Nucleic Acids Research, Vol. 20, No. 14 3575–3583

Characterization of an enhancer upstream from the muscle-type promoter of a gene encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

Martine I.Darville, Isabelle V.Antoine and Guy G.Rousseau*
Hormone and Metabolic Research Unit, Louvain University Medical School and International Institute of Cellular and Molecular Pathology, 75 Avenue Hippocrate, B-1200 Brussels, Belgium

Received May 12, 1992; Revised and Accepted June 17, 1992 GenBank accession no. M26215

ABSTRACT

The muscle-type isozyme of rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is encoded by a mRNA transcribed from the M promoter of a 55-kb gene, which also produces the liver-type isozyme from an alternative promoter. By transient transfection and *in vitro* protein-DNA binding assays we have delineated, within 4.7 kb of 5′ flanking sequence, the M promoter proper and an enhancer located between −1615 and −1809. This enhancer stimulated up to 12-fold the activity of the promoter in the context of an intact 5′ flanking sequence and close to 900-fold the activity of the minimal (+41 to −40) M promoter cloned directly downstream from it. A functional dissection of the enhancer by site-directed mutagenesis and use of oligonucleotides suggested that its activity involves the cooperative effect of six binding sites for *trans*-acting factors clustered within 150 bp. These sites contain either an EF-1A/E4TF1 motif (also known to bind the *ets* oncogene product) or a Sp1 motif, or both. The activity of the enhancer could be demonstrated in L6 myoblasts and myocytes and in FTO2B hepatoma cells. When left within the intact 5′ flanking sequence, however, enhancer activity was inhibited upon differentiation of myoblasts into myocytes.

INTRODUCTION

The expression of genes that encode rate-limiting or regulatory enzymes can be regulated through the use of alternative promoters. Different primary transcripts of the same gene typically encode isozymes that share the same catalytic core but differ by a regulatory domain often corresponding to the first exon, as in glucokinase (1) and pyruvate kinase (2). A case in point is 6-phosphofructo-2-kinase (EC 2.7.1.105, PFK-2)/fructose-2,6-bisphosphatase (EC 3.1.3.46, FBPase-2), a bifunctional enzyme that catalyzes the synthesis and degradation of fructose-2,6-bisphosphate. The latter is the most potent stimulator of 6-phosphofructo-1-kinase, hence of glycolysis (for a review, see ref. 3). There are several isozymes of PFK-2/FBPase-2. The muscle-type (M) isozyme has an amino acid sequence identical to that of the liver-type (L) isozyme, which has 470 residues, except for the first 32 residues which are replaced by a decapeptide in the M isozyme (4). This difference leads to a 10-fold decrease in PFK-2/FBPase-2 activity ratio and a loss of the site of phosphorylation by cyclic AMP-dependent protein kinase which, in the L isozyme, inactivates PFK-2 and activates FBPase-2 (3).

We have characterized a 55-kb rat gene (gene A), containing 15 exons, that encodes the M and L isozymes of PFK-2/FBPase-2 by alternative use of two promoters, referred to as M and L promoters (5). The M and L mRNAs share the same 13 exons. They begin with an additional, coding, exon which is specific for either the M isozyme (first exon of the gene, corresponding to the N-terminal decapeptide) or the L isozyme (second exon of the gene). This organization, together with the strategic position of PFK-2/FBPase-2 isozymes for integrating hormonal and metabolic signals in the control of glycolysis (3), prompted us to investigate the mechanisms involved in the transcriptional regulation of the gene. Work on the L promoter has been presented earlier (6). We have now explored by transient transfection the *cis*-acting elements over 4.7 kb upstream from exon 1, namely upstream from the transcription initiation site of the M-type mRNA. The putative *trans*-acting factors were studied by *in vitro* DNase I footprinting, band-shift, and methylation interference assays. This led to the delineation of the upstream proximal elements of the M promoter and to the identification of a potent enhancer which acts by binding a unique combination of ubiquitous factors.

MATERIALS AND METHODS

Cell cultures

Rat hepatoma FTO2B cells were grown as monolayers in a mixture (1:1) of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, supplemented with 2 mM glutamine and 10% fetal calf serum. Rat hepatoma HTC cells were grown in suspension culture in Swim's-S77 medium supplemented with 10 mM glucose and 10% newborn calf serum. Fibroblastic Rat-1

* To whom correspondence should be addressed

cells were grown in DMEM supplemented with 5% newborn calf serum. Rat (L6) and mouse (C2C12) skeletal muscle cell lines were grown in DMEM supplemented with 20 mM glucose and 20% fetal calf serum. Myogenic differentiation was induced by transferring confluent undifferentiated cultures to DMEM containing 2% horse serum (differentiation medium).

Plasmids and oligonucleotides

All p2KMluc plasmids contain an M promoter fragment cloned upstream from the firefly luciferase reporter gene of pXP2 (7). All constructs with 5′ deletions have the same 3′ end at nucleotide +41 relative to the major cap site of the M-type mRNA (5). This 3′ end was reconstitued by ligating the λ20 *Bam*HI-*Hae*II (−1253/+7) genomic fragment (5) to a double stranded oligonucleotide corresponding to the sequence from +8 to +41 with a 3′ added *Xho*I site, and by cloning this construct into the *Bam*HI and *Xho*I sites of the pXP2 polylinker. All 5′ deleted constructs were derived by standard procedures from the construct described above (−1253/+41) or, for p2KM4700luc and p2KM2400luc, from this construct plus the appropriate fragment of the λ20 genomic clone (5). The numbering of the plasmids corresponds to the position of the restriction site used to make the 5′ end, which was filled in with Klenow fragment or digested by mung bean nuclease (*Hph*I site at −91), and ligated into the *Sma*I site of the pXP2 polylinker. Enhancer fragments were subcloned in one of the remaining restriction sites of the polylinker upstream from the M promoter TATA box (p2KM40luc) or upstream from the herpes simplex virus thymidine kinase (tk) gene promoter, namely the *Bam*HI-*Bgl*II fragment of pBLCAT2 (8), inserted into the *Bgl*II site of pXP2. In the denomination of the plasmids, letters between brackets refer to the restriction sites used to isolate the enhancer fragment.

Synthetic double stranded oligonucleotides with *Hind*III and *Sal*I cohesive ends (underlined) had the following upper strand (with the nucleotides in parentheses added to the original sequence) : Oligo A (−1743/−1718): 5′-AGCTTAGGCGGGCAAAGGCATTGCCG; Oligo B (−1725/−1701): 5′-(AGCTT)CATTGCCGCCCGGAAGTGAGCTGGT(G); Oligo C (−1709/−1677): 5′-(AGCT)TGAGCTGGTTCCCCGCCTTCCTCTCGCGTTTCC(G). Oligonucleotides were inserted into the *Hind*III and *Sal*I sites of p2KM40luc. The resulting constructs were named p2KM[A]40luc, p2KM[B]40luc and p2KM[C]40luc. To make p2KM[A/B]40luc, p2KM[A]40luc was cut at the *Sal*I site, filled in with the Klenow fragment, and cut at the *Bam*HI site. The *Bam*HI-blunt ended *Sal*I fragment was inserted into the *Bam*HI-blunt ended *Hind*III sites of p2KM[B]40luc. To make p2KM[B/C]40luc, the *Bam*HI-blunt ended *Sal*I fragment of p2KM[B]40luc was inserted into the *Bam*HI-blunt ended *Hind*III sites of p2KM[C]40luc. The sequence of these constructs was verified by dideoxy sequencing (9). Other synthetic oligonucleotides were as follows. Oligo NF1: 5′-ATTTTGGCTACAAGCCAATATGAT; oligo USF: 5′-GTAGGCCACGTGACCGGG; oligo Sp1: 5′-GGGGCGGGGC; the latter oligo was used as a concatemer (three copies).

Site-directed mutagenesis

Site-directed mutagenesis was performed by using the Amersham oligonucleotide-directed mutagenesis system version 2. The −1675/−1744 enhancer fragment was isolated from p2KM[HHp]tkluc and cloned into M13mp18. Single stranded template was isolated and hybridized to a 43-mer oligonucleotide in which nucleotides −1722/−1711 (underlined) had been modified as follows: 5′-CTTAGGCGGGCAAAGGCATCATTATTTAAGGGTGAGCTGGTTC. The second strand was extended following the manufacturer's instructions. Mutated clones were identified by dideoxy sequencing (9). The mutated fragment was inserted upstream from the tk promoter. The resulting construct was named p2KM[HHp$_m$]tkluc.

Transfection and enzymes assays

Cells were transfected by the calcium phosphate method (10) with 10 μg of supercoiled test plasmid and 1 or 2 μg of pRSVβgal as an internal control, per 60 mm-diameter dish. FTO2B and Rat-1 cells were incubated overnight with the calcium phosphate precipitate and washed with phosphate-buffered saline before adding fresh medium. The cells were harvested 24 h later. L6 myoblasts were incubated for 5 h with the calcium phosphate precipitate, washed with DMEM and harvested after an additional 24 h in fresh medium as undifferentiated myoblasts. At the time of harvesting myoblasts, the medium of parallel sets of cultures was replaced by differentiation medium and the cells were cultured for an additional 96 h to obtain myocytes. Luciferase and β-galactosidase activities were measured as described (6). Background luciferase activity values obtained with pXP2 were 7 to 15 U in FTO2B cells and 10 to 50 U in L6 cells. Maximal values of 70,000 U in FTO2B cells and 200,000 U in L6 cells were obtained with enhancer test plasmids. Relative activities were calculated from the ratio of luciferase to β-galactosidase activity in each cell extract.

DNA-protein binding assays

Nuclear extracts from HTC, L6, and C2C12 cells were prepared as described (11) with modifications (12) for the muscle cell lines. Skeletal muscle extracts (12) and nuclear extracts from rat liver and whole extracts from FT02B cells (6) were prepared as described. For DNase I footprinting, 10 to 20 μg of proteins were incubated for 20 min at 0°C in 50 μl containing 10 mM Hepes (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 1 μg poly(dIdC), 2% polyvinyl alcohol and 10,000 to 20,000 cpm of 3′ end ^{32}P-labeled probe. Fifty μl of a 5 mM $CaCl_2$, 10 mM $MgCl_2$ solution were added and the samples were treated with various concentrations of DNase I for 1 min at 20°C. The reaction was stopped by adding 100 μl of stop buffer (200 mM NaCl, 20 mM EDTA and 1% sodium dodecyl sulfate). After extraction with phenol-chloroform and ethanol precipitation, samples were analysed on 6 or 8% polyacrylamide sequencing gels. For gel retardation assays, the binding reaction (20 μl) was performed in the same buffer as for DNase I footprinting, except that polyvinyl alcohol was omitted. One to 5 μg of proteins were preincubated for 10 min at 0°C with 1 to 3 μg of poly(dIdC) before addition of competitor and labeled probe (5000 cpm). The incubation was continued for 20 min at 0°C and the samples were electrophoresed on a 5% polyacrylamide gel (40:1) in 6.7 mM Tris (pH 7.9), 3.3 mM sodium acetate, 1 mM EDTA, at 4°C and 200 V with recirculating buffer. Methylation interference assay was performed as described (13)

RESULTS

Localization of *cis*-acting sequences

To delineate the 5′ flanking sequences involved in controlling the M promoter, a fragment of 4.7 kb upstream from the first exon and deletants thereof were linked to the luciferase reporter

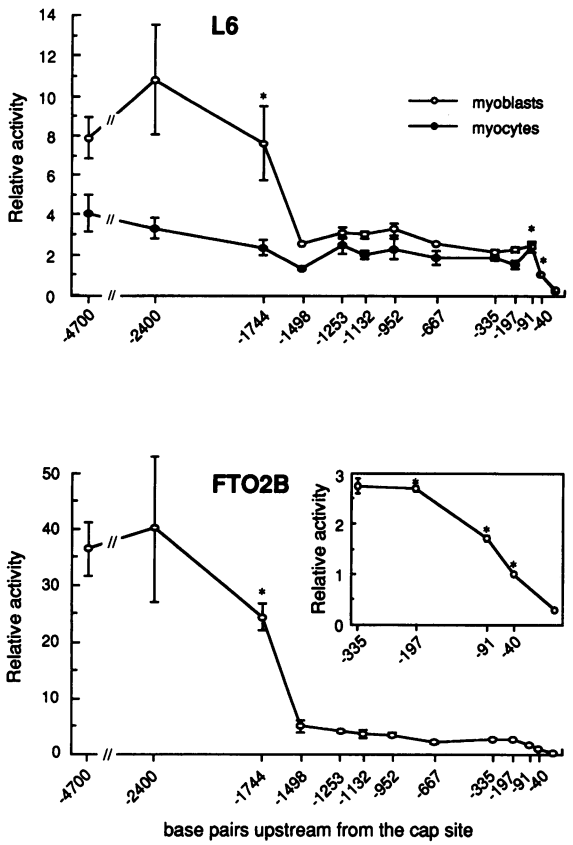

Fig. 1. Analysis by transfection of the region upstream from the M promoter. L6 and FTO2B cells were transfected with 5′ deletions constructs, from −40 to −4700 bp relative to the cap site, linked to the luciferase reporter gene. A value of 1 was assigned to the −40 construct (p2KM40luc) The last point at the right of the abscissa corresponds to the promoterless pXP2. The relative activity values are means ± SEM for 2 to 8 experiments in L6 cells (except one value for the −1498 construct) and 2 or 3 experiments in FTO2B cells. Error bars are not shown when smaller than the size of the experimental point. Asterisks mean that the value is significantly different from that obtained with the immediately shorter construct.

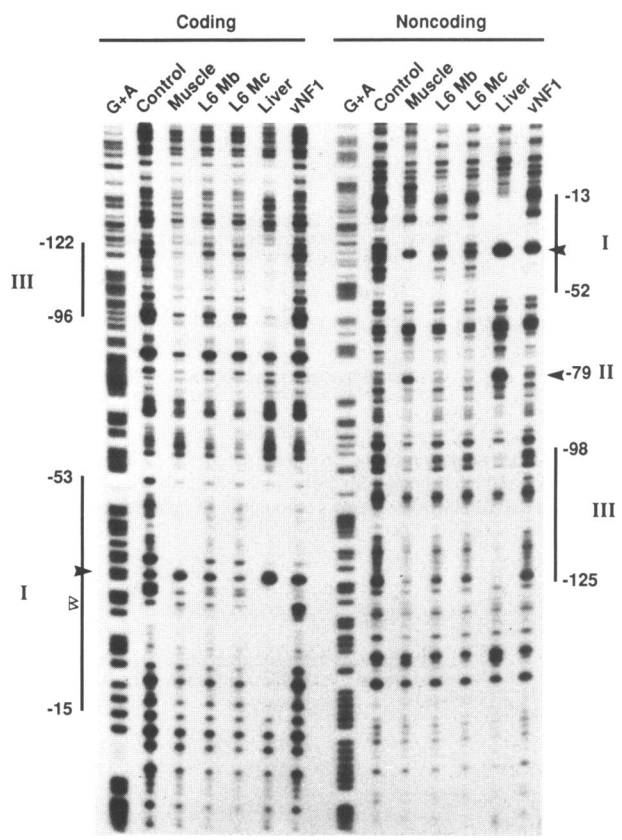

Fig. 2. DNase I footprinting and hypersensitive sites on the proximal region. The fragment from +41 to −197 was labeled on the coding or on the noncoding strand. Vertical bars delineate the footprints (roman numerals) obtained with the extracts indicated above the lines. Arrowheads, DNase I-hypersensitive sites; open arrowheads, hypersensitive sites seen only with vNF1. G+A, sequencing ladder; control, no extract used; Mb, myoblasts; Mc, myocytes; vNF1, purified recombinant NF1.

gene, and their activity was tested by transient transfection. Two types of rat cell lines were used. FTO2B hepatoma cells were chosen because they express a 2.1-kb PFK-2/FBPase-2 mRNA that contains the M-specific, but not the L-specific, exon (14 and our unpublished data). L6 cells were chosen as a skeletal muscle cell line. The 1.9-kb mRNA typical of adult muscle (4) is detectable neither in L6 cells myoblasts, nor in the myocytes obtained from the latter upon serum starvation. Instead, myoblasts express the same mRNA as FTO2B cells. This mRNA disappears when the cells are induced to differentiate into myocytes (our unpublished data).

The data in Fig. 1 show that, in L6 myoblasts, two regions were important for stimulating M promoter activity. The first 40 base pairs (bp), which contain the TATA box, were sufficient for expression of a luciferase activity five times the background. A 2.5-fold increase was obtained by including the first 91 bp of 5′ flanking sequence. No further significant change was seen with longer deletants until they included the region comprised between −1498 and −1744. In this case, promoter activity was further stimulated about 4-fold, an effect undistinguishable from that seen with the deletant ending at −2400 or with the nondeleted (4.7 kb) PFK-2/FBPase-2 gene fragment. After differentiation into myocytes, the same increase as in myoblasts was observed with the sequence between −40 and −91, without further change with the sequence between −91 and −1498. In contrast to myoblasts, however, the stimulatory effect of sequences beyond −1498 was not significantly greater than that of the region −40 to −91, suggesting the presence of differentiation-specific elements upstream from −91.

This finding of a proximal and a distal *cis*-acting element was confirmed with FTO2B cells (Fig. 1). In these cells, the proximal element included 197 bp of flanking sequence since the sequence between −40 and −91 stimulated luciferase activity 1.7-fold and addition of the sequence up to −197 further increased activity 1.7-fold. As to the distal sequence, it was actually three times more active in FTO2B cells than in L6 myoblasts, with a further stimulation reaching up to 12-fold. In FTO2B cells submitted to the protocol of serum starvation used to differentiate myoblasts into myocytes, the distal region was no less active than in FTO2B cells under normal culture conditions (data not shown). This makes unlikely a role of serum *per se* in the difference in activity of the distal region between myocytes and myoblasts.

These experiments delineate the proximal *cis*-acting element, namely the promoter proper, within the first 91 bp of 5′ flanking sequence for L6 cells and within the first 197 bp for FTO2B cells.

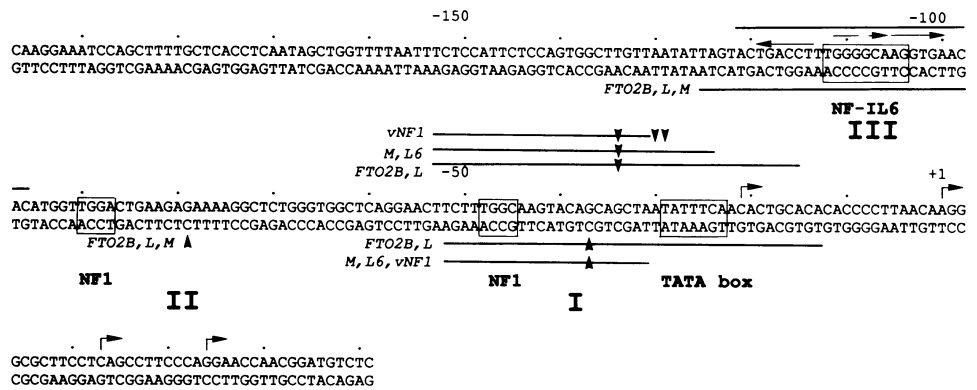

Fig. 3. Nucleotide sequence of the proximal region and localization of the footprints. The DNase I footprints obtained with the various extracts are indicated by horizontal lines and the hypersensitive sites by arrowheads. L, liver; L6, myoblasts and myocytes; M, muscle; vNF1, purified recombinant NF1. Consensus binding sequences are boxed. Horizontal arrows at footprint III indicate hemipalindromes known to bind the thyroid hormone receptor. Broken arrows indicate the transcription start sites.

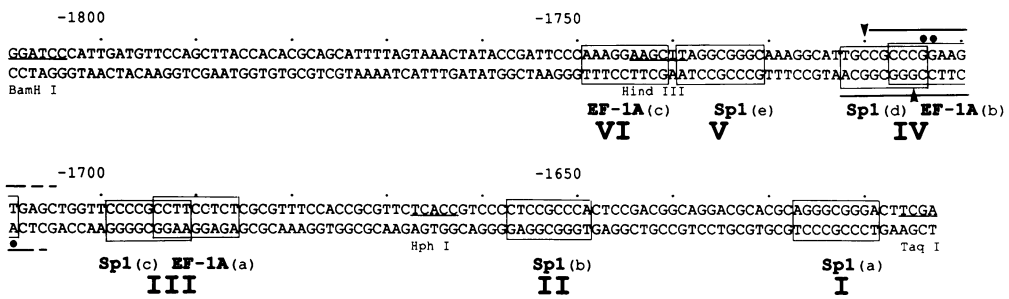

Fig. 4. Nucleotide sequence of the enhancer region and localization of the footprint. The DNase I footprint is indicated by horizontal lines and the hypersensitive sites by arrowheads. Bases where methylation interferes with binding are indicated by a circle. Sequences compatible with the EF-1A or the Sp1 consensus are boxed, with small case letters as referred to in the text. The six potential sites of protein-DNA interaction are numbered I to VI. The restriction sites used to produce the enhancer fragments are underlined.

They also suggest the presence, between −1498 and −1744, of an enhancer which is very active in FTO2B cells, moderately active in L6 myoblasts, and virtually inactive in L6 myocytes.

Trans-acting factors of the promoter region

The factors involved in the activity of the proximal *cis*-acting element were studied by *in vitro* DNase I footprinting (Fig. 2). With nuclear extracts from cell lines and tissues, three sites of protein-DNA interactions (labeled I, II and III in Figs. 2 and 3) were identified within 197 bp of 5′ flanking sequence, based on DNase I hypersensitivity (sites I and II) and on footprints (sites I and III). Site I was seen with extracts not only from FTO2B cells (not shown), L6 cells and rat skeletal muscle, but also from liver. Sites II and III were seen with all these extracts except with those from L6 cells.

Sites I and II contain the sequence $TGG^{C}/_{A}$, the hemipalindrome typical of the consensus for recognizing, and sufficient for binding (15), the ubiquitous proteins of the NF-1 family (16). Muscle and liver protein binding at sites I and II was prevented by competing NF-1 oligonucleotide (not shown) and binding at site I was mimicked by an active form (17) of recombinant liver NF-1 (Fig. 2). The position of the DNase I hypersensitive site on the noncoding strand with respect to the hemipalindrome was the same in sites I and II (Fig. 3). We conclude that these sites most likely correspond to binding of NF-1, and that the affinity of this factor is lower for site II than for site I, consistent with the known role of neighboring sequences in NF-1 binding (18). At site I, extracts from liver and FTO2B cells, but not from muscle and L6 cells, produced on both strands a footprint that extended beyond the TATA box, namely further downstream than with other extracts. This additional interaction might modulate M promoter activity in cells of hepatic origin.

As to the footprint on site III, it contains (Fig. 3) three hemipalindromes reported to bind the thyroid hormone receptor (19). It also includes a sequence that recognizes (18) DBP, a liver-specific protein (20), and NF-IL6 (21) also called IL-6DBP (22) or LAP (23).

The distal *cis*-acting sequence contains an enhancer

To investigate the presence of an enhancer in the distal *cis*-acting sequence identified by 5′ deletion (Fig. 1), fragments of this sequence were cloned in the sense or antisense orientation upstream from the minimal (+41 to −40) PFK-2/FBPase-2 M promoter or from a heterologous (tk) promoter. These constructs were linked to the luciferase reporter gene and tested by transient transfection. FTO2B cells were used first because, in the

Table 1. Enhancer activity of fragments of 5′ flanking sequence and of oligonucleotides in transfected cells

Construct[a]	Binding site(s)[b]	Relative activity[c]					
		Minimal M promoter[d]			tk promoter		
		FTO2B	L6 myoblasts	L6 myocytes	FTO2B	L6 myoblasts	L6 myocytes
B-T	VI to I	873±36 (4)	180±30 (5)	117±31 (3)	14±1.7 (5)	3.3±0.4 (5)	1.8±0.2 (3)
T-B	I to VI	–	–	–	10±2.0 (4)	1.9±0.3 (4)	–
B-H	VI	4.5, 1.1 (2)	0.9±0.1 (4)	1.3 (1)	3.1, 3.1 (2)	1.5, 1.1 (2)	–
H-Hp	V–IV–III	299±36 (4)	42±7 (4)	46±8 (3)	26±2.7 (5)	4.5±0.4 (3)	2.3, 2.5 (2)
Hp-H	III–IV–V	–	–	–	11±5.0 (3)	3.8, 3.3 (2)	–
H-Hp_m	V–III	11±5 (3)	7.6 (1)	4.5 (1)	9.8±2.4 (3)	3.3, 2.3 (2)	2.5, 2.3 (2)
Hp-T	II–I	60±10 (3)	31±6 (4)	35±7 (3)	5.5±1.7 (3)	1.8, 1.9 (2)	2.1, 2.1 (2)
T-Hp	I–II	52±19 (3)	20, 21 (2)	–	–	–	–
A	V	2.0±0.6 (4)	0.8, 1.3 (2)	1.9, 2.1 (2)	–	–	–
B	IV	18±2.6 (3)	5.7±1.0 (3)	9.6±3.8 (3)	–	–	–
C	III	6.1±0.6 (4)	3, 6.9 (2)	5, 7.9 (2)	–	–	–
A/B	V–IV	10±4 (3)	4.1 (1)	9 (1)	–	–	–
B/C	IV–III	25±12 (3)	10.2 (1)	42 (1)	–	–	–

[a]The first eight constructs refer to the restriction fragments (B, *Bam*HI; H, *Hind*III; Hp, *Hph*I; T, *Taq*I) and the last five constructs refer to the oligonucleotides and combinations thereof described in the Methods section (see also Fig. 4). All these constructs were linked to the luciferase reporter gene via the minimal M promoter or the tk promoter and transfected in the cell lines indicated.
[b]See Fig. 4.
[c]Mean ratio ± SEM of the activities obtained with the construct and with the corresponding promoter alone, for the number of independent experiments given in parentheses. –, not determined.
[d]Minimal PFK-2/FBPase-2 M-type promoter (p2KM40luc).

transfection experiments described in Fig. 1, the distal region was more active in these cells than in L6 cells. No enhancer was found in the sequence from −1392 to −1615. Indeed, luciferase activity was not significantly increased when the homologous or the heterologous promoter was 5′ flanked with fragments from −1392 to −1615, from −1392 to −1498, or from −1498 to −1615 (not shown). In contrast, the fragment B-T (−1809/−1615, Fig. 4) was very active under these conditions (Table 1). It behaved as an enhancer because it stimulated 10 to 14-fold the activity of the heterologous promoter in either orientation (compare B-T with T-B). Stimulation was close to 900-fold with the minimal M promoter. This difference in sensitivity of the two promoters was not surprising since the tk promoter, which contains two Sp1 and one NF-1 binding sites (24), was at least 20 times more active than the minimal M promoter in FTO2B cells, 40 times in L6 myocytes, and 70 times in L6 myoblasts. Thus, a lesser degree of stimulation would be expected with the tk promoter.

To localize the enhancer more precisely, three subfragments of B-T were generated by restriction (Fig. 4) at −1675 (*Hhp*I) and at −1744 (*Hind*III). The most distal subfragment (B-H, −1809/−1744) weakly affected transcription, stimulation being about 3-fold with either promoter. The most proximal subfragment (Hp-T, −1675/−1615) was more active. It had about 50% of the potency of the intact enhancer when in front of the tk promoter (6-fold stimulation) and about 10% of the enhancer potency (60-fold stimulation) when in front of the minimal M promoter. The central subfragment (H-Hp, −1744/−1675) was the most active. It was even more potent (26-fold stimulation) than the intact enhancer when combined with the tk promoter and one third as potent (300-fold stimulation) with the minimal M promoter (Table 1). We conclude from these experiments that the distal *cis*-acting element of the gene region studied here contains a potent enhancer localized between −1615 and −1744. Consistent with current models (25), this enhancer probably entails several interacting modules. Indeed, neither one of its active subfragments fully mimicked the activity of the intact enhancer when linked to the minimal M promoter.

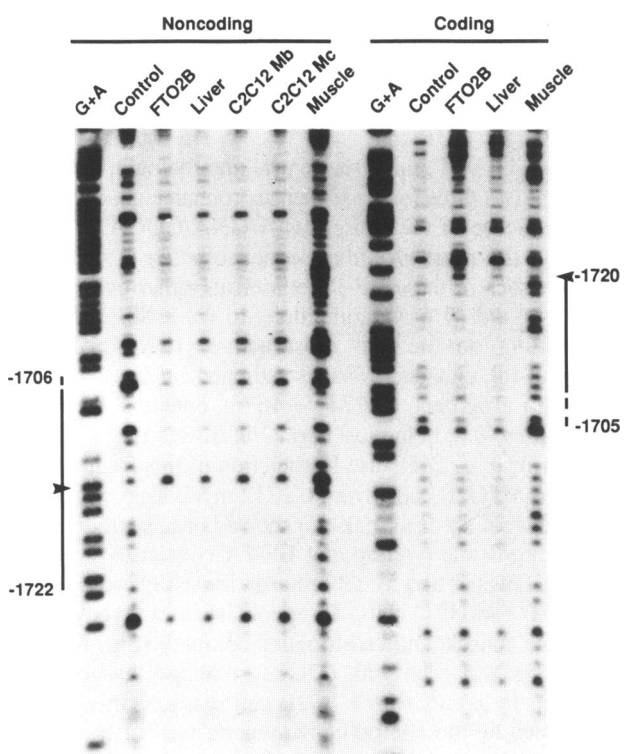

Fig. 5. DNase I footprinting on the middle fragment of the enhancer region. Fragment H-Hp (−1744/−1675) was labeled on the noncoding or on the coding strand. Vertical bars delineate the footprint obtained with the extracts as indicated above the lines. G+A, sequencing ladder; control, no extract used; Mb, myoblasts; Mc, myocytes; arrowheads, DNase I-hypersensitive sites.

Factor binding to the enhancer

Examination of the sequence of the enhancer region (−1615/−1809) shows potential sites for binding two types of ubiquitous factors (Fig. 4). There are five (*a*–*e*) sequences compatible (18) with the Sp1 consensus sequence G/TG/A-

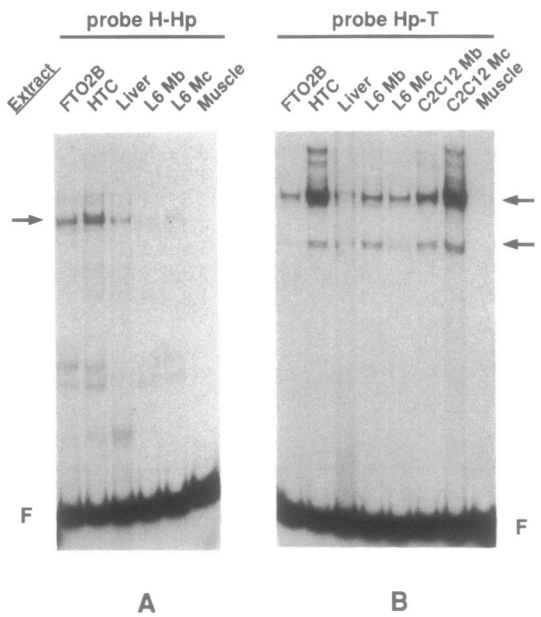

Fig. 6. Tissue distribution of the binding activities in the enhancer region by gel retardation assay. Extracts as indicated above the lines were incubated with (A) the labeled H-Hp fragment (−1744/−1675) or (B) the labeled Hp-T fragment (−1675/−1615). Arrows indicate the bound probe and F the free probe. Mb, myoblasts; Mc, myocytes.

GGCG/TG/AG/AGT, the three central ones being in the reverse orientation. Also, three (*a*−*c*) sequences contain eight consecutive out of the nine bp of the consensus A/GNA/CGGAT/AGT for EF-1A (26), the most proximal sequence being in the reverse orientation. Each of these EF-1A consensus either overlaps with (consensus *a* and *b*) or is contiguous to (*c*) a Sp1 consensus. Factor binding on the two most active (H-Hp and Hp-T) fragments of the enhancer was investigated by *in vitro* assays.

The H-Hp fragment (−1744/−1675) contains the EF-1A consensus *a* and *b* (*c* being lost upon *Hind*III restriction) and the Sp1 consensus *c*, *d*, *e*. DNase I protection of this fragment with extracts from FTO2B cells, from C2C12 myoblasts or myocytes, or from liver (but not from muscle) showed one specific footprint corresponding to the overlapping EF-1A consensus *b* and Sp1 consensus *d* (Figs. 4 and 5). Gel retardation assays with extracts from FTO2B and HTC cells, L6 myoblasts and myocytes, and liver, but not muscle showed a major complex (Fig. 6A). The same complex was seen with C2C12 myoblasts and myocytes (not shown). In assays with FTO2B cell extracts, this complex was prevented by the competing fragment itself or by oligo B, which corresponds to the footprinted region. It was prevented neither by fragment H-Hp_m in which the consensus *b* for EF-1A and *d* for Sp1 had been mutagenized, nor by oligo C which corresponds to the overlapping EF-1A consensus *a* and Sp1 consensus *c*, nor by a Sp1 consensus oligonucleotide (Fig. 7A). When this enhancer fragment was methylated at single purines using dimethylsulfate and subsequently incubated with FTO2B cell extracts, three contact points were detected and they were located within the consensus *b* for EF-1A (Fig. 4). This picture is incompatible with the known pattern of methylation interference produced by Sp1 (27). These three points of methylation interference actually coincided with contact points identified by the same technique in the two EF-1A consensus of the adenovirus E1A core enhancer element using HeLa cell nuclear extracts (26). Thus, the three types of assays used here indicate preferential factor binding *in vitro* to the EF-1A rather than the Sp1 consensus, and to the sense (*b*) rather than the antisense (*a*) EF-1A consensus in the H-Hp fragment.

As to the Hp-T fragment (−1615/−1675), it contains the Sp1 consensus *a* and *b* (Fig. 4). No footprint was seen on this fragment with FTO2B cell extracts, consistent with the reported (27) low sensitivity of this method for detecting Sp1 binding. However, gel retardation assays showed two doublets typical of Sp1 binding with extracts from L6, C2C12, FTO2B or HTC cells, and from liver, but not from muscle (Fig. 6B). With FTO2B cell extracts, these doublets were competed out by an Sp1, but not by an USF, oligonucleotide (Fig. 7B). Sp1 reportedly occurs as two variants (95 and 105 kDa) of the same polypeptide (28). The occurrence of two Sp1 binding sites on this fragment could explain the presence of two doublets. These doublets were inhibited by the zinc-chelating agent, 1,10-phenanthroline, and this was counteracted by $ZnSO_4$ (Fig. 7B), as expected from the requirement for zinc fingers in the DNA binding activity of Sp1 (28). These data are compatible with the binding of a Sp1-like protein on the proximal half of the enhancer. Consistent with this, the enhancer fragment was active in both orientations (Hp-T *versus* T-Hp in Table 1) as is the case for Sp1 binding sites (27).

Role of factor binding in enhancer activity

These *in vitro* binding data suggest that two types of ubiquitous factors, namely EF-1A and a Sp1-like protein, can interact with the enhancer. If factor binding where the consensus are overlapping is mutually exclusive, then the enhancer contains six potential sites of protein-DNA interactions, labeled I to VI in Fig. 4. Further functional dissection of the enhancer was performed by reducing it to portions containing only one or two of these sites. This was achieved by determining how oligonucleotides or the mutagenized enhancer fragment H-Hp_m would stimulate the minimal M promoter in transfection experiments. This promoter was preferred to the tk promoter with which stimulation was less dramatic. The mutation (H-Hp_m) was chosen to destroy the overlapping Sp1 and EF-1A consensus at site IV, because this site was the only one detectable by footprinting.

The results are shown in Table 1. Together with those obtained with the enhancer fragments, these transfection data indicate that *trans*-activation *in vivo* may involve the cooperative binding of factors at each of the six sites. Indeed, oligo A (site V) which contains a single Sp1 consensus stimulated activity 2-fold, while fragment Hp-T which contains two Sp1 sites (I and II) stimulated activity 60-fold, suggesting that cooperative binding occurs at site I and site II. Fragment B-H namely site VI (EF-1A) stimulated promoter activity 3-fold, and oligo C namely site III (Sp1/EF-1A in the same orientation) stimulated 6-fold. Oligo B namely site IV alone (Sp1/EF-1A in opposite orientations) stimulated 18-fold. The individual, cooperative, contribution of sites III, IV and V was demonstrated by the fact that their association two by two in any combination (oligos A/B and B/C, H-Hp_m) stimulated promoter activity 10 to 25-fold while the three together (fragment H-Hp) stimulated 300-fold. When comparing the results obtained with oligos A, B and C to those with oligos A/B and B/C, no clear-cut potentiation of site IV activity by sites V or III was seen, perhaps because sites III and IV are separated by 25 bp and sites IV and V by 27 bp in these

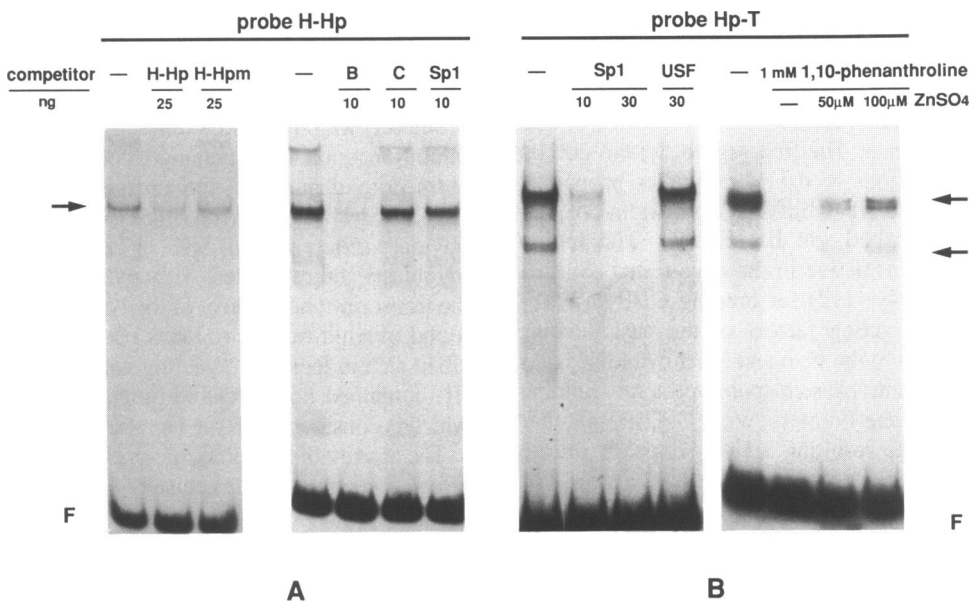

Fig. 7. Competition experiments by gel retardation assay with fragments of the enhancer region. An FTO2B cell extract was incubated with (A) the labeled H-Hp fragment (−1744/−1675) or (B) the labeled Hp-T fragment (−1675/−1615) and the quantity of the following competitors indicated above the lines: fragment itself (H-Hp), mutated fragment (H-Hp_m), oligonucleotides B or C, consensus oligonucleotides Sp1 or USF (see Materials and Methods), or in the presence of 1,10-phenanthroline and $ZnSO_4$. Arrows indicate the bound probe and F the free probe.

constructions, instead of 8 or 9 bp in the enhancer. When the six sites (I to VI) coexisted in their natural context (fragment B-T), stimulation reached close to 900-fold, further supporting a modular model for the function of this enhancer.

This cooperative model would also account for the different sensitivity of the tk promoter and the minimal M promoter to the enhancer fragments (Table 1). The H-Hp fragment (−1744/−1675), which lacks sites I, II, and VI of the enhancer, lost about 60% of the activity of the intact enhancer when tested with the minimal M promoter, but retained full activity with the tk promoter. This is not surprising if one considers that the tk promoter contains two Sp1 and one NF-1 sites which could substitute for the cooperating enhancer binding sites missing from H-Hp fragment. Sp1 promoter sites have indeed been shown to cooperate with other Sp1 sites (29,30). By the same token, the Hp-T enhancer fragment (−1675/ −1615) which contains only sites I and II, the H-Hp_m fragment which contains only sites III and V, and the B-H fragment (−1809/−1744) which contains only site VI, all retained more relative activity with the tk than with the minimal M promoter.

Finally, the transfection experiments showed that the function of the enhancer is not totally independent of distance and orientation effects. Indeed, the 900-fold stimulation seen in FTO2B cells when the enhancer was fused to the minimal M promoter was reduced to a 12-fold stimulation when it was located 1.7 kb upstream from it in the context of the intact 5′ flanking sequence (Fig. 1). Also, the enhancer core, which contains sites III, IV and V, was less active in the reverse (Hp-H) than in the right (H-Hp) orientation (Table 1).

Enhancer activity in myoblasts and in myocytes

As shown in Fig. 1 by transfecting 5′ deletants in L6 cells, when the 5′ flanking sequence was extended beyond −1498 there was an increase in relative luciferase activity in myoblasts but not in myocytes. To investigate whether the enhancer was inactive in myocytes, the activity of fragments of the region between −1498 and −1809 cloned upstream from the tk promoter or from the minimal M promoter was tested by transfection in L6 cells prior to or after differentiation (Table 1).

In myoblasts, the enhancer was functional with the two promoters, albeit less so than in FTO2B cells. This lesser activity is consistent with the data in Fig. 1. The limits of the enhancer were the same as in FTO2B cells since the fragment from −1498 to −1615 was inactive (not shown). The relative activity of the enhancer fragments tested in myoblasts was also in keeping with the corresponding results obtained in FTO2B cells (Table 1). In myocytes, the enhancer and fragments thereof were as active as in myoblasts when tested with the M promoter, suggesting that the *trans*-acting factors specific for the enhancer were still present and active after differentiation. However, the relative activities measured for the enhancer (B-T) and for its middle (H-Hp) fragment when cloned upstream from the tk promoter were lower in myocytes than in myoblasts, as seen with the intact 5′ flanking sequence. This difference was abolished by mutagenesis of site IV (see the H-Hp_m fragment), suggesting that EF-1A is involved in this effect. These data also suggest that sequences present in the tk promoter or in the 5′ flanking sequence outside the minimal M promoter cooperate with the enhancer to stimulate transcription more efficiently in myoblasts than in myocytes. An effect of serum *per se* in this difference is unlikely since stimulation of the tk promoter with the B-T fragment and with its H-Hp subfragment was as strong in the absence as in the presence of serum in FTO2B cells (not shown).

Consistent with the ubiquitous character of the enhancer binding factors described here, the relative activity of the enhancer (B-T) or of its middle (H-Hp) fragment cloned upstream from the tk promoter was as high in Rat-1 fibroblasts as in L6 myoblasts (not shown).

DISCUSSION

We have investigated the functional organization of the PFK-2/FBPase-2 gene A upstream from the cap site for the M-type mRNA. Two main control regions were identified within 4.7 kb of 5′ flanking sequence. The first region, within 200 bp from the cap site, corresponds to the M promoter proper. It contains the TATA box, two NF-1 binding sites, and a protein-binding element that we called site III (Fig. 3). The region containing the NF-I sites was active in the cells tested. Site III contains a consensus (−104/−112) for binding DBP and NF-IL6/LAP, which are *trans*-acting factors of the basic-leucine zipper family. NF-IL6 binds to the IL-6 and other cytokine genes and to genes encoding the acute-phase response proteins. Binding involves homodimers or heterodimers with C/EBP, another member of this family. The resulting activity depends on the nature of the dimer and on the type of cell (20,22,23). For example, while DBP is liver-specific (20), NF-IL6 is constitutively expressed in several hepatoma cell lines (21); LIP, a truncated form of LAP, forms with the latter a dominant negative heterodimer (31). Such features might explain why the M promoter region between −91 and −197 was active in FTO2B cells but inactive in L6 cells. In fact, the footprint on site III of the promoter was not seen with L6 cell extracts.

Site III also includes three hexameric half-sites for the thyroid hormone receptor (19) arranged as inverted palindromes. Two or more copies of this consensus actually bind not only the thyroid receptor but also other members of the nuclear receptors superfamily, i.e. the retinoid, vitamin D_3, and estrogen receptors, in the form of homo- or heterodimers (19,32). The distal half-site corresponds to a perfect consensus (5′ AAAGG-TCA) for NGFI-B, also called nur/77, an early-response protein and orphan member of the nuclear receptor superfamily, which binds to a DNA sequence that contains only one half-site (33). The significance of these receptor sites for the control of M-type mRNA transcription is unknown.

The regulatory sequences of genes expressed in muscle include motifs that are recognized by so-called muscle-specific *trans*-acting factors. The MEF-2, the CarG, the M-CAT, and the E boxes are representative of such motifs (34,35). Among these motifs, only potential E boxes were found in the 1.8 kb of 5′ flanking sequence studied here, at −98 (CACATG), −632 (CAGTTG), −1016 (CATTTG), −1033 (CAGATG) and −1167 (CAGATG). The E box, found in all skeletal muscle genes described to date (36), binds myogenic transcription factors of the basic helix-loop-helix family such as myoD (MEF-I), myogenin, Myf5 and MRF-4 (34). The role of this motif and of muscle-specific factors in M-type mRNA expression remains to be investigated.

The other *cis*-acting region is a 200-bp fragment (−1615/−1809) that can stimulate up to 12-fold the activity of the M promoter proper in the context of the intact flanking sequence and close to 900-fold the activity of the minimal (+41/−40) M promoter cloned directly downstream from it. This distal fragment had all the properties of an enhancer, namely action from a long distance (1.7 kb), activation in both orientations, efficiency with a heterologous promoter, and modular organization. When L6 myoblasts are allowed to differentiate into myocytes, PFK-2/FBPase-2 mRNA expression is inhibited (our unpublished data). Consistent with this observation, the sequences upstream from −91 stimulated transcription from the transfected deletants in L6 myoblasts but not in myocytes. However, this inhibitory effect of differentiation was no longer seen when the enhancer was directly linked to the minimal M promoter, presumably because of distance effects or of the lack of *cis*-acting sequences. Indeed, by using the tk promoter, which provides binding sites for Sp1 and NF-1, some inhibition upon differentiation could be seen. This inhibition seemed to depend on the presence of the enhancer site IV (EF-1A/Sp1). Yet, our protein-DNA binding assays showed no obvious difference in Sp1 or EF-1A content between L6 myoblasts and myocytes. Moreover, the role of these factors in the transcriptional control of the typical (1.9 kb) M-type mRNA found in adult muscle remains open since our muscle extracts, albeit rich in factors interacting with the promoter sites I, II and III, contained no detectable binding activity for the Sp1 sites, which is consistent with the literature (37), or for the EF-1A sites.

The activity of the enhancer appeared to involve the cooperative effect of six binding sites clustered within 150 bp. These six sites were tentatively assigned to two types of ubiquitous factors, Sp1 and EF-1A. Binding of these factors to sites III and IV was considered to be mutually exclusive because of consensus overlap. Although sites V and VI could be functionally separated in the constructs tested, factor binding there might be mutually exclusive in the promoter *in situ*, due to steric hindrance. The Sp1 consensus in site III also contains the core consensus GCGGGG for NGFI-C, an early-response protein which is, like Sp1, a zinc finger transcriptional activator (38). Sp1-like factors reportedly participate to the developmental control of the chicken gene encoding the α-subunit of muscle acetylcholine receptor (39). The promoter region of this gene contains a site for the overlapping binding of Sp1 and of a G stretch-binding protein. Upon differentiation of myoblasts into myotubes, which is concomitant with increased expression of the gene, the abundance of Sp1 appears to increase, while that of the G stretch-binding protein decreases, as if the latter were an inhibitor displaced by Sp1 (39). However, these interactions take place at −50 in the acetylcholine receptor gene i.e. immediately upstream from the TATA box, as is often the case for Sp1 binding (29). Because Sp1 is a typical proximal promoter element, one cannot rule out the occurrence of an unidentified promoter in the vicinity of the enhancer described in this paper. This additional promoter need not contain a TATA box since the latter is often absent from GC-rich promoters, where it is replaced by an initiator (Inr) sequence (40). It might determine the synthesis of the PFK-2/FBPase-2 mRNA expressed in FTO2B cells and L6 myoblasts. Indeed, although this mRNA contains exon 1, which is specific for the M isozyme, it appears to be slightly longer than the M-type mRNA (14) and therefore could contain (an) additional exon(s) upstream from exon 1. Even if (a) cryptic cap site(s) were present near the GC boxes, our data show that the B-T fragment contains regions that displayed enhancer properties. Thus, the enhancer could act on several promoters of the PFK-2/FBPase-2 gene A. Indeed, Sp1 can act *in vivo* from enhancer sites that are as far as 1.8 kb from the promoter and exhibit synergistic interaction with promoter-proximal binding sites. This appears to rest on the ability of Sp1 to engage in homologous or heterologous protein-protein interactions through DNA looping (30). Although Sp1 is ubiquitous, the potency of the enhancer may depend on the cell type because of the large tissue differences in Sp1 concentration and of possible effects of posttranslational modifications on Sp1 activity (37).

Binding site redundancy and cooperation with other factors as described here is a known feature of Sp1 (29,30). To our

knowledge, however, this is the first example of an association of Sp1 sites with EF-1A sites. EF-1A does not appear to be tissue-specific since it is present in human HeLa (cervix adenocarcinoma), 293 (embryonic kidney) and HepG2 (hepatoma) cell lines, in rat liver, and in mouse L (fibroblast) cells (26). It transactivates the adenovirus E1A enhancer element I by binding to two cooperating motifs located at −300 and −270 (26). EF-1A also binds to a similar motif at −140 in the adenovirus E4 transcriptional control region, in which case it was termed E4TF1 (41). E4TF1 was purified from HeLa cells and shown to consist of at least two distinct subunits identified as a 60 kDa DNA-binding peptide and a 53 kDa transcriptional modulator peptide (42). EF-1A also binds to the PEA-3 site of the polyoma virus A enhancer (26). The latter site may be involved in the induction of this polyoma enhancer by the *ets* oncogene product, a potential member of the cell signalling network. Binding site IV in the enhancer described here contains a perfect consensus for the *ets*-1 binding sequence (18), and binding sites III and VI contain seven consecutive out of the eight bp of this consensus. E4TF1 binding sites have been described in the N-*ras* gene promoter region (43) and potential *ets* binding sites are found in the promoters of the so-called early response genes and of genes that are highly expressed in transformed cells (44). Cell transformation, or stimulation by mitogens, is often accompanied by an increased PFK-2 activity and fructose-2,6-bisphosphate concentration. This might contribute to the so-called Warburg effect, namely the abnormally high glycolytic flux seen under these conditions (45,46). Whether this phenomenon involves increased transcription of the PFK-2/FBPase-2 gene studied here remains to be established. Still, the identification in this gene of an enhancer liable to possible control by the oncogene products of the *ets* family offers interesting working hypotheses.

ACKNOWLEDGEMENTS

We thank Th. Lambert for secretarial assistance and F.Lemaigre and S.Durviaux for liver and FTO2B cell extracts, R.Roeder for helpful comments and L.Hue for continued interest. We also gratefully acknowledge the following generous gifts: FTO2B cells from G.Schütz (Heidelberg), L6 cells from C.Pinset (Paris), C2C12 cells from H.Bloemendal (Nijmegen), Rat-1 cells from J.Wyke (Glasgow), purified vNFI from R.Cortese and R.De Francesco (Heidelberg), and pRSVβgal from J.Martial (Liège). This work was supported by the Belgian State-Prime Minister's Office−Science Policy Programming (Incentive Program in Life Sciences grant 20), by the Belgian Cancer Association, and by the Interuniversity Poles of Attraction.

REFERENCES

1. Magnuson, M.A. (1990) *Diabetes*, **39**, 523−527.
2. Tremp, G.L., Boquet, D., Ripoche, M.A., Cognet, M. , Lone, Y.C., Jami, J. Kahn, A. and Daegelen, D. (1989) *J. Biol. Chem.*, **264**, 19904−19910.
3. Hue, L. and Rider, M.H. (1987) *Biochem. J.*, **245**, 313−324.
4. Crepin, K.M., Darville, M.I., Hue, L. and Rousseau G.G. (1989) *Eur. J. Biochem.*, **183**, 433−440.
5. Darville, M.I., Crepin, K.M., Hue, L. and Rousseau, G.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6543−6547.
6. Lemaigre, F.P., Durviaux, S.M. and Rousseau, G.G. (1991) *Mol. Cell. Biol.* **11**, 1099−1106.
7. Nordeen, S.K. (1988) *BioTechniques*, **6**, 454−457.
8. Luckow, B. and Schütz, G. (1987) *Nucleic Acids Res.*, **15**, 5490.
9. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
10. Wigler, M., Pollicer, A., Silverstein, S. and Axel, R.A. (1978) *Cell*, **14**, 725−731.
11. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.
12. Zahradka, P., Larson, D.E. and Sells, B.H. (1989) *Exp. Cell Res.*, **185**, 8−20.
13. Chodosh, L.A., Baldwin, A.S., Carthew, R.W. and Sharp P.A. (1988) *Cell*, **53**, 11−24.
14. Cifuentes, M.E., Espinet, C., Lange, A.J., Pilkis, S.J. and Hod, Y. (1991) *J. Biol. Chem.*, **266**, 1557−1563.
15. Paonessa, G., Gounari, F. , Frank, C. and Cortese, R. (1988.) *EMBO J.*, **7**, 3115−3123.
16. Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) *Nature*, **334**, 218−224.
17. Gounari, F., De Francesco, R., Schmitt, J., van der Vliet, P.C., Cortese, R. and Stunnenberg, H. (1990) *EMBO J.*, **9**, 559−566.
18. Faisst, S. and Meyer, S. (1992) *Nucleic Acids Res.*, **20**, 3−26.
19. Brent, G.A., Moore, D.D. and Larsen P.R. (1991) *Annu. Rev. Physiol.*, **53**, 17−35.
20. Mueller, C.R., Maire, P. and Schibler, U. (1990) *Cell*, **61**, 279−291.
21. Akira, S., Isshiki, H., Sugita, T.,Tanabe, O., Kinoshita, S., Nishio, T., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) *EMBO J.*, **9**, 1897−1906.
22. Poli, V., Mancini, F.P. and Cortese R. (1990) *Cell*, **63**, 643−653.
23. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. and Schibler, U. (1990) *Genes Dev.*, **4**, 1541−1551.
24. McKnight, S.L (1982) *Cell*, **31**, 355−365.
25. Dynan, W.S (1989) *Cell*, **58**, 1−4.
26. Bruder, J.T. and Hearing, P. (1989) *Mol. Cell. Biol.*, **9**, 5143−5153.
27. Gidoni, D., Dynan, W.S. and Tjian, R. (1984) *Nature*, **312**, 409−413.
28. Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987) *Cell*, **51**, 1079−1090.
29. Segal, R. and Berk, A.J. (1991) *J. Biol. Chem.*, **266**, 20406−20411.
30. Su, W., Jackson, S.,Tjian, R. and Echols, H. (1991) *Genes Dev.*, **5**, 820−826.
31. Descombes, P. and Schibler, U. (1991) *Cell*, **67**, 569−579.
32. Glass, C.K., DiRenzo, J.,Kurokawa, R. and Han, Z. (1991) *DNA Cell Biol.* **10**, 623−638.
33. Wilson, T.E., Paulsen, R.E., Padgett, K.A. and Milbrandt, J. (1992) *Science*, **256**:107−110.
34. Olson, E.N (1990) *Genes Dev.*, **4**, 1454−1461.
35. Tuil, D., Clergue, N., Montarras, D., Pinset, C., Kahn, A. and Phan-Dinh-Tuy, F. (1990) *J. Mol. Biol.*, **213**, 677−686.
36. Thompson, W.R., Nadal-Ginard, B. and Mahdavi, V. (1991) *J. Biol. Chem.*, **266**:22678−22688.
37. Saffer, J.D., Jackson, S.P. and Annarella, M.B. (1991) *Mol. Cell. Biol.*, **11**, 2189−2199.
38. Crosby, S.D., Puetz, J.J., Simburger, K.S., Fahrner, T.J. and Milbrandt, J. (1991) *Mol. Cell. Biol.*, **11**, 3835- 3841.
39. Piette, J., Klarsfeld, A. and Changeux J.P., (1989) *EMBO J.*, **8**, 687−694.
40. Smale, S.T. and Baltimore, D. (1989) *Cell*, **57**:103−113.
41. Watanabe, H., Imai, T., Sharp, P.A. and Handa, H. (1988) *Mol. Cell. Biol.*, **8**:1290−1300.
42. Watanabe, H., Wada, T. and Handa H. (1990) *EMBO J.*, **9**:841−847.
43. Thorn, J.T., Todd, A.V., Warrilow, D., Watt, F., Molloy, P.L. and Iland, H.J. (1991) *Oncogene*, **6**, 1843−1850.
44. Gutman, A. and Wasylyk, B. (1991) *Trends in Genetics*, **7**, 49−54.
45. Bosca, L., Rousseau, G.G. and Hue, L.(1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6440−6444.
46. Bosca, L., Mojena, M. Ghysdael, J., Rousseau, G.G. and Hue L. (1986) *Biochem. J.*, **236**, 595−599.