After 2 min at 20°C, 50 μl of an aqueous solution of 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was added together with 15 to 55 mU of DNase I (Boehringer) in 1 to 3 μl of water. After 1 min, the reaction was stopped with 100 μl of an aqueous solution of 200 mM NaCl, 20 mM EDTA, and 1% sodium dodecyl sulfate (SDS). Nucleic acids were extracted with a 1:1 mixture of phenol and chloroform, ethanol precipitated, and analyzed on a 6% denaturing polyacrylamide gel. In some experiments the protein-DNA interaction was performed under the same conditions but in a final volume of 20 μl devoid of polyvinyl alcohol. In this case, DNA was digested by the addition of 1 μl of DNase I (150 to 300 mU) diluted in 25 mM $CaCl_2$. Essentially the same results were obtained under both conditions. For footprinting of purified proteins no poly(dI-dC) was added in the incubation.

For band-shift assays, purified vNFIBD (see below) was incubated in a final volume of 20 μl under the same conditions as for DNase I footprinting but with 1,000 cpm of ^{32}P-labeled DNA probe and 6 to 50 times less vNFIBD. After 20 min at 0°C, the samples were loaded and run on a nondenaturing 4% polyacrylamide gel as previously described (4).

Plasmids. All pPLLuc plasmids contain an L promoter fragment cloned upstream of the firefly luciferase gene in the polylinker of pXP2 (20). The 3′ end of all the deleted promoter constructs corresponds to the *Alu*I site at +87 (+1 is the major cap site [7]) cloned in the *Sma*I site of the polylinker. Only pPLLuc36 contains an additional *Kpn*I site and a blunt-ended *Ban*II site in the polylinker between the promoter and the luciferase gene. The numbering of the pPLLuc plasmids corresponds to the position of the restriction site used to make the 5′ deletion: *Ban*II, −36; *Bst*NI, −71; *Spe*I, −111; *Dpn*I, −138; *Stu*I, −219; *Alu*I, −237; *Acc*I, −360; *Hpa*II, −444; *Eco*RV, −809; and *Hin*dIII, −2663. All these sites were cloned in the blunt-ended *Sal*I site of the polylinker, with the exceptions of pPLLuc2663, in which the 5′ end is cloned in the *Hin*dIII site of the polylinker, and of pPLLuc360 and pPLLuc36, in which the 5′ end is cloned in the *Sma*I site of the polylinker. pPFKLuc2663 contains the *Hin*dIII (−2663)-*Hae*III (+4) L promoter fragment cloned in the *Hin*dIII and *Sma*I sites of pXP2. pTZP2663 contains the *Hin*dIII-*Bgl*II fragment, which contains the −2663 to +4 promoter region, of pPFKLuc2663 cloned in the *Hin*dIII-*Bam*HI sites of pTZ19R (Pharmacia).

RESULTS

Binding of nuclear proteins to the PFK-2/FBPase-2 L promoter. As shown below, we found that full promoter activity in transfection experiments is conferred by the 360 bp upstream from the cap site. To delineate the functionally important promoter regions, we searched for potential protein-binding sites in this 360-bp fragment. DNase I footprinting experiments were performed on an *Acc*I (−360)-*Bgl*II (+4) fragment isolated from pPFKLuc2663 and end labeled on the noncoding strand at the *Bgl*II site. Three footprints were visualized in the presence of liver nuclear extract (Fig. 1A): footprint I, from −43 to −66; footprint II, from −78 to −102; and footprint III, from −114 to −132. Protection was not complete at the downstream border (−78) of footprint II (see Fig. 3, compare lanes 1 and 2). Footprints II and III were interrupted by a DNase I-hypersensitive site (arrows in Fig. 1A). On the coding strand, the coordinates of footprints I, II, and III were, respectively, −42 to −63, −79 to −98, and −112 to −128 (Fig. 1B). Note that the hypersensitive sites (arrows in Fig. 1B) in footprints II and III were again observed.

To determine whether these footprints were liver specific, we performed footprinting experiments with spleen nuclear extracts. With these extracts three footprints with the same coordinates as with the liver extract were observed (Fig. 1A). However, footprints I and II were detected at higher protein concentrations with spleen than with liver extract, and the hypersensitive site in footprint III was never observed with spleen extract.

The same region of the promoter, i.e., the *Acc*I (−360)-*Xho*I (+4) fragment of pTZP2663, was labeled at the other end on the coding strand (*Acc*I site). This enabled us to visualize three additional footprints with liver extract: foot-

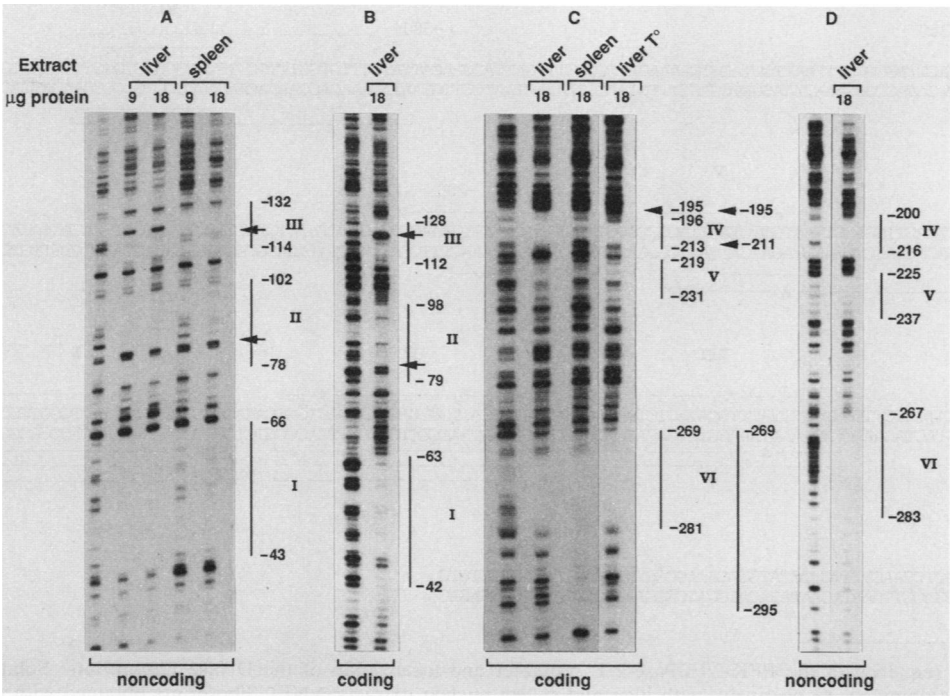

FIG. 1. DNase I footprinting on the PFK-2/FBPase-2 L promoter. (A and B) *Acc*I-*Bgl*II fragment isolated from pPFKLuc2663, labeled at the *Bgl*II site on the noncoding (A) or coding (B) strand. (C and D) *Acc*I-*Xho*I fragment from pTZP2663, labeled at the *Acc*I site on the coding (C) or noncoding (D) strand. Vertical bars delineate the footprints obtained with liver or spleen nuclear extract, as indicated above the lanes. Liver T°, Heated (65°C, 5 min) extract. Arrows point to DNase I-hypersensitive sites.

print IV, from −196 to −213, with a DNase I hypersensitive site at −195 (arrow); footprint V, from −219 to −231; and footprint VI, from −269 to −281 (Fig. 1C). The downstream boundary of footprint VI was ill-defined under these conditions. We never observed total protection against DNase I in the footprint V region, even at higher protein concentrations (not shown). On the noncoding strand, the coordinates of footprints IV, V, and VI were, respectively, −200 to −216, −225 to −237, and −267 to −283 (Fig. 1D).

With spleen extracts, DNase I protection experiments using the same probe showed a different picture. Footprint IV was replaced by hypersensitive sites at −195 and −211 (arrows in Fig. 1C). Footprint V was absent, and the region from −269 to −295 that overlaps with footprint VI was protected (Fig. 1C). A summary of the footprinting data on the PFK-2/FBPase-2 L promoter is shown in Fig. 2.

Footprints I and II are due to NF-I-related proteins. Examination of the nucleotides corresponding to footprints I and II (Fig. 2) shows that each contains on the coding strand a TGGC sequence (−58 and −95) that is found (23) in the palindromic binding motif TGGC/ANNNNNGCCAA for nuclear factor I (NF-I). It is known that half of this palindrome suffices to bind NF-I (22). We therefore performed a footprinting experiment with liver nuclear extract (Fig. 3) in the presence of a competing oligonucleotide, 5′-ATTTTGGCTACAAGCCAATATGAT-3′, that contains an NF-I-binding site (underlined). Addition of this NF-I oligonucleotide inhibited footprints I and II (Fig. 3, lanes 3 and 4). This effect was specific, since footprint III was not prevented and because an unrelated oligonucleotide, 5′-GTAGGCCACGTGACCGGG-3′, which contains an upstream stimulatory factor (USF)-binding site (underlined), had no effect on footprints I, II, and III (Fig. 3, lane 5). If footprints I and II are due to NF-I, then they should also be visualized by using purified NF-I instead of nuclear extracts. We therefore incubated the DNA probe with purified vNFIBD which corresponds to the N-terminal 240 amino acids, i.e., the DNA-binding domain, of liver NF-I (10). vNFIBD produces footprints identical to those seen with intact NF-I (6). A footprint (Fig. 3, lane 8) identical to footprint II (Fig. 3, lane 7), including the hypersensitive site (arrow), was obtained. vNFIBD also produced a footprint whose 5′ boundary (−66) coincided with that of footprint I but which extended further downstream to −30. The region from −30 to −66 protected by vNFIBD contains not only a TGGC sequence on the coding strand at −58, i.e., within the boundaries of footprint I delineated with liver extract, but also a TGGA sequence on the noncoding strand at −34, downstream of footprint I (Fig. 2). Our interpretation was that pure vNFIBD enabled us to detect two neighboring NF-I sites in this region in which a crude extract showed only one footprint. This was confirmed by a band-shift experiment (Fig. 4). When a probe encompassing nucleotides −71 to −10 was incubated with increasing concentrations of purified vNFIBD, this assay showed two major retarded complexes, the lower one appearing at a lower vNFIBD concentration. A faint, slower-migrating complex was also seen in some experiments, but its intensity did not increase with increases in protein concentration. The two major retarded bands were due to vNFIBD, since they were prevented by the addition of 10 ng of NF-I oligonucleotide (not shown). This result suggests that the −71 to −10 region contains at least two NF-I-binding sites, which confirms the footprinting data. The most proximal of these sites was detected only with vNFIBD, either because its affinity is too low to be detected with crude extracts or because of the

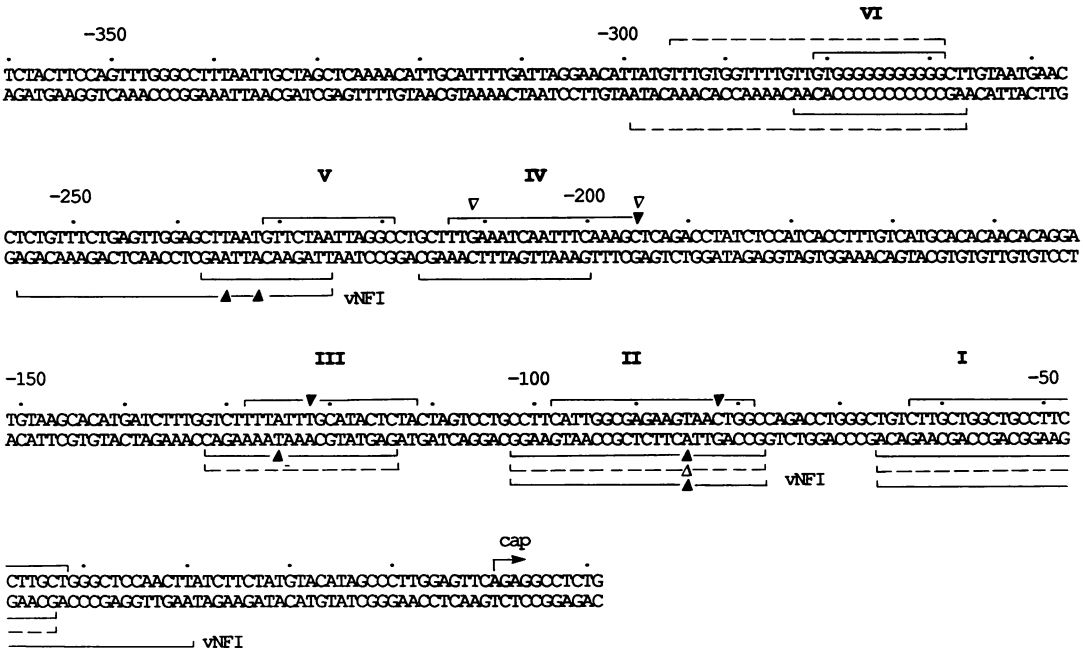

FIG. 2. Nucleotide sequence of the PFK-2/FBPase-2 L promoter and localization of the DNase I footprints. Solid and dotted lines delineate the footprints obtained, respectively, with liver and spleen nuclear extracts. vNFI, Footprints obtained with purified vNFIBD. DNase I-hypersensitive sites detected with liver (▲) and spleen (△) nuclear extracts. Spleen extract was not tested in footprinting on the coding strand between −150 and +1.

smaller size of vNFIBD compared with NF-I, which might not bind at the site because of steric hindrance.

vNFIBD produced another footprint containing two hypersensitive sites (arrows) located from −225 to −255 and therefore overlapping with footprint V (Fig. 3, lane 10). This region contains the half NF-I palindromic motif TGGA at −242 (Fig. 2). This NF-I site was not detected with liver nuclear extract, presumably because of low affinity or be-

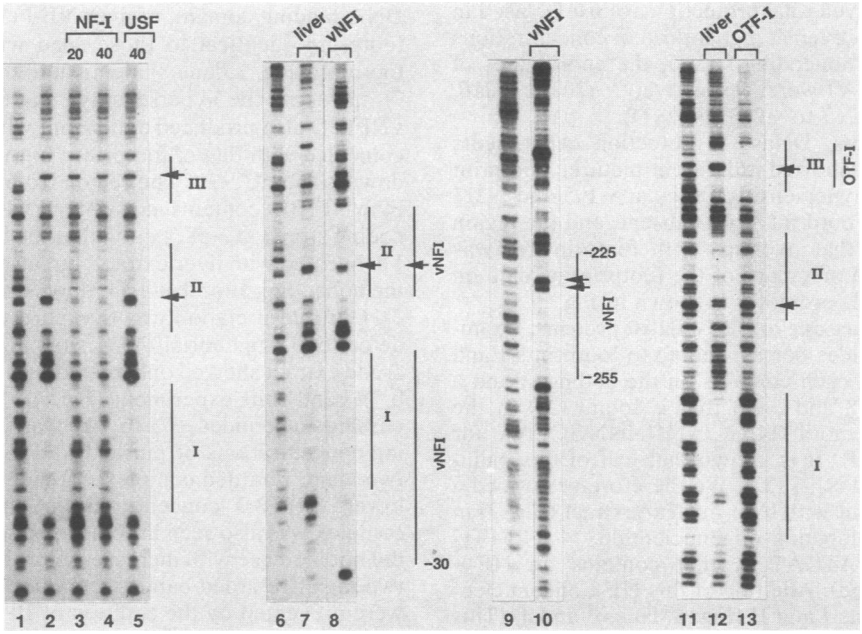

FIG. 3. DNase I footprinting on the PFK-2/FBPase-2 L promoter. Lanes 1 to 8, Footprinting on the same fragment as in Fig. 1A without (lanes 1 and 6) or with (lanes 2 to 5 and 7) 18 μg of liver nuclear protein or with 2 μl of purified vNFIBD (lane 8). NF-I and USF (lanes 3 to 5), Competing oligonucleotides (in nanograms). USF, Upstream stimulatory factor. Lanes 9 and 10, Footprinting on the fragment shown in Fig. 1D without (lane 9) or with (lane 10) 2 μl of purified vNFIBD; lanes 11 to 13, footprinting on the fragment shown in Fig. 1B without (lane 11) or with (lane 12) 18 μg of liver nuclear protein or with 100 fmol of purified OTF-I (lane 13).

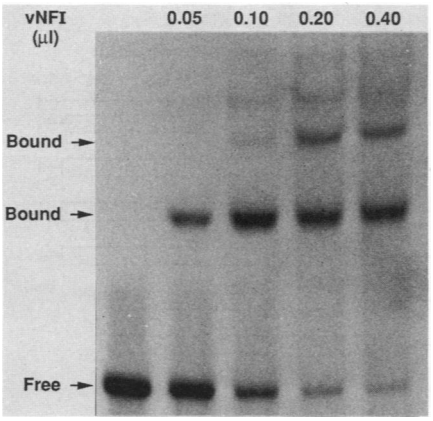

FIG. 4. Band-shift experiment of purified vNFIBD at increasing concentrations with a 78-bp *Hin*dIII-*Sty*I fragment labeled on the noncoding strand at the *Hin*dIII end and isolated from pPLLuc71.

cause of competition with the factor producing footprint V, whose binding site overlaps with the NF-I site.

Footprint III contains an octamer sequence but shows a liver-specific pattern. Footprint III contains the canonical octamer sequence ATTTGCAT at −125 (Fig. 2) and was therefore suspected to be due to the ubiquitous octamer transcription factor I (OTF-I; also called Oct-1, OBP100, NF-A1, and NFIII [see reference 28 for a review]). Surprisingly, a hypersensitive site interrupted the footprint III seen with liver but not that seen with spleen extract (Fig. 1A). As far as we know, such a hypersensitive site has never been described in footprints obtained with any octamer factor. Furthermore, OTF-I purified from HeLa cells produced on our promoter fragment a footprint III with the same boundaries as with liver or spleen nuclear extract but with no hypersensitive site (Fig. 3, lane 13). The hypersensitive site therefore seems to be liver specific. Indeed, we detected it with rat hepatoma Fa32 cell extract, but not with rat pituitary GC cell extract (not shown).

Proteins involved in footprints IV, V, and VI. Footprint IV was detected with liver but not with spleen nuclear extract (Fig. 1C). The sequence on the noncoding strand (−196) CTTTGAAATTGATTTCAAAGC (−216) resembles (underlined) the human transferrin gene promoter sequence 5′-CTTTGACCTTGAGCCCAG-3′, which is known to bind the transcription factor Tf-LF2 (21). The footprint IV region also contains on each strand a sequence GCTTTGAAAT that resembles (underlined) the core sequence TCTTTGACCT found in the binding sites for the liver-specific factors Tf-LF1, Tf-LF2, and LF-A1 (21). The central part of footprint IV and the hypersensitive site at −195 persisted when the assay was performed with a liver extract heated at 65°C for 5 min (Fig. 1C). Since Tf-LF2, but not Tf-LF1 or LF-A1, is thermostable (21), footprint IV might be due to Tf-LF2 or to a related protein.

Footprint V, always weak with liver nuclear extract and absent with spleen nuclear extract, contains the sequence (−236) TTAATGTTCTAATTAGGC (−219), which is similar (underlined) in its central part to the 5′-GGTATGATTTTGTAATGGTA-3′ sequence of the mouse albumin gene known to bind transcription factors DBP and C/EBP (18). The latter is heat stable. Since heated liver nuclear extract (65°C for 5 min) did not produce footprint V (Fig. 1C), the participation of C/EBP in footprint V is unlikely. The disappearance of footprint V on heating might explain why the boundaries of footprint IV changed under those conditions (Fig. 1C), since the lack of footprint V is expected to modify DNase I sensitivity in this region. Identification of the protein responsible for footprint V with DBP is only tentative, since the thermosensitivity of DBP has not been reported and this footprint also contains a palindrome, GCNTAATNNNNNNATTANGC, that bears no relationship to the DBP-binding site on the albumin gene.

Footprint VI corresponds to a poly(dG) stretch. It was detected with liver and spleen extracts but extended further upstream to −295 with spleen extract (Fig. 1C). The sequence between −281 and −295 is T rich. To our knowledge, no poly(dT)-binding site for a spleen-specific factor has been reported on another gene. With rat pituitary GC cell extracts and with HeLa cell extracts, the boundaries of footprint VI were identical to those seen with liver extract (not shown). The footprint observed with liver extract persisted after the extract was heated at 65°C for 5 min, in which case the boundaries were better defined (Fig. 1C).

Delineation of transcriptional regulatory elements within the PFK-2/FBPase-2 L promoter. To localize the regulatory elements of the PFK-2/FBPase-2 L promoter, we performed transfection experiments in the ATIII-SV40 hepatoma cell line. The L promoter (from −2663 to +87) and 5′ deletions thereof were cloned upstream of the luciferase-coding sequences. The constructs were cotransfected with pRSVβgal as an internal control, and the results of luciferase activity were normalized for β-galactosidase activity.

The results are shown in Fig. 5. It can be seen that all the transcriptionally active sequences were located within the 360 bp upstream of the cap site, since addition of nucleotides −360 to −2663 did not modify promoter activity significantly. The 5′ deletions, performed within the proximal 360 bp and chosen to remove the footprinted sequences described above, showed that discrete regions had a clear-cut effect on transcription. Basal promoter activity (pPLLuc36) was increased twofold by the addition of the sequence that includes footprint I (pPLLuc71) and threefold by the addition of the sequence that also contains the footprint II region (pPLLuc111). A further increase in transcriptional activity was observed with pPLLuc138. This construct, which contains the footprint III region, had an activity two- to threefold higher than that seen with pPLLuc111. Addition of the sequence up to −219, which includes the liver-specific footprint IV (pPLLuc219), further increased transcription 1.5-fold. No further enhancement of transcriptional activity was observed with pPLLuc237, which contains the footprint V region. Finally, addition of the sequence from −237 to −360 (pPLLuc360), which includes footprint VI, yielded the highest promoter activity obtained in our assay system.

To investigate the effects of L promoter deletions in isolated hepatocytes, we transfected these cells with some of our pPLLuc constructs (Table 1). With pPLLuc36, pPLLuc71, pPLLuc111, and pPLLuc138 we observed effects comparable with those seen in transfection experiments performed on ATIII-SV40 cells. Basal promoter activity (pPLLuc36) increased two- to threefold on addition of the sequence that includes footprint I (pPLLuc71). Activity was again doubled when the sequence containing footprint II was present in the construct (pPLLuc111). Addition of the sequence that includes footprint III (pPLLuc138) further stimulated (twofold) transcriptional activity. Unlike ATIII-SV40 cells, sequences upstream of −138 did not increase promoter activity in isolated hepatocytes. This may be consistent with our observation that the viability of isolated hepatocytes was severely affected on transfection. Since this could affect

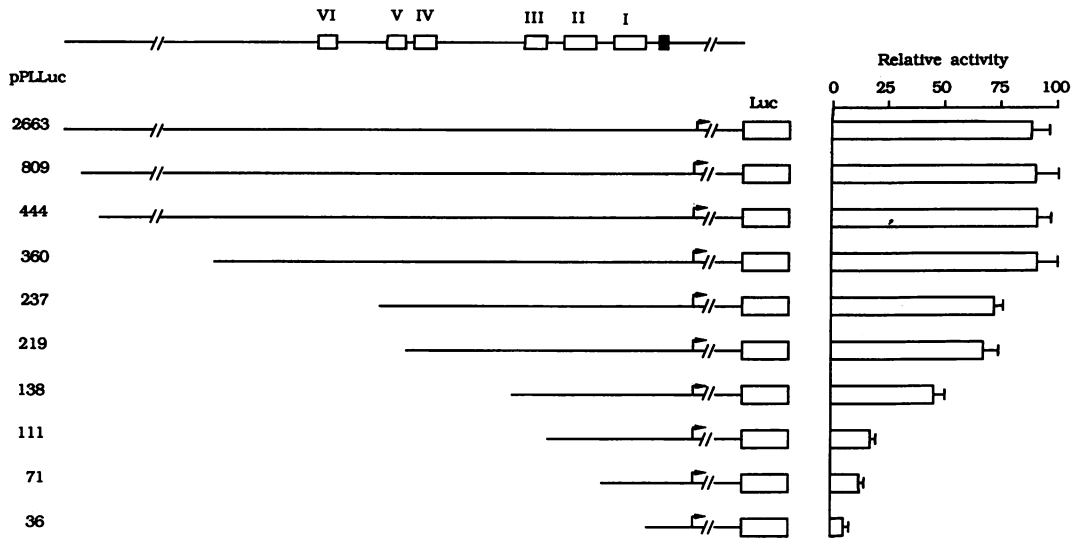

FIG. 5. Transcriptional activity from the PFK-2/FBPase-2 L promoter in pPLLuc constructs transfected in ATIII-SV40 cells. The promoter constructs and the positions of the footprints are schematized on scale. The black box refers to the TATA box. For each transfection, luciferase activity (test plasmid) was corrected for β-galactosidase activity (control plasmid). The whole series of constructs was tested concomitantly in four independent experiments. To correct for variation between experiments (see Materials and Methods), a value of 100% was assigned in each experiment to the activity of the construct that gave the highest ratio of luciferase to β-galactosidase. The data shown are means ± standard errors of the mean of these normalized values.

some cellular functions before others, one possibility is that the factors that bind upstream of −138 are more readily affected than those that bind downstream. Another possibility is that the activity of sequences upstream of −138 depends on factors, e.g., the liver-specific factor responsible for footprint IV, that are no longer present or active in cultured hepatocytes. Indeed, dedifferentiation is a known feature of hepatocytes in culture.

Finally, to analyze the activity of the L promoter in a nonhepatic cell line, we performed transfection experiments in rat pituitary GC cells. pPLLuc360, which produced a maximal activity in cells of hepatic origin, yielded in GC cells 1,193 ± 11 relative units (mean ± standard error of the mean for three experiments; luminescence units corrected for β-galactosidase activity). pPLLuc2663, which also produced maximal activity in cells of hepatic origin, yielded in GC cells 261 ± 28 relative units, i.e., a value 4.5-fold lower than that produced by pPLLuc360. This indicates that in GC cells the −360 to −2663 region functions as a negative regulatory element. This is consistent with the fact that we could not detect L PFK-2/FBPase-2 mRNA by Northern (RNA) blot analysis of GC cell RNA (not shown).

TABLE 1. Promoter activity of transfected pPLLuc constructs in isolated hepatocytes

Construct	Relative activity[a] in expt:	
	1	2
pPLLuc36	10	9
pPLLuc71	27	20
pPLLuc111	42	40
pPLLuc138	84	94
pPLLuc219	ND[b]	100
pPLLuc237	100	80
pPLLuc360	99	79

[a] Relative activity is expressed as described in the legend to Fig. 5.
[b] ND, Not determined.

DISCUSSION

We have demonstrated in vitro DNA-protein interactions on the L promoter of the gene encoding the L and M PFK-2/FBPase-2 isozymes and have delineated by transfection some of its transcriptional regulatory elements. A promoter fragment that is reduced to the 360 bp upstream of the cap site sufficed to confer maximal transcriptional activity in our assay systems. Six footprints were detected within this region with liver nuclear extracts.

The first two footprints upstream of the cap site were assigned to NF-I. Our DNase I and gel-shift experiments with purified NF-I suggested the presence of two additional NF-I-binding sites; one was between footprint I and the cap site, and another was just upstream of footprint V. NF-I actually refers to a family of proteins (25, 27) originating from alternative splicing of the same transcript (27) or from different genes (24). These proteins contain a highly conserved N terminus and a variable C terminus (17). Our transfection experiments demonstrated that the proteins which bind between footprint III and the TATA box, i.e., most likely NF-I, together stimulate transcription threefold. NF-I has been implicated in the transcriptional control of genes expressed in the liver (22). Thus, NF-I is a good candidate for participating in the control of transcription from the L PFK-2/FBPase-2 gene promoter in vivo.

Another protein that appears to mediate transcriptional stimulation of the L promoter is the one involved in footprint III. This footprint contains a sequence known to bind a family of octamer proteins. These include the ubiquitous Oct-1 factor, the lymphocyte-specific Oct-2A and Oct-2B factors, and the factors Oct-3 to Oct-10, which are detected in various mouse tissues and embryo at different stages of development (30). The Oct factors are transcriptional acti-

vators, but in F9 cells the octamer factor NF-A3, which is probably identical to Oct-4 (29), can mediate repression (13). The PFK-2/FBPase-2 L promoter octamer sequence stimulated transcription more than twofold. The footprint pattern seen with liver extract differed from that obtained with purified HeLa Oct-1. One interpretation is that the liver octamer protein differs from Oct-1. If so, these two proteins should, however, be very similar, since in band-shift assays an immunoglobulin heavy-chain gene fragment bearing the octamer sequence is retarded identically with a liver extract and with purified HeLa Oct-1 (30). Another interpretation is that the liver-specific pattern of footprint III results from the binding of a complex consisting of Oct-1 associated with another liver protein(s). In this case, the sequences located near the octamer consensus on the PFK-2/FBPase-2 L promoter could play a role in the formation of the postulated Oct-1-protein complex, since only one complex was visualized in the band-shift experiment mentioned above with the immunoglobulin promoter fragment.

A third type of factor that *trans*-activates the promoter studied here is the one responsible for the liver-specific footprint IV. We have tentatively assigned this footprint to Tf-LF2. The sequence protected contains the remarkable palindrome 5′-GCTTTGAAATNNATTTCAAAGC-3′. This palindrome is poorly conserved in the Tf-LF2-binding site of the human transferrin gene promoter sequence 5′-AGTCT GTCTTTGACCTTGAGCCCAGCT-3′. Since Ochoa et al. (21) did not mention that Tf-LF2 binding required a palindrome, further study is in order to determine whether this palindrome is the actual Tf-LF2 consensus or whether footprint IV is due to another protein.

The sequence upstream of footprint IV conferred 25% additional activity to the L promoter. This activity was due to the region between −219 and −360. We identified in this region a binding site for a DBP-like protein (footprint V), the 5′ side of which overlaps with an NF-I-binding site, and a binding site for a heat-stable poly(dG)-binding protein. Assuming that the NF-I site (−230 to −242) is lost in the pPLLuc237 construct, then either NF-I or the poly(dG)-binding protein or both are candidates for mediating transcriptional stimulation conferred by the region from −219 to −360. Clark et al. (2) have listed the longest poly(dG) stretches contained in polymerase II-transcribed genes. The same group of investigators detected poly(dG)-binding proteins in several tissues (14) but did not look for the presence of such proteins in liver. Although the function of these proteins is unknown, the erythrocyte-specific poly(dG)-binding BGP1 factor seems to be involved in nucleosome positioning, without having a transcriptional activity (14). Further work is needed to determine the role of the heat-stable poly(dG)-binding factor that accounts for footprint IV. It is known that not all specific protein-DNA interactions can be identified by footprinting. Thus, factors other than the poly(dG)-binding protein or NF-I which were not detected here could be involved in the activity of the sequence upstream from footprint V. For instance, the sequence (−261) TGAACCT (−255), which was not footprinted in our experiments, is a motif reported to bind the liver-specific *trans*-acting factor LF-A1 (21).

Expression of the PFK-2/FBPase-2 liver isozyme is controlled by insulin (3) and by thyroid (31) and glucocorticoid hormones. The latter stimulate transcription of the isozyme (16). Thus, the DNA-binding proteins and sequences delineated here might be involved in the hormonal regulation of this promoter, whether these sequences were active or not under the basal conditions of our assays. A case in point is the sequence around footprint V which we describe here as transcriptionally inactive. This sequence contains the motif (−241) GGAGCTTAATGTTCT (−227), which is compatible (underlined) with GGTACANNNTGTTCT, described as a glucocorticoid response element (1).

L PFK-2/FBPase-2 mRNA content is much higher in liver than in the other tissues in which it is detectable (5). Transcriptional and/or posttranscriptional regulation could explain this relative tissue specificity. Our transfection experiments shed light on this question. L promoter activity in liver cells was positively regulated by *cis*-acting sequences that bind tissue-specific factors (i.e., footprint IV and the liver-specific pattern of footprint III). Furthermore, transfection of L promoter constructs in pituitary tumor cells pointed to the existence of a negative regulatory element located between −360 and −2663. Taken together, these data provide evidence for positive regulation and derepression of PFK-2/FBPase-2 L mRNA synthesis in the liver as well as for negative regulation in other tissues.

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