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The Distal Enhancer Implicated in the Developmental Regulation of the Tyrosine Aminotransferase Gene Is Bound by Liver-Specific and Ubiquitous Factors

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Tyrosine aminotransferase gene expression is confined to parenchymal cells of the liver, is inducible by glucocorticoids and glucagon, and is repressed by insulin. Three enhancers control this tissue-specific and hormone-dependent activity, one of which, located at −11 kb, is implicated in establishing an active expression domain. We have studied in detail this important regulatory element and have identified a 221-bp fragment containing critical enhancer sequences which stimulated the heterologous thymidine kinase promoter more than 100-fold in hepatoma cells. Within this region, we have characterized two essential liver-specific enhancer domains, one of which was bound by proteins of the hepatocyte nuclear factor 3 (HNF3) family. Analyses with the dedifferentiated hepatoma cell line HTC suggested that HNF3α and/or -γ, but not HNF3β, are involved in activating the tyrosine aminotransferase gene via the −11-kb enhancer. Genomic footprinting and in vitro protein-DNA binding studies documented cell-type-specific binding of ubiquitous factors to the second essential enhancer domain, which by itself stimulated the thymidine kinase promoter preferentially in hepatoma cells. These results will allow further characterization of the role of these enhancer sequences in developmental activation of the tyrosine aminotransferase gene.

Differential gene expression is the basis of cellular diversity in complex organisms. Work from many laboratories has established that gene activity is regulated by the combinatorial action of sequence-specific DNA-binding proteins which, together with the basic transcription apparatus, control the frequency of transcription initiation at the promoter. Binding sites for transcriptional activator proteins, which mediate cell-type-specific, hormone-dependent, and developmentally regulated gene expression, can be found in the vicinity of the transcription start site and in enhancer sequences located several kilobases upstream or downstream (33, 43). More recently, cofactors or adaptors which couple activator proteins to the basal transcription factors by protein-protein interaction have been described (reference 42 and references therein).

Studies on liver-specific genes have led to the identification of several liver-enriched transcriptional activators, the majority of which can be grouped into four families (see references 14 and 61 for reviews): hepatocyte nuclear factor 1 (HNF1) proteins, which contain an extra large homeodomain; HNF3 proteins, which have a DNA binding domain of high homology to the *Drosophila* protein fork head; HNF4-related proteins, which belong to the steroid hormone receptor superfamily; and C/EBP-like proteins, which contain a basic DNA binding domain adjacent to a leucine zipper. With the exception of the HNF3 proteins, which bind to DNA as monomers, all of these factors bind as dimers or heterodimers, thus increasing the complexity of possible protein-DNA interactions. Recently, HNF4 has been shown to regulate expression of the HNF1α gene, suggesting a hierarchical order of liver-specific transcription factors (37, 65).

Tyrosine aminotransferase (TAT) is a gluconeogenic enzyme which has been studied as a classical system for more than 20 years (reviewed in reference 51). The TAT gene is switched on in the parenchymal cells of the liver shortly after birth, when hormone levels change dramatically (26, 28). Gene activity is induced by glucocorticoids and by glucagon, acting via its intracellular mediator cyclic AMP (cAMP) (25, 29, 58), and is repressed by insulin (60). Our analyses have defined three enhancers which are responsible for the liver-specific and hormone-inducible expression of the TAT gene. The glucocorticoid-inducible enhancer at −2.5 kb contains a glucocorticoid response element and binding sites for liver-specific transcription factors of the HNF3 and C/EBP families (24, 32, 49, 56). In the enhancer at −3.6 kb, a cAMP response element (CRE) synergizes with a liver-specific element, which is bound by HNF4 (7, 49, 70). This enhancer also mediates repression by insulin (19). The liver-specific enhancer, located at −11 kb, activates the TAT gene constitutively (52).

Analyses in transgenic mice have revealed that developmentally regulated expression can be seen only when the enhancer at −11 kb is part of the transgene (3). Furthermore, chromatin studies have established that this enhancer is located in a DNase I-hypersensitive site in fetal liver, before the TAT gene is expressed (62), indicating that it may be important to constitute an active expression domain. Therefore, we have undertaken a detailed analysis of the properties of this enhancer. Gene transfer experiments defined as the minimal control region a 221-bp fragment containing two essential domains, one of which is bound by HNF3 proteins. Genomic footprinting and in vitro protein-DNA binding studies documented hepatoma cell-specific binding of ubiquitous proteins to the second liver-specific enhancer domain.

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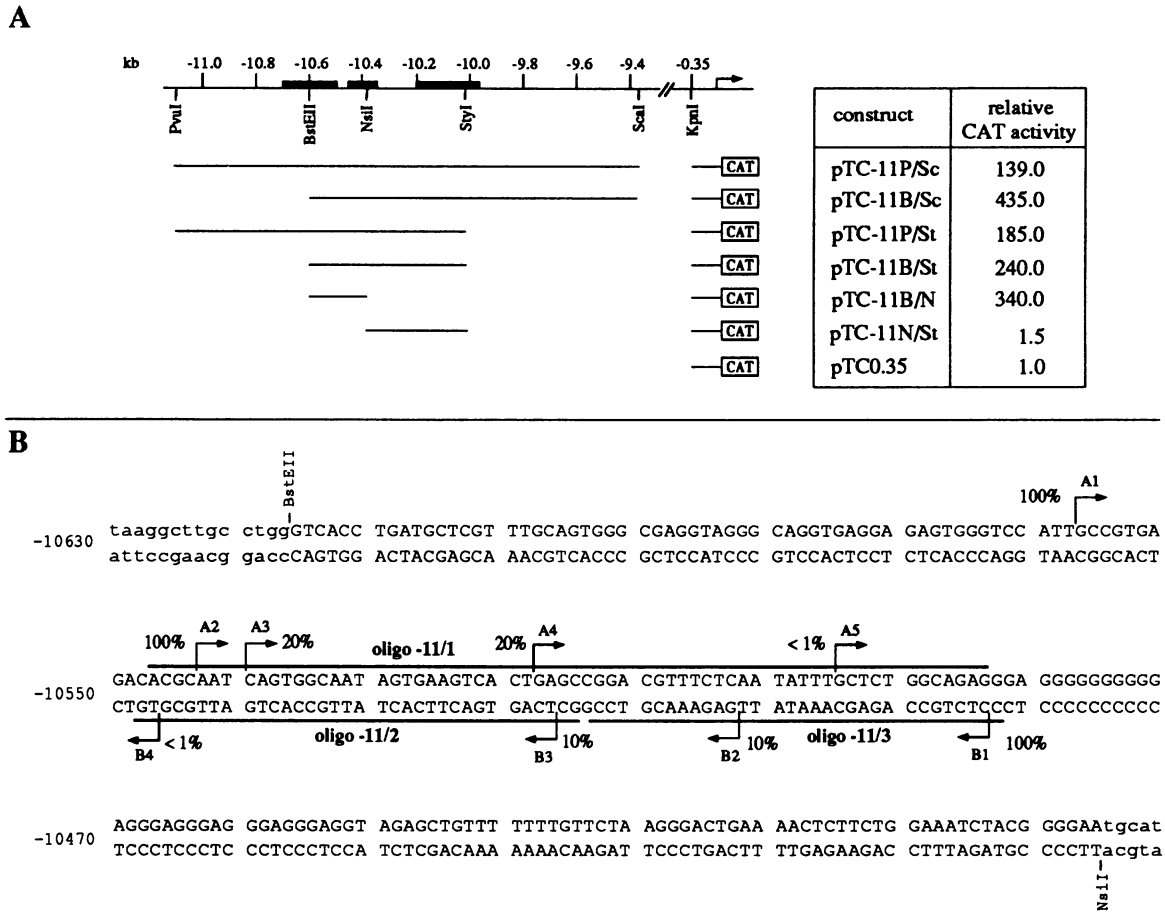

FIG. 1. Characterization of essential enhancer domains. Different regions from the TAT enhancer at −11 kb were cloned in front of the TAT promoter driving the bacterial CAT gene (see Materials and Methods for details). The resulting constructs were transiently transfected into the hepatoma cell line FTO-2B. (A) Constructs analyzed. The enhancer region is depicted at the top, with DNase I-hypersensitive regions (described in reference 52) marked by black bars and relevant restriction sites indicated underneath. Restriction fragments that were cloned in position −351 of the TAT promoter are represented by horizontal lines below. Average relative CAT activities from two independent transfection experiments are shown at the right. The activity of the TAT promoter construct (pTC0.35) was set at 1, corresponding to 0.12 pmol of acetylated chloramphenicol per min per mg of protein. (B) Sequence of the 221-bp enhancer fragment (pTC-11B/N in A) and summary of results of transfection experiments with 5′ and 3′ deletion constructs, which were generated by unidirectional exonuclease III digestion of the 221-bp fragment. Selected deletion endpoints are indicated by arrows above (5′ deletions) and below (3′ deletions) the sequence, pointing to the remaining sequences, and are designated A1 to A5 and B1 to B4, respectively. The activity of each deletion construct is given as percentage of the activity of the wild-type construct pTC-11B/N. Horizontal lines indicate three oligonucleotides (−11/1, −11/2, and −11/3) which were subsequently cloned in front of the heterologous TK promoter (see Table 1). In all transfection experiments, a Rous sarcoma virus long terminal repeat-driven luciferase expression vector (15) was cotransfected as internal reference.

gATERIALS AND METHODS

Plasmid constructions. Synthetic oligonucleotides used in plasmid constructions and gel retardation experiments were synthesized on an Applied Biosystems synthesizer. The constructs depicted in Fig. 1A contain different restriction fragments from the −11-kb enhancer region cloned in front of the TAT promoter driving the chloramphenicol acetyltransferase (CAT) gene (pTC0.35 [52]). The following restriction sites were used (Fig. 1): *Pvu*I (−11116 bp), *Bst*EII (−10616 bp), *Nsi*I (−10395 bp), *Sty*I (−10030 bp), and *Sca*I (−9382 bp). Construct pTC-11P/Sc corresponds to pTC-11HS in reference 52. 5′ and 3′ deletion constructs were generated by unidirectional exonuclease III digestion of the 221-bp fragment of construct pTC-11B/N, cloned in either orientation into the *Xba*I site in the polylinker of pTC0.35. Deletion endpoints were determined by sequencing. All constructs containing enhancer sequences cloned in front of the thymidine kinase (TK) promoter are based on pBLCAT5 (6). For the analysis of clustered point mutations, the corresponding wild-type and mutated oligonucleotides (see Fig. 2 and 7) were cloned as *Hin*dIII-*Bam*HI fragments in the sense orientation into the polylinker of pBLCAT5. In constructs pTTC-11/1, pTTC-11/2, and pTTC-11/3, the following oligonucleotides were cloned in the sense orientation into the *Bam*HI site of pBLCAT5 (the sequence of the upper strand is given): −11/1, 5′-GATCCGCAATCAGTGGCAATAGTG AAGTCACTGAGCCGGACGTTTCTCAATATTTGCTCT GGCAGAGA-3′; −11/2, 5′-GATCCACGCAATCAGTGGC AATAGTGAAGTCACTGAGCCA-3′; and −11/3, 5′-GATC CGACGTTTCTCAATATTTGCTCTGGCAGATA-3′. In constructs pTTC$(-11/3)_4$ and pTTC$(-11/3\text{mut})_4$, four head-to-tail copies of oligonucleotides −11/3 and −11/3mut (5′-GATCCGACGTTTATACATATTTTATCTGGCAGATA-3′), respectively, are inserted in the sense orientation into the

*Bam*HI site of pBLCAT5. All constructions containing oligonucleotides were verified by plasmid sequencing (8) using T7 polymerase and the Sequenase (U.S. Biochemical) protocol. Plasmids were grown in *Escherichia coli* HB101 and purified by alkaline lysis and two successive CsCl gradients followed by dialysis.

Cell culture and transfections. The rat hepatoma cell lines FTO-2B (35, 36) and HTC (64) were cultured in a 1:1 (vol/vol) dilution of Dulbecco's modified Eagle medium and Ham F12 medium. XC fibrosarcoma (63) cells were grown in Dulbecco's modified Eagle medium. Media were supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4), and 2 mM glutamine. Cells were grown at 37°C in 5% CO_2. Electroporations (10, 18) were performed with a Gene Pulser and Capacitance Extender from Bio-Rad. Cells were trypsinized, washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, and resuspended in phosphate-buffered saline at a concentration of 2.2×10^7 cells per ml (FTO-2B) or 1.1×10^7 cells per ml (XC and HTC). A 450-μl volume of cell suspension was mixed with 50 μl of TE (10 mM Tris [pH 7], 1 mM EDTA) containing 10 μg of plasmid DNA plus 1 μg of reference plasmid RSVlucA/H. In this construct, provided by A. Hecht, the firefly luciferase gene (15) is driven by the Rous sarcoma virus promoter and enhancer. Cells were immediately transferred into electroporation cuvettes (Bio-Rad) and pulsed at room temperature with 960 μF and 250 V. After 5 to 10 min, the cells were diluted in medium containing charcoal-stripped fetal calf serum and plated.

CAT and luciferase assays. Cells were harvested 48 h after electroporation, washed twice with phosphate-buffered saline, harvested in 1 ml of 150 mM NaCl–40 mM Tris (pH 7.4)–1 mM EDTA, and collected by low-speed centrifugation. Extracts were prepared in 250 μl of 250 mM Tris (pH 7.8)–1 mM EDTA–0.2% saponin–1 mM dithiothreitol by three cycles of freezing and thawing and were cleared by centrifugation at 10,000 × *g* at 4°C for 20 min. CAT assays (23) were performed in a total volume of 200 μl with 250 mM Tris (pH 7.8), 5 mM EDTA, 1 mM acetyl coenzyme A (Pharmacia), and 1.25 μCi of [^{14}C]chloramphenicol (New England Nuclear) per ml (final concentrations). After thin-layer chromatography, the unacetylated chloramphenicol and the acetylated forms were quantified by liquid scintillation counting to determine the conversion rate. Luciferase activity was determined in 30 μl of freshly prepared extract as described previously (15).

Genomic footprinting. Methylation of intact cells and of protein-free DNA, purification of the DNA, restriction digestions, and cleavage with piperidine were performed as described previously (2). For analysis of the upper strand, 25 μg of piperidine-cleaved DNA was subjected to a linear amplification reaction as described elsewhere (55), using a primer corresponding to the upper strand of the TAT sequences from bp −10718 to −10693. Amplifications were performed with 10 U of *Taq* polymerase (Perkin-Elmer) in 30 cycles with 1 min of denaturation at 92°C, 1 min of annealing at 64°C, and elongation for 1.7 min at 72°C. For analysis of the lower strand, 30 μg of genomic DNA was cut by *Dra*II, purified, and subsequently cleaved with piperidine at the methylated guanines. The purified DNAs were subsequently analyzed on a 6% sequencing gel, electroblotted onto GeneScreen, baked at 80°C, covalently cross-linked by UV irradiation, and hybridized as described elsewhere (2), using a cDNA probe of high specific activity (71) that recognizes TAT sequences from bp −10562 to −10677.

Nuclear extract preparation and DNase I footprinting. Nuclear extracts used in DNase I footprinting experiments and in gel mobility shift assays with labeled oligonucleotide −11/2A were prepared according to Dignam et al. (16), with modifications (72). Gel mobility shift experiments with oligonucleotide −11/3 were carried out with extracts prepared as described elsewhere (59). DNase I footprinting experiments were performed as described in reference 41, using 50 μg of nuclear extract or bovine serum albumin (BSA) and 500 ng of poly(dI) · poly(dC) as nonspecific competitor DNA. The binding reaction was performed for 10 min on ice in 25 mM HEPES (pH 7.8)–12.5 mM $MgCl_2$–20% glycerol–100 mM KCl–0.1% Nonidet P-40–1 mM dithiothreitol. Protein binding was assayed on restriction fragments that were excised from the constructs containing wild-type or mutated (M4 and M7) sequences of the enhancer from bp −10562 to −10507 (Fig. 2). Purified DNA was separated on a 6% polyacrylamide–50% urea sequencing gel.

Gel mobility shift analyses. In gel shift assays with oligonucleotide −11/2A (5′-GATCTGCCGTGAGACACGCAAT CAGTGGCG-3′), 7.5 μg of protein was incubated for 10 min on ice with 10 fmol of labeled oligonucleotide in the presence of 500 ng of poly(dA) · poly(dT) in 10 mM HEPES (pH 7.9)–70 mM KCl–6 mM $MgCl_2$–4 mM spermidine–10% glycerol–0.5 mM dithiothreitol. Protein-DNA complexes were separated in 4% polyacrylamide gels at 15 V/cm in 0.5× TBE (4.4 mM Tris-borate, 4.4 mM boric acid, 1.0 mM EDTA) buffer. In gel shift assays with oligonucleotide −11/3 (see above) 5 μg of extract was preincubated 10 min on ice with 500 ng of poly(dI) · poly(dC) in 20 mM HEPES (pH 7.9)–4% Ficoll–2 mM $MgCl_2$–40 mM KCl–0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (pH 8)–0.5 mM dithiothreitol. After addition of 10 fmol of labeled oligonucleotide and incubation for 10 min on ice, the protein-DNA complexes were separated on an 8% polyacrylamide gel at 15 V/cm in 0.3× TBE (2.64 mM Tris-borate, 2.64 mM boric acid, 0.6 mM EDTA). Specific antisera directed against HNF3α, -β, and -γ (38, 39) were kindly provided by V. Prezioso and J. Darnell. The oligonucleotides used in competition experiments correspond to the HNF3 and HNF4 binding sites from the transthyretin gene (11), the CCAAT box element from the aldolase B promoter and a mutant thereof (67), the C/EBP site from the hepatitis B virus enhancer (40), the D element from the albumin promoter (12, 45), and enhancer domain −11/2B (5′-GATC TGTGGCAATAGTGAAGTCACTGAGCCG-3′).

Methylation interference studies. End-labeled oligonucleotides were partially methylated with dimethylsulfate (DMS) by standard procedures (44). The amounts of DNA and protein used for 10 analytical gel retardation reactions were loaded onto a preparative 4% polyacrylamide gel and, after separation, visualized by autoradiography. Material corresponding to bound and free fractions was cut out of the gel, and the DNA was recovered by isotachophoresis (53). Precipitated DNA was cleaved at the methylated purines by incubation for 15 min at 92°C in 10 mM phosphate buffer (pH 6.8)–1 mM EDTA and, after addition of 100 mM NaOH, for a further 30 min at 92°C. Following ethanol precipitation, equal amounts of radioactive material resuspended in formamide loading buffer were separated on a 15% sequencing gel.

RESULTS

A 221-bp fragment of the rat TAT gene enhancer at −11 kb confers full enhancer activity and contains two essential domains. A 1.8-kb fragment from the TAT enhancer at −11 kb

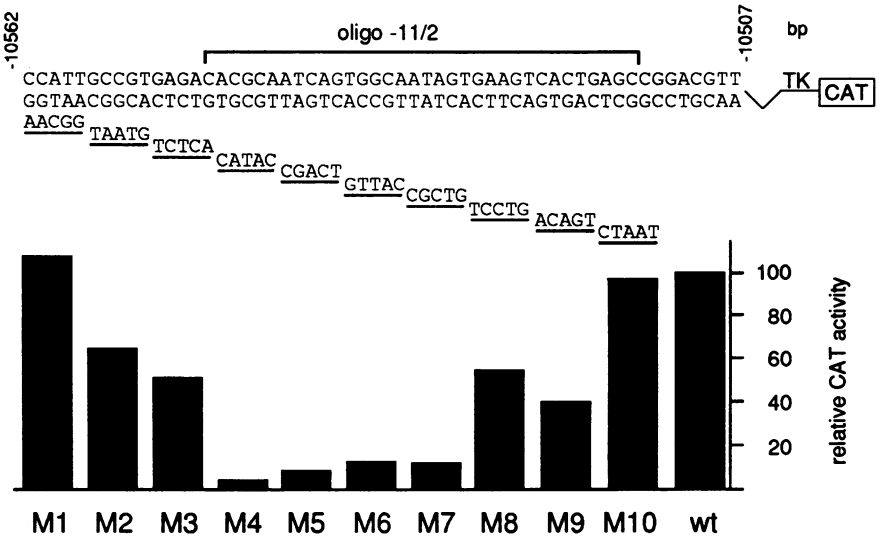

FIG. 2. Analyses of clustered point mutations in enhancer domain −11/2. Clusters of five transversions were introduced into the TAT sequences from bp −10562 to −10507, generating mutants M1 to M10. The corresponding oligonucleotides were cloned in front of the TK promoter, and the resulting constructs transfected into FTO-2B hepatoma cells. At the top, the sequence of the wild-type oligonucleotide is given; below, the transversions introduced in the various mutated oligonucleotides are indicated for the upper strand. The bar diagram depicts average relative CAT activities obtained in two independent transfection experiments; the activity of the wild-type construct was arbitrarily set at 100.

activates the TAT promoter more than 100-fold in transient transfection experiments (52) (pTC-11P/Sc in Fig. 1A). To define the essential sequences within this important regulatory region, we tested the activities of different subfragments. Figure 1A depicts the results obtained with the rat hepatoma cell line FTO-2B, which shows high levels of TAT gene expression. Full enhancer activity was conferred by a 221-bp fragment containing sequences from bp −10616 to −10395 (pTC-11B/N in Fig. 1A; compare with Fig. 1B). The increase in activity of constructs −11B/N and −11B/Sc in comparison with −11P/Sc and −11P/St possibly indicates that a negative regulatory region is located upstream of restriction site *Bst*EII. The 221-bp fragment was further dissected by analysis of 5′ and 3′ deletions (pTC-11A and pTC-11B series, respectively). Figure 1B summarizes the results obtained with selected deletion clones and indicates the oligonucleotides that were subsequently tested for enhancer activity (Table 1). The deletion endpoints A2 and B1 defined 60 bp as critical enhancer sequences within which activity was lost in two steps (Fig. 1B).

To test enhancer activity in the context of a heterologous promoter, the 221-bp fragment and three oligonucleotides that contain important enhancer sequences as defined by the 5′ and 3′ deletion series (Fig. 1B) were cloned in front of the herpes simplex virus TK promoter. The resulting constructs are designated pTTC-11B/N, pTTC-11N/B, pTTC-11/1, pTTC-11/2, and pTTC-11/3. The 221-bp fragment strongly stimulated the TK promoter in both orientations; constructs pTTC-11/1 and pTTC-11/2 also showed more than 100-fold stimulation, whereas construct pTTC-11/3 was only weakly active (Tables 1 and 2).

The results presented in Fig. 1 and Table 1 document that a 221-bp fragment containing sequences from bp −10616 to −10395 activated the TAT and TK promoters >100-fold and consists of two domains. The first, defined by an oligonucleotide encompassing 35 bp from the center of the 221-bp region (−11/2), stimulated the TK promoter by itself >100-fold. The second enhancer domain is characterized by two 3′

TABLE 1. Strong stimulation of the heterologous TK promoter by a 35-bp region

Construct[a]	Relative CAT activity[b]
pBLCAT5	1
pTTC-11B/N	135
pTTC-11N/B	143
pTTC-11/1	163
PTTC-11/2	145
pTTC-11/3	5

[a] The 221-bp fragment in both orientations or oligonucleotide −11/1, −11/2, or −11/3 (see Fig. 1B) was cloned in front of the TK promoter in pBLCAT5.
[b] Results of a representative transfection experiment in FTO-2B hepatoma cells. The activity of pBLCAT5 is set at 1 and corresponds to 0.85 pmol min^{-1} mg^{-1}.

TABLE 2. Hepatoma cell specificity of both enhancer domains

Construct[a]	Relative CAT activity[b]		
	FTO-2B	XC	HTC
pBLCAT5	1.0	1.0	1.0
pTTC-11B/N	238.0	3.8	3.4
pTTC-11/2	146.0	8.0	11.0
$pTTC(-11/3)_4$	55.0	3.0	2.3
$pTTC(-11/3mut)_4$	1.5	ND	ND
SV40	74.0	32.0	46.0

[a] The 221-bp fragment, oligonucleotide −11/2, or a construct containing four copies of oligonucleotide −11/3 or −11/3mut (containing 5-bp exchanges) was cloned in front of the TK promoter in pBLCAT5. The construct designated SV40 contains five copies of the simian virus 40 enhancer in front of the TK promoter (46).
[b] Results of representative transfection experiments in the indicated cell lines. The activity of pBLCAT5 is set at 1 in each cell line, corresponding to absolute values of 0.9 (FTO-2B cells), 102.5 (XC cells), and 4.3 (HTC cells) pmol min^{-1} mg^{-1}. ND, not determined.

deletion constructs, between the endpoints of which 90% of enhancer activity is lost (deletion endpoints B1 and B2 in Fig. 1B). Together, these results also suggest that sequences outside the two essential domains, designated −11/2 and −11/3, may contribute to the activity of the enhancer in its natural position at −11 kb. Whereas in the context of the heterologous TK promoter, full enhancer activity is conferred by domain −11/2 alone (Table 1), deletion of domain −11/3 greatly reduces stimulation of the TAT promoter (Fig. 1B). These results are underscored by the observation that oligonucleotides −11/1 and −11/2 activate the TAT promoter 10- to 20-fold less efficiently than does the 221-bp enhancer core fragment (data not shown).

Clustered point mutations identify important sequences in enhancer domain −11/2. To identify individual motifs in enhancer domain −11/2, we introduced clusters of point mutations and cloned the different oligonucleotides in front of the TK promoter (Fig. 2). Transient transfection experiments in FTO-2B hepatoma cells documented significantly reduced stimulation by the four mutants M4 to M7 and two- to threefold reductions in activity of the adjacent mutants (M2, M3, M8, and M9). Mutant M4 showed loss of >95% of the activity of the wild-type construct, but interestingly, four of the five base pairs that have been exchanged belong to a region which can be deleted in the 221-bp fragment without having any effect (compare deletion endpoints A1 and A2 in Fig. 1B). This result suggests that the enhancer is composed of several enhancer motifs which are in part functionally redundant if tested in close proximity to the promoter.

Both enhancer domains are preferentially active in hepatoma cells. Previously, we have shown that the activity of the −11-kb enhancer is restricted to hepatoma cells (52). We now wanted to determine how this cell type specificity is determined by the individual subdomains of the enhancer. Table 2 summarizes the results of transient transfection experiments in three different rat cell lines. The 221-bp fragment strongly stimulated the TK promoter in FTO-2B hepatoma cells but was almost inactive in the fibrosarcoma cell line XC. The sequences representing domain −11/2 were also at least 15-fold more active in FTO-2B cells than in XC cells. Because one copy of enhancer domain −11/3 was only weakly active (Table 1), we tested the activity of tandem repeats of it. Four copies of the corresponding oligonucleotide −11/3 strongly stimulated the TK promoter in FTO-2B hepatoma cells but not in XC fibroblasts. Mutations at five positions, which were protected in in vivo and in vitro footprinting experiments (Fig. 3 and 4), abolished this activity [compare pTTC$(-11/3)_4$ and pTTC$(-11/3\text{mut})_4$ in Table 2]. Thus, both subdomains of the TAT enhancer at −11 kb are preferentially active in differentiated hepatoma cells.

In the dedifferentiated rat hepatoma cell line HTC, steady-state TAT mRNA levels are 5- to 10-fold lower than in FTO-2B cells and liver (52). In HTC cells, all constructs derived from the −11-kb TAT enhancer showed less than 10% of the activity observed in the differentiated hepatoma cell line FTO-2B (Table 2), correlating with greatly reduced DNase I hypersensitivity in HTC cells (52).

Genomic footprinting reveals hepatoma cell-specific in vivo protein-DNA interactions at mutation-sensitive enhancer domains. We performed genomic footprinting experiments to look for protein-DNA interactions in vivo which might correlate with the activity and mutation sensitivity of the enhancer in transient transfection experiments. The experiments revealed a 16-bp duplication within the enhancer in the H4EIIEC-derived hepatoma cell line FTO-2B compared with rat genomic DNA of another source (48), so that DNA

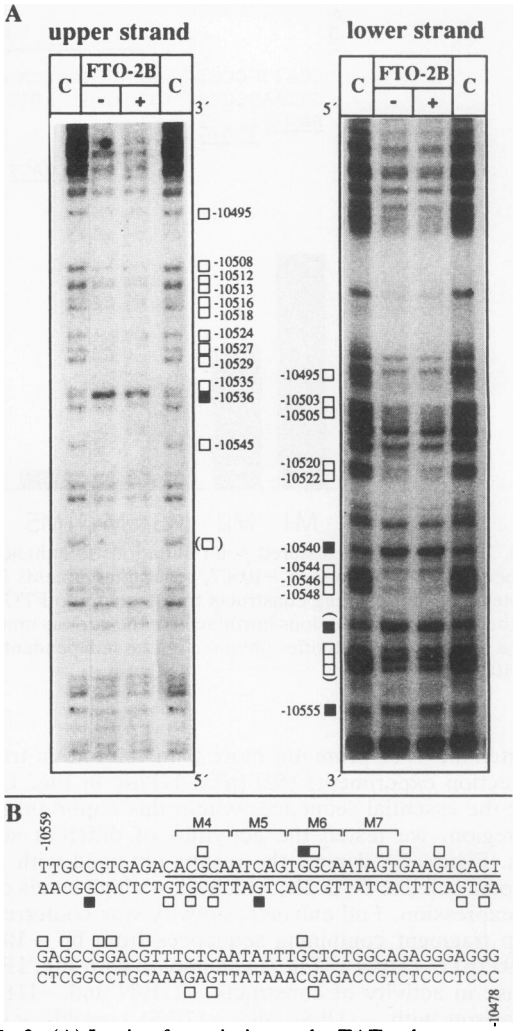

FIG. 3. (A) In vivo footprinting at the TAT enhancer at −11 kb. FTO-2B cells were cultured overnight in serum-free medium and then induced for 2 h with 10^{-6} M forskolin and 3×10^{-7} M dexamethasone (+) or treated with 0.1% ethanol as a solvent control (−). Intact cells were reacted with DMS and then processed separately for the upper and lower strands as described in Materials and Methods. As a control (C), purified genomic DNA was reacted in vitro with DMS. In each lane, 30 μg of DNA was cut by *Dra*II and cleaved by piperidine for analyses of the upper strand; for the lower strand, the DNA was cleaved with piperidine and then linearly amplified by using *Taq* polymerase and a primer corresponding to the upper strand from bp −10718 to −10693. All samples were subsequently separated on a 6% polyacrylamide gel by electrophoresis, electroblotted onto a nylon membrane, covalently cross-linked, and hybridized against a cDNA probe corresponding to the upper strand from bp −10718 to −10693. Altered DMS reactivity of guanine residues is marked by solid squares for enhancements and open squares for protections. Numbers indicate the positions in base pairs relative to the start site of transcription. The analysis has revealed a sequence duplication in FTO-2B cells compared with rat DNA from other cell lines. Changes in DMS reactivity in the duplicated region, which corresponds to bp −10554 to −10539, are in parentheses. XC fibroblasts show a DMS reactivity comparable to that of the naked control DNA (data not shown). (B) TAT sequences from bp −10559 to −10478 and summary of the in vivo footprints observed in FTO-2B hepatoma cells. Enhancements and protections are depicted as in panel A. The relative positions of the critical mutants depicted in Fig. 2 and sequences contained in oligonucleotides −11/2 and −11/3 (see Tables 1 and 2) are indicated by horizontal brackets above and lines between the sequences, respectively.

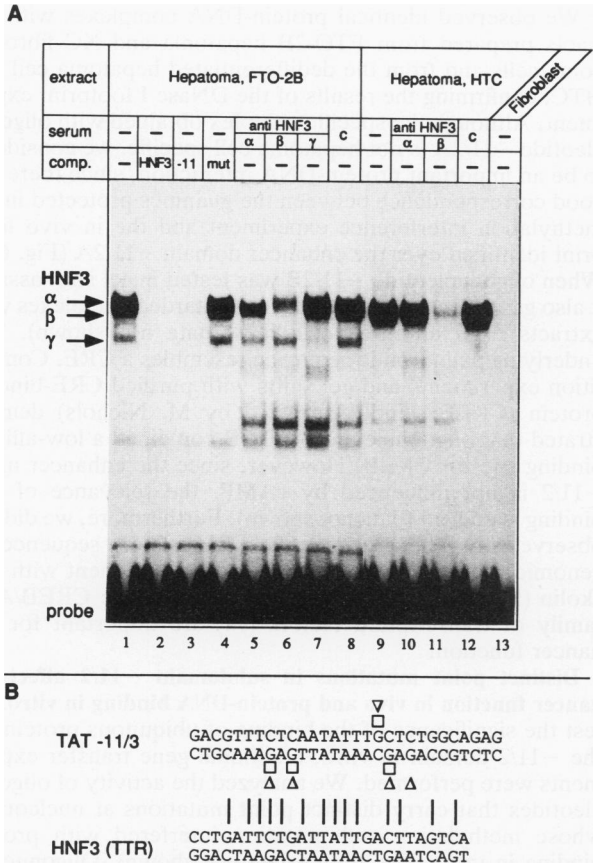

FIG. 4. Binding of HNF3 proteins to enhancer domain −11/3. (A) Protein extracts (5 μg) from FTO-2B (lanes 1 to 8), HTC (lanes 9 to 12), and XC (lane 13) cells were incubated with 10 fmol of labeled oligonucleotide −11/3 in the presence of 500 ng of poly(dI-dC) as nonspecific competitor and subsequently separated in an 8% polyacrylamide gel. Competition experiments were performed by preincubation of the extract with a 100-fold molar excess of unlabeled oligonucleotide −11/3 (lane 3) or −11/3mut (lane 4) or an oligonucleotide containing an HNF3 binding site from the transthyretin gene (11) (lane 2). For immunoshift experiments (lanes 5 to 8 and 10 to 12), extracts were preincubated with the indicated antiserum or preimmune serum as a control (c). Arrows point at the specific HNF3 complexes. (B) Sequence comparison of TAT oligonucleotide −11/3 and the HNF3 binding site from the transthyretin (TTR) gene. Homologous positions are indicated by vertical lines between the sequences. Open squares at the TAT sequence mark guanosines that are protected from methylation in vivo (Fig. 3); triangles represent guanosines whose methylation interferes with complex formation in vitro (data not shown).

from DMS-treated XC fibroblasts cannot be directly aligned with the genomic DNA from FTO-2B cells. In Fig. 3, we therefore present methylation of protein-free DNA isolated from FTO-2B cells as a control. Fibroblasts, however, did not show any differences in DMS reactivity in comparison with naked DNA, suggesting that no protein-DNA interactions occur in fibroblasts in vivo (data not shown).

On both strands, several prominent differences in DMS reactivity were evident between uninduced FTO-2B cells and the control. Induction with both dexamethasone and forskolin did not result in any additional changes, supporting previous conclusions that this enhancer is not involved in hormonal control of the TAT gene (52). The in vivo footprints were found within the two essential enhancer domains −11/2 and −11/3, which are underlined in Fig. 3B. A cluster of protections and an enhancement was located at sequences whose mutation resulted in loss of >90% of enhancer activity (M4 and M5 in Fig. 2). No differences were observed outside the functionally relevant enhancer sequences. Thus, the cell-type-specific activity of the enhancer correlates with in vivo footprints in hepatoma cells at essential enhancer motifs.

Proteins of the HNF3 family bind to enhancer domain −11/3. In recent years, several liver-specific transcription factors have been described and cloned (14, 61). We were interested in determining whether any of these proteins bind to the TAT enhancer at −11 kb at those sites determined by in vivo footprinting experiments. Therefore, we performed in vitro protein-DNA binding studies with the enhancer subdomains −11/2 and −11/3. Oligonucleotide −11/3, containing one of the two essential enhancer domains, was used in gel mobility shift assays. As a control, we used a mutated oligonucleotide that contains transversions at guanines protected from methylation in vivo (Fig. 3) and was inactive in gene transfer experiments (Table 2).

DNA binding reactions with nuclear extracts from FTO-2B hepatoma cells, but not from XC fibroblasts, resulted in two specific protein-DNA complexes (Fig. 4A, lanes 1 and 13). Formation of these complexes could be inhibited by an excess of the wild-type but not the mutated oligonucleotide (lanes 1, 3, and 4). An HNF3 binding site derived from the transthyretin promoter (11) also competed for formation of both complexes (lane 2). With the help of antisera directed against the liver-specific factors HNF3α, -β, and -γ (kindly provided by V. Prezioso and J. Darnell) (38, 39), we could show that all three HNF3 proteins interacted with oligonucleotide −11/3 from the TAT gene enhancer, with HNF3α and -β contained in the prominent upper shift and HNF3γ corresponding to the lower shift (lanes 5 to 8). The underlying sequence shows significant homology to the HNF3 motif of the transthyretin gene, and methylation interference studies documented a good correspondence to the in vivo footprint, as indicated by open triangles and squares, respectively (Fig. 3 and 4B and data not shown).

Gel mobility shift assays with extracts prepared from HTC cells demonstrated that HNF3β DNA-binding activity was present; however, HNF3α and -γ could not be detected in this dedifferentiated hepatoma cell line (Fig. 4A, lanes 9 to 12). Four copies of the HNF3 binding site from the TAT enhancer stimulated the TK promoter >50-fold in FTO-2B hepatoma cells, which contain all three HNF3 proteins, but were inactive in HTC cells [pTTC(−11/3)$_4$ in Table 2]. Thus, the HNF3β DNA-binding activity observed in HTC cells is apparently not sufficient to activate the HNF3 binding site from the TAT enhancer in gene transfer experiments.

Ubiquitous proteins bind to the cell-type-specific enhancer domain −11/2. We performed in vitro DNase I footprinting experiments to detect proteins binding to the enhancer domain −11/2. As shown in Fig. 5, nuclear extracts derived from hepatoma cells as well as from fibroblasts gave an extended footprint over the mutation-sensitive region defined in transient transfection experiments (Fig. 2), even though this enhancer domain was at least 15-fold less active in fibroblasts than in FTO-2B hepatoma cells (pTTC-11/2 in Table 2). Analyses of the mutated templates M4 and M7 indicated that the DNase I footprint reflects the binding of two proteins to adjacent enhancer motifs, the mutation of

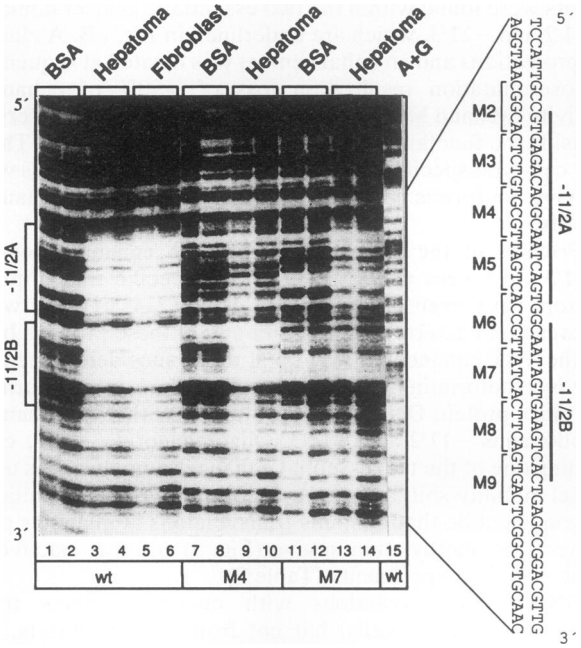

FIG. 5. Binding of two ubiquitous proteins to enhancer domain −11/2. Fifty micrograms of nuclear extract of the indicated source or BSA as a control was incubated with restriction fragments end labeled at the upper strand and corresponding to the wild-type (lanes 1 to 6) or mutant M4 (lanes 7 to 10) or M7 (lanes 11 to 14) sequence of enhancer domain −11/2 (see Fig. 2). After digestion with DNase I (6.25 ng/ml in lanes 1, 7, and 11; 12.5 ng/ml in lanes 2, 8, and 12; 75 ng/ml in lanes 3, 5, 9, and 13; 125 ng/ml in lanes 4, 6, 10, and 14), the DNA was purified and electrophoresed in a 6% denaturing polyacrylamide gel. Footprints are indicated by brackets on the left and designated −11/2A and −11/2B, corresponding to the 5′ and 3′ enhancer motif, respectively. A+G, purine-specific sequencing lane of the wild-type template. On the right, the sequence of enhancer domain −11/2 is depicted; the DNase I footprints are indicated by vertical lines. The mutated positions in M2 to M9 (see Fig. 2) are indicated by brackets.

which resulted in loss of the corresponding protein-DNA interactions.

We subsequently performed gel mobility shift assays with labeled oligonucleotides −11/2A and −11/2B, which correspond to the distal and proximal DNase I footprints at the enhancer motif −11/2. As illustrated in Fig. 6A, we observed a specific protein-DNA complex with oligonucleotide −11/2A, which was not formed by a mutated oligonucleotide, containing a triple-point mutation at base positions that were protected in methylation interference experiments (Fig. 6A to C). Competition with several different binding sites, including the adjacent enhancer motif −11/2B, did not affect complex formation (Fig. 6A and D, lanes 2). Oligonucleotide −11/2A contains a striking homology to a CCAAT box motif found in the aldolase B promoter, which is bound by a liver-enriched protein (67). Reciprocal competition experiments with the two binding sites and the corresponding mutants as controls established that the protein binding to the TAT enhancer is distinct from the factor interacting with the aldolase B promoter (Fig. 6D, lanes 1 to 6, and data not shown). Formation of complex −11/2A was also not competed for by the C/EBP binding sites from the hepatitis B virus enhancer and from the albumin promoter (Fig. 6D, lanes 7 and 8).

We observed identical protein-DNA complexes with extracts prepared from FTO-2B hepatoma and XC fibrosarcoma cells and from the dedifferentiated hepatoma cell line HTC, confirming the results of the DNase I footprint experiment. Although the specific complex obtained with oligonucleotide −11/2A is not hepatoma cell specific, we consider it to be an important protein-DNA interaction, since there is a good correspondence between the guanines protected in the methylation interference experiment and the in vivo footprint identified over the enhancer domain −11/2A (Fig. 6C). When oligonucleotide −11/2B was tested in gel shift assays, it also gave a comparable pattern of retarded complexes with extracts from all three cell lines (data not shown). The underlying palindromic sequence resembles a CRE. Competition experiments and gel shifts with purified CRE-binding protein (CREB; kindly provided by M. Nichols) demonstrated that oligonucleotide −11/2B contained a low-affinity binding site for CREB. However, since the enhancer motif −11/2 is not influenced by cAMP, the relevance of this binding is unclear (data not shown). Furthermore, we did not observe any changes at this CRE-related sequence in genomic footprinting analysis following treatment with forskolin (Fig. 3). Possibly other members of the CREB/ATF family of transcription factors (74) are important for enhancer function.

Distinct point mutations in subdomain −11/2 affect enhancer function in vivo and protein-DNA binding in vitro. To test the significance of the binding of ubiquitous proteins to the −11/2 domain in vitro, additional gene transfer experiments were performed. We analyzed the activity of oligonucleotides that carry distinct point mutations at nucleotides whose methylation and mutation interfered with protein binding in vitro (Fig. 6 and data not shown). Oligonucleotides were cloned in front of the TK promoter and transfected into FTO-2B hepatoma cells. Figure 7 illustrates that the triple-point mutation, which abolished formation of the specific complex obtained with oligonucleotide −11/2A (Amut), reduced the stimulation of the TK promoter by this enhancer domain to less than 10% of wild-type activity. Mutation of the palindromic motif (Bmut) resulted in a fourfold reduction. Thus, distinct point mutations in enhancer domain −11/2 interfere with the binding of ubiquitous proteins in vitro and hepatoma cell-specific enhancer function in vivo.

In addition, we tested the effect of mutating two guanines at bp −10535 and −10536, which showed altered reactivity to DMS in vivo (Fig. 3) and which lie exactly between the two DNase I footprints illustrated in Fig. 5 (Cmut in Fig. 7). Mutation of the respective guanines in enhancer domain −11/2 results in loss of more than 90% of wild-type activity. Gel mobility shift assays with an oligonucleotide containing sequences from bp −10526 to −10546 have failed to reveal any in vitro protein-DNA complex sensitive to mutation of, and corresponding to the in vivo footprint at, guanines −10535 and −10536 (data not shown).

DISCUSSION

We have studied in detail a constitutive liver-specific enhancer located approximately 11 kb upstream of the promoter of the rat TAT gene. Contained within a 221-bp fragment are important regulatory sequences which stimulated the heterologous TK promoter more than 100-fold in differentiated hepatoma cells. We have identified an HNF3 binding site as an essential enhancer motif (−11/3), the deletion of which resulted in loss of 90% of enhancer

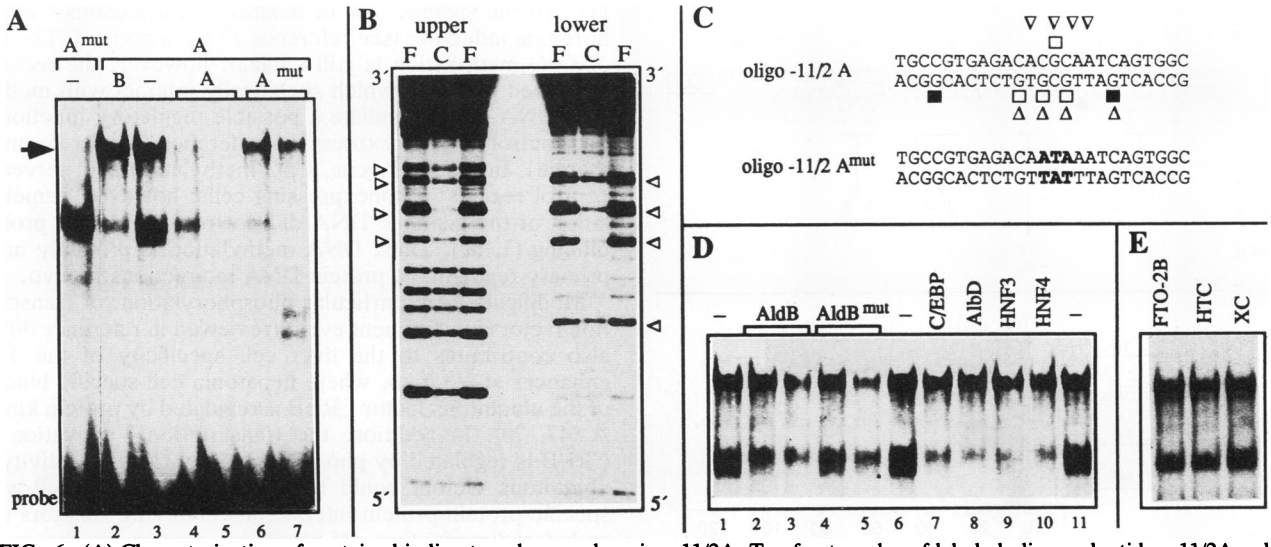

FIG. 6. (A) Characterization of proteins binding to enhancer domain −11/2A. Ten femtomoles of labeled oligonucleotide −11/2A, which corresponds to the 5′ half of enhancer domain −11/2 (lanes 2 to 7), or oligonucleotide −11/2Amut (lane 1; see panel C) was incubated with 7.5 μg of extract prepared from FTO-2B hepatoma cells in the presence of 300 ng of poly(dA-dT) and no oligonucleotide (lanes 1 and 3) or a 40-fold (lanes 4 and 6) or 200-fold (lanes 2, 5, and 7) molar excess of the indicated unlabeled oligonucleotides. A, A^{mut}, and B denote oligonucleotides −11/2A, −11/2Amut, and −11/2B, respectively. Protein-DNA complexes were subsequently separated from the unbound probe in a 4% polyacrylamide gel. The arrow points to the specific complex. (B) Results of methylation interference experiments for the upper and lower strand of oligonucleotide −11/2A. Preparative gel mobility shift assays with partially methylated oligonucleotide and FTO-2B extracts were performed. DNA from the specific complex (lanes C) and the unretarded fraction (lanes F) was isolated and cleaved at methylated purines as described in Materials and Methods. The purified DNA fragments were separated in a 15% denaturing polyacrylamide gel. Triangles point at purines whose methylation strongly interferes with complex formation. (C) Summary of the methylation interference pattern of complex −11/2A and comparison with the in vivo footprint (see Fig. 3). In the sequence of oligonucleotide $-11/2A^{mut}$, the triple-point mutation is shown in boldface letters. (D) Gel mobility shift experiments with FTO-2B extracts, performed as for panel A in the absence (−) or presence of a 40-fold (lanes 2 and 4) or 200-fold (lanes 3, 5, and 7 to 10) molar excess of oligonucleotides corresponding to a CCAAT box motif from the aldolase B promoter or a mutant thereof (lanes 2 to 5), the C/EBP binding site from the hepatitis B virus enhancer (lane 7) or the albumin promoter (lane 8), and HNF3 and HNF4 binding sites from the transthyretin promoter (lanes 9 and 10). (E) Assay in which extracts prepared from FTO-2B, HTC, and XC cells were incubated with oligonucleotide −11/2A as described for panel A.

activity. A second essential domain (−11/2) is constituted by a 35-bp fragment, which itself conferred liver cell-specific activity to the TK promoter. Thus, the enhancer has a modular structure with at least two liver cell-specific domains. These enhancer motifs are distinct from the essential elements of the liver-specific TAT enhancer at −3.6 kb, in which an HNF4 binding site and a CRE synergize to confer cell-type-specific and cAMP-inducible activity (7, 47, 49, 70). However, HNF3 proteins are also involved in the activation of the glucocorticoid-inducible enhancer of the TAT gene in hepatoma cells (49).

Our analysis of transgenic mice carrying reporter genes with different TAT upstream sequences suggests that the enhancer at −11 kb is required for regulated expression (3). We have shown here that the liver-specific factors HNF3α, -β, and -γ (38, 39) are capable of interacting in vitro with an essential element of this enhancer, giving rise to a methylation interference pattern which shows a good correspondence to the in vivo footprint observed in hepatoma cells. HNF3 binding sites have also been identified in the distal enhancers of the albumin, α-fetoprotein, and phosphoenolpyruvate carboxykinase genes (21, 31, 73). In each case, the enhancers are required for high levels of liver-specific expression in transgenic mice (9, 27, 54). In the mouse, the HNF3α, -β, and -γ genes are expressed at day 9, when the liver bud has just formed (34). This fact is compatible with the observation that the TAT enhancer at −11 kb is characterized by a DNase I-hypersensitive site in fetal liver which appears prior to the hypersensitive sites corresponding to the other regulatory regions of the TAT gene (62). Thus, we propose that one or all of the HNF3 proteins may be involved in opening the TAT expression domain.

HNF3 proteins contain a conserved DNA binding domain with high homology to the *Drosophila* protein fork head, which is implicated in the regulation of endoderm development (39, 68). The HNF3 proteins bind to the same DNA sequences in vitro; however, differences in affinity have been observed among them (39). The HNF3 motif in the TAT gene enhancer shows a significant sequence homology to the strong HNF3 binding site in the transthyretin gene promoter, and it interacts with all three proteins. It is, however, unclear whether this interaction is more selective in vivo. Analyses in the dedifferentiated hepatoma cell line HTC indicate different functional roles of the three HNF3 proteins. Whereas in FTO-2B cells, which express all three factors, four tandem repeats of the HNF3 motif stimulated the TK promoter at least 50-fold, the same construct was inactive in the HTC cell line even though it contains high levels of HNF3β DNA-binding activity. Since we also did not observe any in vivo footprint at the HNF3 motif in HTC cells (data not shown), it appears that HNF3β does not bind in vivo. One possible explanation is that in HTC cells, HNF3β is lacking some modification which is essential for its activity. Recently, two putative casein kinase I phosphorylation sites have been described (53a). Another, more speculative interpretation of the results obtained with the

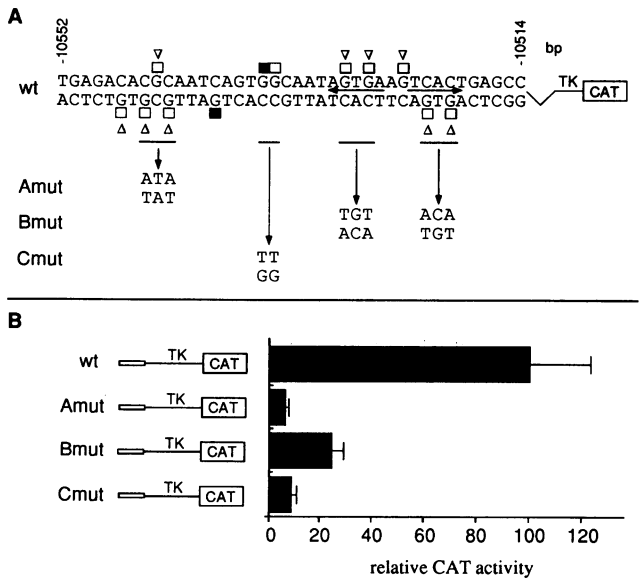

FIG. 7. Mutation of the binding sites for the ubiquitous proteins results in reduced enhancer activity. FTO-2B cells were transfected with constructs containing either the wild-type (wt) sequence of enhancer domain −11/2 or distinct mutants thereof in front of the TK promoter driving the CAT gene. (A) Mutations and relationship to in vivo and in vitro protein-DNA interactions. Open and filled squares correspond to guanosines that show reduced and enhanced reactivity to DMS treatment in vivo, respectively; triangles indicate guanosines whose methylation interferes with protein binding in vitro (Fig. 3B and 4B and data not shown); horizontal arrows outline the palindromic sequence underlying enhancer motif −11/2B. (B) Results of transfection experiments in FTO-2B cells, showing the relative CAT activities of the different constructs. The activity of the wild-type construct was set at 100. The mean values and standard deviations of three independent experiments are given.

HTC cell line is that HNF3α and/or -γ but not HNF3β are involved in activating the TAT gene via the enhancer at −11 kb. Functional differences between the three HNF3 proteins are also suggested by the differential expression of the respective genes in hepatoma × fibroblast hybrids, in which expression of HNF3β and -γ but not HNF3α is down-regulated (50).

The second liver-specific enhancer domain (−11/2) interacts in vitro with two ubiquitous proteins, whereas in vivo protein binding seems to be restricted to hepatoma cells, as evidenced from genomic footprinting experiments. Although it cannot be excluded that the corresponding liver-enriched factors have not been detected in the in vitro analyses, the identification of distinct point mutations which abolish protein binding as well as enhancer activity strongly suggests a role of the corresponding proteins in enhancer function. Cell-type-specific in vivo binding of ubiquitous proteins has been observed before (1). These data suggest that transcription factors which are ubiquitously expressed can also contribute to tissue-specific expression patterns. Several different mechanisms, which could act on the level either of the regulatory protein or of the target DNA, can be envisaged.

Changes in chromatin structure and/or DNA modifications could influence the binding of factors to their target DNA. A function of chromatin structure in the control of gene activity is suggested by several observations, including the repression of in vitro transcription by nucleosomes and histone H1 and the specific loss of positioned nucleosomes during hormone induction (see reference 17 for a review). The role of CpG methylation is still unclear; however, the recently described proteins, which specifically interact with methylated DNA, could mediate a possible regulatory function in the control of gene expression (reference 5 and references therein). In the TAT gene, CpG methylation is observed at control regions in nonexpressing cells; however, demethylation of the genomic DNA did not restore in vivo protein binding (1, 69). Thus, DNA methylation is probably not a primary regulator of protein-DNA interactions in vivo.

Modification, in particular phosphorylation, of transcription factors is a frequent event (reviewed in reference 30). It also contributes to the liver cell specificity of the TAT enhancer at −3.6 kb, where hepatoma cell-specific binding of the ubiquitous factor CREB is regulated by protein kinase A (47, 70). In addition, the transcriptional activation by CREB is regulated by phosphorylation (22). The activity of ubiquitous factors could be controlled also by cell-type-specific protein-protein interactions involving adaptors (20) or heterodimerization. Of particular interest are the examples of dominant repressors, which have been described for several groups of cell-type-specific transcription factors such as the helix-loop-helix, POU, and C/EBP families (4, 13, 57, 66). Cloning of the proteins binding to the enhancer domain −11/2 will help to elucidate whether protein modification and/or specific protein-protein interactions restrict the binding in vivo to hepatoma cells.

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REFERENCES

1. **Becker, P. B., S. Ruppert, and G. Schütz.** 1987. Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. Cell **51**:435–443.
2. **Becker, P. B., and G. Schütz.** 1988. Genomic footprinting, p. 1–19. *In* J. K. Setlow (ed.), Genetic engineering, principles and methods, vol. 10. Plenum Press, New York.
3. **Beermann, F., E. Hummler, E. Schmid, and G. Schütz.** Perinatal activation of a tyrosine aminotransferase fusion gene does not occur in albino lethal mice. Mech. Dev., in press.
4. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
5. **Bird, A.** 1992. The essentials of DNA methylation. Cell **70**:5–8.
6. **Boshart, M., M. Klüppel, A. Schmidt, G. Schütz, and B. Luckow.** 1991. Reporter constructs with low background activity utilizing the cat gene. Gene **110**:129–130.
7. **Boshart, M., F. Weih, A. Schmidt, R. E. K. Fournier, and G. Schütz.** 1990. A cyclic AMP response element mediates repression of tyrosine aminotransferase gene expression by the tissue-specific extinguisher locus Tse-1. Cell **61**:905–916.
8. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
9. **Cheyette, T. E., T. Ip, S. Faber, Y. Matsui, and R. Chalkley.** 1992. Characterization of the factors binding to a PEPCK gene upstream hypersensitive site with LCR activity. Nucleic Acids Res. **20**:3427–3433.
10. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic

Acids Res. 15:1311–1326.
11. **Costa, R. H., D. R. Grayson, and J. E. Darnell.** 1989. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α1-antitrypsin genes. Mol. Cell. Biol. 9:1415–1425.
12. **Descombes, P. M., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler.** 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes Dev. 4:1541–1551.
13. **Descombes, P. M., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67:569–579.
14. **De Simone, V., and R. Cortese.** 1991. Transcriptional regulation of liver-specific gene expression. Curr. Opin. Cell Biol. 3:960–965.
15. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7:725–737.
16. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475–1489.
17. **Felsenfeld, G.** 1992. Chromatin as an essential part of the transcriptional mechanism. Nature (London) 355:219–224.
18. **Fromm, M., L. P. Taylor, and V. Walbot.** 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. USA 82:5824–5828.
19. **Ganss, R., F. Weih, and G. Schütz.** Unpublished data.
20. **Glass, C. K., O. V. Devary, and M. G. Rosenfeld.** 1990. Multiple cell type-specific proteins differentially regulate target sequence recognition by the α retinoic acid receptor. Cell 63:729–738.
21. **Godbout, R., R. Ingram, and S. M. Tilghman.** 1988. Fine-structure mapping of the three mouse α-fetoprotein gene enhancers. Mol. Cell. Biol. 8:1169–1178.
22. **Gonzalez, G. A., and M. R. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680.
23. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
24. **Grange, T., J. Roux, G. Rigaud, and R. Pictet.** 1991. Cell-type-specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor. Nucleic Acids Res. 19:131–139.
25. **Granner, D. K., and E. G. Beale.** 1985. Regulation of the synthesis of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase by glucocorticoid hormones, p. 89–138. *In* G. Litwack (ed.), Biochemical actions of hormones XII. Academic Press, New York.
26. **Greengard, O.** 1970. The developmental formation of enzymes in rat liver, p. 53–85. *In* G. Litwack (ed.), Mechanisms of hormone action, vol. 1. Academic Press, New York.
27. **Hammer, R. E., R. Krumlauf, S. A. Camper, R. L. Brinster, and S. M. Tilghman.** 1987. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. Science 235:53–58.
28. **Hargrove, J. L., and D. K. Granner.** 1985. Biosynthesis and intracellular processing of tyrosine aminotransferase, p. 511–532. *In* P. Christen and P. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
29. **Hashimoto, S., W. Schmid, and G. Schütz.** 1984. Transcriptional activation of the rat liver tyrosine aminotransferase gene by cAMP. Proc. Natl. Acad. Sci. USA 81:6637–6641.
30. **Hunter, T., and M. Karin.** 1992. The regulation of transcription by phosphorylation. Cell 70:375–387.
31. **Ip, T. V., D. Poon, D. Stone, D. K. Granner, and R. Chalkley.** 1990. Interaction of a liver-specific factor with an enhancer 4.8 kilobases upstream of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. 10:3770–3781.
32. **Jantzen, H.-M., U. Strähle, B. Gloss, F. Stewart, W. Schmid, M. Boshart, R. Miksicek, and G. Schütz.** 1987. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 49:29–38.
33. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58:799–839.
34. **Kästner, K., and G. Schütz.** Unpublished data.
35. **Killary, A. M., and R. E. K. Fournier.** 1984. A genetic analysis of extinction: trans-dominant loci regulate expression of liver-specific traits in hepatoma hybrid cells. Cell 38:523–534.
36. **Killary, A. M., T. G. Lugo, and R. E. K. Fournier.** 1984. Isolation of thymidine kinase deficient rat hepatoma cells by selection with bromo-deoxyuridine, Hoechst 33258, and visible light. Biochem. Genet. 22:201–213.
37. **Kuo, C. J., P. B. Conley, L. Chen, F. M. Sladek, J. E. Darnell, Jr., and G. R. Crabtree.** 1992. A transcriptional hierarchy involved in mammalian cell-type specification. Nature (London) 355:457–461.
38. **Lai, E., V. R. Prezioso, E. Smith, O. Litvin, R. H. Costa, and J. E. Darnell, Jr.** 1990. HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. 4:1427–1436.
39. **Lai, E., V. R. Prezioso, W. Tao, W. S. Chen, and J. E. Darnell, Jr.** 1991. Hepatocyte nuclear factor 3α belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes Dev. 5:416–427.
40. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. 2:786–800.
41. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741–752.
42. **Lewin, B.** 1990. Commitment and activation at Pol II promoters: a tail of protein-protein interactions. Cell 61:1161–1164.
43. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science 236:1237–1245.
44. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65:499–560.
45. **Mueller, C. R., P. Maire, and U. Schibler.** 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell 61:279–291.
46. **Müller, G., S. Ruppert, E. Schmid, and G. Schütz.** 1988. Functional analysis of alternatively spliced tyrosinase gene transcripts. EMBO J. 7:2723–2730.
47. **Nichols, M., F. Weih, W. Schmid, C. DeVack, E. Kowenz-Leutz, B. Luckow, M. Boshart, and G. Schütz.** 1992. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J. 11:3337–3346.
48. **Nitsch, D.** 1991. Ph.D. thesis. Fachbereich Biologie, Universität Heidelberg, Heidelberg, Germany.
49. **Nitsch, D., M. Boshart, and G. Schütz.** Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. Proc. Natl. Acad. Sci. USA, in press.
50. **Nitsch, D., M. Boshart, and G. Schütz.** 1993. Extinction of tyrosine aminotransferase gene activity in somatic cell hybrids involves modification and loss of several essential transcriptional activators. Genes Dev. 7:308–319.
51. **Nitsch, D., S. Ruppert, G. Kelsey, A. Schedl, F. Weih, A. F. Stewart, U. Strähle, C. DeVack, A. Reik, M. Boshart, and G. Schütz.** 1991. Hormonal and liver-specific control of expression of the tyrosine aminotransferase gene, p. 223–234. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal control regulation of gene transcription. Elsevier Science Publishers B.V., The Netherlands.
52. **Nitsch, D., A. F. Stewart, M. Boshart, R. Mestril, F. Weih, and G. Schütz.** 1990. Chromatin structures of the rat tyrosine aminotransferase gene relate to the function of its *cis*-acting elements. Mol. Cell. Biol. 10:3334–3342.
53. **Öfverstedt, L.-G., K. Hammarström, N. Balgobin, S. Hjertén, U. Pettersson, and J. Chattopadhyaya.** 1984. Rapid and quantitative recovery of DNA fragments from gels by displacement electro-

phoresis (isotachophoresis). Biochim. Biophys. Acta **782:**120–126.

53a. **Pani, L., D. G. Overdier, A. Porcella, X. Qian, E. Lai, and R. H. Costa.** 1992. Hepatocyte nuclear factor 3β contains two transcriptional activation domains, one of which is novel and conserved with the *Drosophila* fork head protein. Mol. Cell. Biol. **12:**3723–3732.

54. **Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter.** 1987. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev. **1:**268–276.

55. **Reik, A., G. Schütz, and A. F. Stewart.** 1991. Glucocorticoids are required for establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer. EMBO J. **10:**2569–2576.

56. **Rigaud, G., J. Roux, R. Pictet, and T. Grange.** 1991. In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell **67:**977–986.

57. **Ron, D., and J. F. Habener.** 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev. **6:**439–453.

58. **Schmid, E., W. Schmid, D. Mayer, B. Jastorff, and G. Schütz.** 1987. Transcriptional activation of the tyrosine aminotransferase gene by glucocorticoids and cAMP in primary hepatocytes. Eur. J. Biochem. **165:**499–506.

59. **Schreiber, E., P. Matthias, M. M. Müller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res. **27:**6419.

60. **Schubart, U. K.** 1986. Regulation of gene expression in rat hepatocytes and hepatoma cells by insulin: quantitation of messenger ribonucleic acid's coding for tyrosine aminotransferase, tryptophan oxygenase, and phosphoenolpyruvate carboxykinase. Endocrinology **119:**1741–1749.

61. **Sladek, F. M., and J. E. Darnell, Jr.** 1992. Mechanisms of liver-specific gene expression. Curr. Opin. Genet. Dev. **2:**256–259.

62. **Stewart, A. F., and G. Schütz.** Unpublished data.

63. **Svoboda, J.** 1960. Presence of a chicken tumor virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma virus. Nature (London) **186:**980–981.

64. **Thompson, E. B., G. M. Tomkins, and J. F. Curran.** 1966. Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. Proc. Natl. Acad. Sci. USA **56:**296–303.

65. **Tian, J.-M., and U. Schibler.** 1991. Tissue-specific expression of the gene encoding hepatocyte nuclear factor 1 may involve hepatocyte nuclear factor 4. Genes Dev. **5:**2225–2234.

66. **Treacy, M. N., X. He, and M. G. Rosenfeld.** 1991. I-POU: a POU-domain protein that inhibits neuron-specific gene activation. Nature (London) **350:**577–584.

67. **Tsutsumi, K.-I., K. Ito, and K. Ishikawa.** 1989. Developmental appearance of transcription factors that regulate liver-specific expression of the aldolase B gene. Mol. Cell. Biol. **9:**4923–4931.

68. **Weigel, D., and H. Jäckle.** 1990. The fork head domain: a novel DNA binding motif of eukaryotic transcription factors? Cell **63:**455–456.

69. **Weih, F., D. Nitsch, A. Reik, G. Schütz, and P. B. Becker.** 1991. Analysis of CpG methylation and genomic footprinting at the tyrosine aminotransferase gene: DNA methylation alone is not sufficient to prevent protein binding in vivo. EMBO J. **10:**2559–2567.

70. **Weih, F., A. F. Stewart, M. Boshart, D. Nitsch, and G. Schütz.** 1990. In vivo monitoring of a cAMP-stimulated DNA-binding activity. Genes Dev. **4:**1437–1449.

71. **Weih, F., A. F. Stewart, and G. Schütz.** 1988. A novel and rapid method to generate single stranded DNA probes for genomic footprinting. Nucleic Acids Res. **16:**1628.

72. **Wildeman, A. G., P. Sassone-Corsi, T. Grundström, M. Zenke, and P. Chambon.** 1984. Stimulation of in vitro transcription from SV40 early promoter by the enhancer involves a specific trans-acting factor. EMBO J. **3:**3129–3133.

73. **Zaret, K. S., J.-K. Liu, and C. M. DiPersio.** 1990. Site-directed mutagenesis reveals a liver transcription factor essential for the albumin transcriptional enhancer. Proc. Natl. Acad. Sci. USA **87:**5469–5473.

74. **Ziff, E. B.** 1990. Transcription factors: a new family gathers at the cAMP response site. Trends Genet. **6:**69–72.