A C/EBP-Binding Site in the Transferrin Promoter Is Essential for Expression in the Liver but Not the Brain of Transgenic Mice

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The gene for the iron-binding protein transferrin is transcribed at a high level in liver hepatocytes but is also active in several other cell types, including oligodendrocytes in the brain. Enhancer elements between bp –560 and –44 of the transferrin gene promoter specifically activated transcription from a heterologous promoter in transgenic mouse liver and brain. Within this region, a potent *cis*-acting element between bp –98 and –83 was found to be essential for gene activity in both cultured hepatocytes and transgenic mouse liver. The –98 to –83 element contains a CCAAT sequence and is specifically bound by a nuclear factor from mouse liver that is homologous to rat liver C/EBP (CAAT enhancer-binding protein). Point mutations within this binding site inhibit factor binding and abolish transcription in transfected hepatoma cells. When placed in the context of the 3,000-bp transferrin promoter, the C/EBP binding site mutation causes a complete loss of transcription in transgenic mouse liver; however, transgene expression in the brain of the same animals was unaffected. These results suggest a modular structure for the transferrin promoter and demonstrate that deletions or specific point mutations can be used to generate transgene promoters with an activity more restricted than that of their endogenous counterparts.

Most eucaryotic genes are expressed in more than a single tissue or cell type, and many are expressed in a stage-specific pattern during development. In some cases, multiple tissue-specific enhancer elements that are presumed to co-operate with proximal promoter elements to elicit complex expression patterns can be identified in the promoters of these genes. We have focused our attention on the transferrin gene since it contains a promoter that directs expression to several highly differentiated tissues and this expression can be regulated by hormonal and nutritional effectors (10, 24, 26).

The mammalian transferrin (Tf) gene encodes an iron-binding protein that is secreted by liver and circulates at ~2 to 4 mg/ml in the blood, where it functions to deliver iron to cells throughout the organism (34). Several other tissues, including brain oligodendrocytes (3), choroid plexus (45), Sertoli cells (24), and mammary gland (10), express Tf in the adult. In most of these cases, Tf is produced in an environment that is partially sequestered from access to blood Tf, and the synthesized Tf may play a role in local iron homeostasis.

The Tf promoter has been isolated from chicken (12), human (30), and mouse (25) genomic DNAs. The human promoter has been extensively studied by expression of promoter-fusion constructs in transfected cells and by analyzing protein-DNA interactions. Multiple positive and negative elements were postulated on the basis of the deletion studies, and some of these corresponded to sites of high-affinity protein-DNA interactions (7). In addition to elements in the proximal promoter that are partially responsible for hepatoma cell specificity, attention has also been focused on

a far-upstream 5' sequence between kb –3.3 and –3.6 that stimulates transcription in human hepatoma cells but not in HeLa or Sertoli cells (4, 21, 37). The human Tf promoter has also been fused to chloramphenicol acetyltransferase (CAT) and analyzed in transgenic mice. The results indicated that the proximal promoter region between –152 and +46 was poorly expressed in all tissues, compared with high levels of brain and liver expression with constructs containing sequences out to bp –652 or –1152 (1). These results suggest that the proximal promoter region of the human Tf gene is not sufficient for tissue-specific expression in transgenic mice.

Our previous results using transgenic mice differ somewhat from the human promoter studies. We used a fusion gene between the mouse Tf promoter and the human growth hormone gene (hGH) to show that the major control elements required for liver-specific expression of the mouse promoter lie within 139 bp of the start site of transcription (25). Differences between our work with the mouse promoter and the results from studies with the human promoter may reflect species specific promoter differences or the influence of the different reporter constructs used (CAT versus hGH).

The proximal promoter regions of both human and mouse Tf contains a CCAAT consensus sequence that binds to members of the C/EBP (CAAT enhancer-binding protein) family of transcription factors (35). In this study, we characterize the C/EBP binding site in the mouse Tf promoter centered around bp –91 and show that this site is essential for expression in transiently transfected hepatoma cell cultures. We then examine the role of the C/EBP binding site in directing tissue-specific expression in transgenic mice by specifically mutating this site in the context of the intact 3-kb promoter. The results demonstrate that the interaction of C/EBP or related factors with the CCAAT site at bp –91 is essential for expression in transgenic mouse liver but is not required for oligodendrocyte expression in mouse brain.

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MATERIALS AND METHODS

Construction of CAT fusion genes for transient transfections. (i) **-3000TfCAT.** A 3,050-bp *EcoRI*-*Bam*HI DNA fragment covering Tf sequences from positions -3000 to +50 (25) was cloned into the *Bam*HI-*Bgl*II vector of plasmid pMMTVCAT (33) that was lacking the mouse mammary tumor virus control sequences. The *Eco*RI site of the fragment and the *Bam*HI site of the vector were filled in with Klenow DNA polymerase.

(ii) **-581TfCAT.** -3000TfCAT was digested with *Hind*III. The fragment containing Tf sequences from -581 to +50 fused to the CAT gene and all vector sequences to a *Hind*III site within the polylinker was isolated and religated at the *Hind*III sites to generate -581TfCAT.

(iii) **-326TfCAT, -180TfCAT, and -83TfCAT.** A *Pvu*II fragment from position -326 of the Tf promoter to position +150 within the CAT gene was isolated from -581TfCAT. This fragment was either used directly (-326TfCAT) or recut with *Hin*FI (-180TfCAT), *Fok*I (-98TfCAT), or *Fsp*I (-83TfCAT). Restriction sites were filled in with Klenow DNA polymerase. The DNA fragments were recut within the CAT gene with *Xho*I and ligated into a *Hind*III (blunt)-*Xho*I-cut CAT vector that was derived from pMMTVCAT. Plasmid DNAs for transient transfection experiments were purified by centrifugation in two successive CsCl gradients.

Construction of Tf-hGH fusion genes. (i) **-98TfhGH.** A DNA fragment from positions -98 to +50 of the Tf gene was isolated from -98TfCAT by digestion with *Pvu*II and *Sau*3A. The fragment was fused to the 2.2-kb *Bam*HI-*Eco*RI hGH gene fragment cloned into the Bluescript KS+ vector (Stratagene).

(ii) **(-44 to -560)TfhGH.** A 516-bp *Hae*III fragment from positions -560 to -44 of the Tf 5' region was cloned in inverse orientation into a unique *Hinc*II site at position -83 in the hGH promoter. For production of transgenic mice, DNA fragments containing the fusion genes were isolated from vector sequences by restriction digestion and separation on agarose gels. DNA fragments were extracted by electroelution, extracted twice with phenol-chloroform (1:1), extracted with chloroform, and precipitated with ethanol.

Site-directed mutagenesis. Tf DNA sequences were mutagenized with the Amersham oligonucleotide-directed in vitro mutagenesis system. A 631-bp *Hind*III-*Bam*HI fragment (positions -581 to +50) of the Tf gene region was cloned into M13 mp19. Mutated DNA was identified by restriction mapping, and the mutations were confirmed by DNA sequencing.

Cell growth, cell transfection, and CAT assay. C2Rev7 cells (14) were grown in 10-cm-diameter tissue culture dishes (Falcon) in Ham's F12 medium (GIBCO) supplemented with 5% fetal calf serum at 37°C in 5% CO₂. Cells were harvested by trypsinization, diluted 1:5, and fed with 10 ml of fresh medium twice a week. Cells were transfected by the calcium phosphate coprecipitation method as described by Theisen et al. (44), with the following modifications. C2Rev7 cells were harvested by trypsinization, and 1.5×10^6 cells were plated on a 10-cm-diameter tissue culture dish. The DNA-CaPO₄ suspension was immediately added to the cells. Cells were incubated for 8 h at 37°C. Thereafter, cells were washed with 1× EDTA, fed with fresh medium, and incubated for 48 h at 37°C. Cells were harvested and lysed in 150 µl of 0.25 M Tris-Cl (pH 7.8) by freeze-thaw. The lysate was cleared by centrifugation in a microcentrifuge, and the cell extract was stored at -20°C. CAT activity was measured by

incubation of 50 µl of extract in a total volume of 180 µl containing 0.25 M Tris-Cl (pH 7.8), 4 mM acetyl coenzyme A, and 0.2 µCi of [¹⁴C]chloramphenicol (58 mCi/mmol). The reaction was terminated by extraction of chloramphenicol with ethyl acetate, and the acetylated derivatives were separated from unmodified chloramphenicol by thin-layer chromatography on silica gel (Whatman) with chloroform-methanol (95:5). The conversion value was determined by scintillation counting.

Production of transgenic mice. Fusion gene DNA was microinjected into the male pronucleus of (C57 × SJL)F₂ mouse eggs to produce transgenic mice (6). Transgene copy number was determined by dot blot analysis using a nick-translated hGH gene fragment to probe samples of DNA isolated from mouse tails (36). The structure of each of the integrated transgenes was determined by restriction analysis and Southern blotting.

RNA analysis. Transgene mRNA concentration was determined by solution hybridization as described by Idzerda et al. (25, 26). Total nucleic acid was isolated from mouse tissues and hybridized in solution to a single-stranded ³²P-labeled RNA probe. After hybridization, samples were treated with RNase to digest unhybridized probe. Nucleic acid was precipitated with trichloroacetic acid and collected on Whatman GF/C glass fiber filters. Radioactivity was determined by scintillation counting. For Northern (RNA) blots, RNA was purified from total nucleic acid by precipitation with an equal volume of 4 M LiCl. One to 5 µg of RNA was denatured at 68°C in 6.6% formaldehyde-20 mM morpholine propanesulfonic acid (MOPS; pH 7.0)-1 mM EDTA, 5 mM sodium acetate-50% formamide, electrophoresed through a 1% agarose gel (23), and transferred to a nitrocellulose filter (Schleicher & Schuell). The filter was hybridized with a mixture of a nick-translated 0.35-kb *Pvu*II-*Pst*I Tf DNA fragment and a 0.3-kb *Ssp*I-*Bgl*II hGH gene fragment. Filters were exposed to Kodak XAR-5 films for autoradiography.

S1 nuclease mapping. Transcriptional start sites of transgene mRNAs were determined by S1 nuclease mapping as described by Theisen et al. (44). RNA samples were hybridized in 10 µl of 400 mM NaCl-40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)-1 mM EDTA-80% formamide at 43°C for 12 h to an excess of a 228-bp *Dde*I fragment from positions -152 to +76 of the fusion genes. After hybridization, probes were diluted with 250 µl of 300 mM NaCl-30 mM sodium acetate (pH 4.5)-3 mM ZnSO₄-100 µg of denatured and sonicated salmon sperm DNA per ml and digested with 100 U of S1 nuclease at 25°C for 2 h. S1-resistant DNA fragments were analyzed on polyacrylamide-urea sequencing gels. Gels were exposed to Kodak XAR-5 films.

Preparation of nuclear extracts. Protein extracts from mouse liver nuclei were prepared as described by Dignam et al. (15). Fresh liver tissue was homogenized with a Polytron (Kinematica) in 3 ml of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol) per g. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 10 min at 3,500 rpm in a Sorvall HB4 rotor. The crude nuclear pellet was washed with 2 ml of buffer A and centrifuged for 20 min at 14,500 rpm (25,000 × g) in a Sorvall SS34 rotor. The pellet was resuspended in 3 ml of buffer C (20 mM HEPES [pH 7.9], 25% glucose, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride 0.5 mM dithiothreitol per 10⁹ nuclei. Nuclei were homogenized in a Dounce homogenizer (10

strokes, type B pestle), and the suspension was stirred at 4°C for 30 min. After centrifugation for 30 min at 14,500 rpm in a Sorvall SS34 rotor, the supernatant was dialyzed for 5 h against 50 volumes of buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). The extract was cleared by centrifugation for 20 min at 14,500 rpm in an SS34 rotor, frozen in aliquots in liquid N₂, and stored at -70°C.

Protein-DNA binding assays. Protein-DNA binding reactions were performed as described by Borgmeyer et al. (5), with the following modifications. In a total volume of 50 µl, 3 to 30 µg of liver nuclear proteins was preincubated with 80 ng of poly(dI-dC) (Pharmacia) in 10 mM HEPES (pH 8.0)–0.1 mM EDTA–100 mM NaCl for 10 min at 25°C; 0.5 to 2 ng of ³²P-labeled fragment was added, and the mixture was incubated for 20 min at 25°C. For gel retardation assays (17), samples were loaded on 5% polyacrylamide gels and the bound DNA was separated from the nonbound fraction by electrophoresis. For DNase footprint assays (41), samples were chilled on ice. Then 1 µl of 0.1 M MgCl₂ and 5 µl of serial dilutions of DNase I (0.1 mg/ml) were added, and the samples were incubated for 3 min at room temperature. The reaction was stopped by addition of 1 µl of 0.5 M EDTA. Samples were loaded on 5% polyacrylamide gels to separate bound and nonbound DNA fraction. DNA fragments were isolated from the gel by electroelution. DNA was purified by phenol extraction and ethanol precipitation and analyzed on denaturing polyacrylamide-urea sequencing gels.

In situ hybridization. Ten-micrometer frozen sections of mouse brain were thaw mounted and hybridized to ³⁵S-labeled RNA probes as previously described (8).

RESULTS

Deletion analysis identifies a sequence between -98 and -83 which is required for Tf promoter activity in cultured cells. Putative *cis*-acting transcriptional elements within the mouse Tf promoter were identified by examining the activity of Tf-CAT fusion constructs after transient transfection into C2Rev7 rat hepatoma cells (14). These cells were chosen for the transfection experiments since they express their endogenous Tf gene and would therefore be expected to contain tissue-specific factors necessary for the specific activation of the transfected Tf promoter. The deletion constructs are shown in Fig. 1A. The *EcoRI*-*Bam*HI fragment from positions -3000 to +50 was fused with a promoterless gene for CAT, and Tf sequences were then deleted by using appropriate restriction sites. Figure 1B shows the activities of the individual deletion mutations. In agreement with previous results demonstrating high activity of the -3000Tf_hGX (hGX designates the hGH gene with a frameshift mutation that prevents production of active hormone) and -581Tf_hGX transgenes in transgenic mouse liver (25), the -3000- and -581TfCAT constructs show a high transient activity in C2Rev7 cells. Deletion to -326 leads to a significant loss of activity, but further deletion to -180 restores activity. Deletion of 15 bp between -98 and -83 drops the transcriptional activity of the Tf promoter to a level that is comparable to the level of a transfected lysozyme promoter-CAT gene (44) used as a negative control in these cells (see Fig. 4C). These experiments indicate that after transient transfection into C2Rev7 cells, the Tf promoter region between -98 and +50 is sufficient for high-level transcription of the fusion gene. The deletion studies suggest that a major

FIG. 1. Transcriptional activity of the mouse Tf promoter after transient transfection into C2Rev7 cells. (A) Deletion mutations were constructed as described in Materials and Methods. Plasmid DNA was transfected into C2Rev7 cells, and transcriptional activity was assessed by measuring the activity of CAT in cell extracts 48 h after transfection. (B) CAT activities of extracts from cells transfected with various Tf promoter-CAT fusion genes. Averages and ranges of values for duplicate transfections are shown.

regulatory element for the transcriptional control of the mouse Tf gene is located between bp -98 and -83.

A C/EBP-related protein binds to the -98 to -83 region of the Tf promoter. We used a mouse liver nuclear protein extract (15) to study the interaction of the mouse Tf gene control element(s) with DNA-binding proteins. The -581 to +50 fragment was isolated and digested with different restriction endonucleases to generate the subfragments that are shown in Fig. 2A. ³²P-labeled DNA fragments were analyzed for specific interaction with liver nuclear proteins by gel retardation (17) as described in Materials and Methods. Figure 2 summarizes these experiments. As shown in Fig. 2B (lanes 7 to 9), only the 230-bp *Hinf*I-*Bam*HI fragment bound to nuclear protein(s) in the liver extract. To further localize the binding site(s) on this fragment, three subfragments of the 230-bp fragment were generated and analyzed by gel retardation. Figure 2C shows that the 133-bp *Hinf*I-*Hae*III fragment contains the binding region from the 230-bp fragment (lanes 3 and 4). In contrast, neither the 150-bp *Fok*I-*Bam*HI (lanes 5 and 6) nor the 70 bp *Hinf*I-*Fok*I (lanes 7 and 8) fragment is bound. This result leads to the conclusion that a nuclear factor(s) binds to the Tf promoter between -180 and -44. Restriction digest of the DNA with *Fok*I at position -98 abolishes binding to this sequence. Figure 2D shows the specificity of the DNA-protein interaction in a competition experiment. Binding of protein to the radioactive 230-bp *Hinf*I-*Bam*HI fragment DNA was competed for with an excess of unlabeled *Hinf*I-*Bam*HI fragment (lanes 5 and 6). In contrast, neither the *Hinf*I-*Fok*I nor the *Fok*I-*Bam*HI fragment competed for specific binding (lanes 7 to 10).

FIG. 2. In vitro binding of mouse liver nuclear proteins to the Tf promoter region. (A) Restriction map of the mouse Tf gene promoter. Only recognition sites of restriction enzymes that were used for the isolation of subfragments for DNA binding assays or for the construction of deletion mutations are indicated. The transcriptional start site, +1, is indicated by an arrow. Fragments for in vitro binding assays are shown below the map. Lengths of fragments are given in base pairs. (B) DNA (0.5 ng) of the 257-bp *StyI-HinfI* (lanes 1 to 3), 144-bp *HindIII-StyI* (lanes 4 to 6), and 230-bp *HinfI-BamHI* (lanes 7 to 9) fragments was either directly loaded (lanes 1, 4, and 7) or incubated with bovine serum albumin (BSA) (lanes 2, 5, and 8) or 20 μ g of mouse liver nuclear protein (lanes 3, 6, and 9) prior to electrophoresis on a 5% polyacrylamide gel. For incubation conditions, see Materials and Methods. (C) DNA binding reactions with the 230-bp *HinfI-BamHI* (lanes 1 and 2), 133-bp *HinfI-HaeIII* (lanes 3 and 4), 150-bp *FokI-BamHI* (lanes 5 and 6), and 70-bp *HinfI-FokI* (lanes 7 and 8) fragments. DNA (0.5 ng) was incubated with BSA (lanes 1, 3, 5, and 7) or 20 μ g of mouse liver nuclear protein (lanes 2, 4, 6, and 8) prior to electrophoresis on a 5% polyacrylamide gel. (D) Competition experiment in which 0.5 ng of radioactive *HinfI-BamHI* fragment DNA was incubated with BSA (lane 1) or 20 μ g of liver nuclear protein (lanes 2 to 10). Gel retardation was determined in the presence of no competitor (lanes 1 and 2), 0.8 μ g of poly(dI-dC) (lane 3), 1.2 μ g of poly(dI-dC) (lane 4), 40 and 80 fmol of *HinfI-BamHI* fragment (lanes 5 and 6), 40 and 80 fmol 150-bp *FokI-BamHI* fragment (lanes 7 and 8), and 40 and 80 fmol of 70-bp *HinfI-FokI* fragment (lanes 9 and 10). Binding reactions were as described in Materials and Methods. Bound and nonbound DNA fragments were separated by electrophoresis on a 5% polyacrylamide gel.

To further characterize the binding site(s) within the -180 to -44 region, DNase footprinting was performed. Figure 3A shows as an example the DNase I footprint pattern on the noncoding strand of the *HinfI-HaeIII* fragment. Only a

FIG. 3. Structure of the -98 to -83 binding site. (A) DNase footprint of the Tf upstream region. *HinfI-HaeIII* fragment DNA (0.5 ng) was 3' end labeled at the *HinfI* site and incubated with 20 μ g of liver nuclear extract. After incubation, samples were digested with DNase I as described in Materials and Methods. Bound and nonbound DNA fragments were separated on a 5% polyacrylamide gel. The bound and free DNA fragments were isolated from the gel and denatured, and equal amounts of radioactive DNA were loaded on a 5% polyacrylamide-urea sequencing gel. The first lane on the left displays a Maxam-Gilbert G ladder as a size marker. The next lane contains free DNA, and the third contains DNA from the shifted band. The footprint on the noncoding strand of the Tf DNA is indicated on the right. (B) Summary of DNA binding data. The DNase footprint border on the noncoding DNA strand is indicated by a bracket. Recognition and cut sites for restriction endonuclease *FokI* are shown. (C) Homology between the C/EBP binding site of the mouse Tf promoter, the human and chicken Tf promoters, and the C/EBP binding site in the herpes simplex virus (HSV) *tk* promoter. Conserved nucleotides in all four binding sites are boxed.

single footprint could be identified under these conditions. The center of the DNase-protected region contains the palindromic sequence ATTGGGCAAT (Fig. 3B). Restriction endonuclease *FokI* produced nonbinding subfragments and makes a staggered cut within this palindrome. However, in the -98TfCAT reporter gene, the palindrome was regenerated as a result of fusion of the filled-in fragments to a *HindIII* site in the vector (see Materials and Methods). Binding studies with the -98TfCAT reporter gene showed that specific binding to the -98 to -83 region was restored (data not shown), consistent with the activity of this construct in C2Rev7 hepatocytes.

The palindromic binding site of the mouse Tf promoter is highly conserved in the human and chicken Tf genes (Fig. 3C), and binding of a nuclear factor to this region of the human gene has also been shown by Brunel et al. (7). The ATTGGGCAAT motif is also homologous to the dyad CAAT box of the herpes simplex virus *tk* promoter, and these sequences have been identified as binding sites for the transcription factor C/EBP (20, 29). Since the C/EBP transcription factor is unusually heat stable, we tested the binding of mouse liver nuclear extracts that had been heated to 95°C for 5 min and found that binding activity was

unaffected (data not shown). Further support for the interaction of the Tf promoter region with a C/EBP-like protein comes from experiments demonstrating that pure C/EBP expressed from a cloned bacterial expression vector binds to this Tf element with very high affinity and gives the same DNase footprint as shown in Fig. 3A (35).

Mutation of the C/EBP binding site eliminates transcriptional activity from the Tf promoter in cultured cells. The influence of C/EBP binding on the transcriptional activity of the entire Tf promoter region was examined by introducing a specific mutation into the C/EBP binding sequence. In vitro mutagenesis was used to mutate the wild-type sequence ATTTGGGCAAT to ATTTGTCTAGA, altering bases that have been shown to participate in C/EBP recognition (35) and creating an *Xba*I site to allow identification of mutant transgenes. Binding experiments with the wild-type or mutated version of the 230-bp *Hinf*I-*Bam*HI Tf fragment are shown in Fig. 4. Incubation of mutated DNA with liver nuclear extract followed by gel retardation showed no specific binding of nuclear factors (Fig. 4A, lanes 5 to 8), whereas wild-type DNA specifically bound proteins in the extract (lanes 1 to 4). In a competition experiment with radioactive wild-type DNA as tracer (Fig. 4B), excess mutated DNA fragment did not function as a specific competitor (lanes 7 to 10); wild-type DNA competed specifically for binding of the nuclear factor (lanes 3 to 6). Therefore, the mutation clearly inhibits binding of the C/EBP-like protein to the Tf promoter element in vitro.

The transcriptional activity of the mutated Tf promoter was tested by introducing the C/EBP binding site mutation into -581TfCAT (Fig. 1), generating -581Tf_{mut}CAT. After transient transfection into C2 Rev7 hepatocytes, this mutated vector gave essentially no CAT activity (Fig. 4C). Cells that were transfected with the -581TfCAT wild-type reporter gene as a positive control (581-wt) show the expected high level of CAT activity. These experiments demonstrate that mutation of the C/EBP binding site leads to a loss of transcriptional activity of the Tf promoter in transiently transfected cells.

The proximal location of the C/EBP site on the Tf promoter suggested that it might participate as an essential basal element of the promoter rather than as a tissue-specific enhancer. Attempts to demonstrate that the C/EBP site participated in tissue-specific expression were not convincing with cultured cells. The C2 line of hepatocytes and a mouse hepatocyte cell line, FMH202 (provided by Dieter Paul), were also active expressors of the same CAT constructs that were active in the C2Rev7 cells despite the observation that neither the C2 nor the FMH202 cells expressed the endogenous Tf gene (data not shown). Transgenic mice were therefore used to determine the role of the C/EBP binding site in tissue-specific expression from the Tf promoter under more physiological conditions.

Sequences upstream from the Tf promoter can confer tissue-specific activity on a heterologous promoter in transgenic mice. We have previously shown (25) that a DNA fragment between positions -581 and +50 of the mouse Tf gene is sufficient to direct tissue-specific expression of Tf-hGH transgenes to transgenic mouse liver and brain. Further deletion to position -139 retained liver-specific activity of the transgene (although at a lower overall level), while no brain-specific expression could be detected. To further characterize regulatory elements within this region of the Tf gene, we examined their influence on the expression of a heterologous promoter in transgenic mice.

We constructed a (-44 to -560)Tf-hGH transgene (Fig.

FIG. 4. In vitro binding and transient transfection experiments with the mutated Tf promoter. The C/EBP binding site was mutated as described in Materials and Methods. (A) Radiolabeled DNA (0.5 ng) of wild-type (lanes 1 to 4) and mutated (lanes 5 to 8) *Hinf*I-*Bam*HI fragments (see Fig. 2) was either directly loaded (lanes 1 and 5) or incubated with BSA (lanes 2 and 6), 5 μ g of liver nuclear protein (lanes 3 and 7), or 20 μ g of liver nuclear protein (lanes 4 and 8) prior to electrophoresis. Only the bound DNA fragments are shown. (B) Competition experiment. Radioactive wild-type *Hinf*I-*Bam*HI fragment (0.5 ng) was incubated with BSA (lane 1) or 20 μ g of liver nuclear protein (lanes 2 to 10). Competitor DNA was added as follows: no competitor (lanes 1 and 2); 10, 20, 40, and 80 fmol of wild-type *Hinf*I-*Bam*HI fragment DNA (lanes 3 to 6), and 10, 20, 40, and 80 fmol of mutant *Hinf*I-*Bam*HI fragment DNA (lanes 7 to 10). Binding reactions were as described in Materials and Methods. Bound and nonbound DNA fragments were separated by electrophoresis on 5% polyacrylamide gels; only the bound DNA region is shown. (C) Effect of the C/EBP binding site mutation on the transcriptional activity of the Tf promoter after transient transfection of C2Rev7 cells. The activity of the -581TfCAT wild-type construct (581-wt) was set at 100%. The C/EBP mutation was introduced into the -581TfCAT construct to generate -581_{mut}TfCAT. CAT activity in C2Rev7 cells transfected with the mutant construct (581-mut) was determined and expressed relative to wild-type activity. LYSCAT 2000 indicates the relative activity in extracts from cells that were transfected with a chicken lysozyme promoter-CAT construct (44) as a negative control.

5A) to investigate whether regulatory sequences of the Tf gene can act as a tissue-specific enhancer. A 516-bp *Hae*III DNA fragment from positions -560 to -44 was cloned in inverse orientation into a unique *Hinc*II site 5' of the hGH gene, whose promoter was truncated at -83 (see Materials and Methods). The truncated hGH promoter by itself shows no expression in transgenic mouse tissues (22). Transgenic mice were generated, and animals containing between 1 and 20 transgene copies per cell in their genomes were analyzed for expression of the transgene in liver. Figure 5B shows that all animals except 1706-6 expressed the (-44 to -560)

FIG. 5. Activity of the enhancer construct (-44 to -560)TfhGH in transgenic mouse liver. (A) Map of the (-44 to -560)TfhGH transgene. A 516-bp *Hae*III (Ha) DNA fragment (black bar) from positions -560 to -44 of the Tf gene upstream region was cloned in inverse orientation into a unique *Hinc*II site (Hi) 5' of the hGH gene promoter (hatched bar). The transgene was isolated as a *Hind*III (Hd)-*Eco*RI (E) fragment and used to generate transgenic mice. (B) Transcriptional activity of the (-44 to -560)TfhGH transgene in livers of transgenic mice. Tissue was obtained by partial hepatectomy, and the level of transgene mRNA (molecules per cell; indicated above each bar) was determined by solution hybridization. The number of transgenes incorporated into the genome of each founder was quantitated by dot blot as follows: 1705-1F, 3 copies; 1706-4M, 2 copies; 1706-6F, 1 copy; 1706-10F, 1 copy; 1707-3M, 3 copies; 1707-8M, 4 copies; 1707-9F, 2 copies; and 1708-8M, 20 copies.

TfhGH transgene in their livers. As analyzed by Southern blotting, the 1706-6 founder did not contain an intact transgene copy in its genome (data not shown). Expression levels, determined by solution hybridization, ranged from 23 to 50,000 molecules per liver cell, with the highest levels correlating with the higher transgene copy number. Two of the (-44 to -560)TfhGH animals were sacrificed, and their tissues were analyzed for transgene expression. As shown in Table 1, transgene activity was high in the liver but also detectable in brain from animals 1705-1 and 1707-8. Transgene mRNA was present in the mammary gland of female mouse 1705-1, in keeping with the known expression of the endogenous Tf gene in mouse mammary gland (10). All other analyzed tissues showed no transgene activity. As shown by Northern blot analysis on liver and brain RNA from mouse 1707-8 (see below), the transcripts from the transgene migrate as a 1.2-kb mRNA as expected, indicating that they are correctly initiated and processed. These experiments demonstrate that sequence elements between -560 and -44 of the Tf promoter act as a tissue-specific enhancer element by activating a heterologous promoter only in Tf-expressing tissues of transgenic mice.

Tf promoter sequences from -98 to +50 are sufficient for liver-specific expression in transgenic mice. To analyze the activity of the -98 to -83 DNA sequence element *in vivo*,

TABLE 1. Tissue-specific expression of the (-44 to -560)TfhGH gene in transgenic mice

Tissue	Transgene mRNA (molecules/cell) ^a	
	1705-1F	1707-8M
Liver	1,445	10,148
Brain	24	83
Heart	<1	<1
Lung	<1	<1
Duodenum	<1	<1
Thymus	<1	<1
Kidney	<1	<1
Mammary gland	145	
Testis		<1

^a Determined by solution hybridization.

we fused the -98 to +50 fragment of the transferrin promoter with the hGH gene to generate transgene -98TfhGH. Transgenic animals containing between one and five transgene copies were analyzed. Table 2 shows the results of solution hybridization experiments to determine the expression of the -98TfhGH transgene in liver and brain. In five of eight animals, transgene expression in the liver is clearly detectable, with no expression evident in brain. Expression levels vary between 136 and 3,936 molecules per cell. Southern blot analysis showed that the mice (1560-1, 1563-7, and 1564-5) that did not express the transgene contained rearranged or incomplete transgene copies in their genomes (data not shown). Although high levels of transgene mRNA can be detected in liver, the -98TfhGH gene is not expressed at detectable levels in testis, heart, kidney, or intestine (data not shown). The -98TfhGH transgene is also not expressed in the brain (Table 2), where the endogenous gene, the (-44 to -540)TfhGH transgene, and the -581 TfhGH transgene are all active. These results and those summarized in Table 1 strongly suggest that elements within a 55-bp region (-98 to -44) are sufficient to elicit high levels of liver-specific expression and can do so when placed 5' of the heterologous hGH promoter.

Mutation of the C/EBP binding sequence eliminates liver expression but does not affect expression in brain. The effect of the C/EBP binding site mutation on the tissue-specific activity of the Tf promoter region in transgenic mice was examined by using the -3000Tf_{mut}hGX construct shown in Fig. 6A. This transgene differs from the wild-type construct (25) by only the 5 bp that have been mutated within the C/EBP binding region. Transgenic mice were generated, and integration of intact transgenes in the genome of positive

TABLE 2. Expression of the -98TfhGH transgene in liver^a

Animal	Gene copy ^b	hGH mRNA (molecules/cell) ^c
1560-1	2	<1 ^d
1560-2	1	136
1562-1	5	3,936
1563-7	1	<1 ^d
1564-3	4	1,429
1564-5	1	<1 ^d
1564-6	1	806

^a In all animals, levels were <1 molecule per cell in brain.

^b Copies of the transgene per diploid mouse genome.

^c Determined by solution hybridization.

^d Southern blot analysis indicated that this animal did not contain an intact copy of the transgene.

FIG. 6. Analysis of $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene activity in mouse liver. (A) Map of the mutated transgene. The palindromic sequence within the C/EBP binding site of the 631-bp *HindIII*-*Bam*HI fragment (-581 to $+50$) was mutagenized with the Amersham oligonucleotide-directed in vitro mutagenesis system. A unique *Xba*I restriction site was generated by the mutagenesis. The mutated *HindIII*-*Bam*HI fragment was fused with Tf sequences from -3000 (*Eco*RI) to -581 (*Hind*III) and the modified hGH gene, hGX (25), as the reporter gene. (B) Activities of the endogenous Tf gene and the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene in animal 2089-1, 2089-6, 2091-3, 2091-6, and 2092-6 or the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene as a control in animals 698-2-3 and 696-4-12 were determined by solution hybridization. Transgene copy numbers are as follows: 698-2-3, 8; 696-4-12, 2; 2089-1, 15; 2089-6, 3; 2091-3, 12; 2091-6, 5; and 2092-6, 3.

animals was examined by Southern blotting (data not shown). Transgene activity in the livers of five animals was analyzed by solution hybridization after partial hepatectomy. Figure 6B shows that in contrast to two $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ control animals (698-2-3 and 696-4-12) that expressed high levels of both endogenous Tf mRNA and transgene mRNA, animals that contained the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene showed no expression of transgene mRNA in liver. The expression of the mutated transgene in other mouse tissues, including the brain, where the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ wild-type gene is active (25), was determined in six transgenic animals. Table 3 summarizes the results of solution hybridization experiments with tissues from these animals. As shown in Fig. 6 the mutated transgene is not transcribed in mouse liver. All other tissues, except brain, also contained no transgene mRNA. In contrast, the brain tissue of all animals showed expression levels of the transgene between 116 and 1,758 molecules per cell. These levels are in the range of activities found for the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ wild-type gene (25). This finding leads to the conclusion that mutation of the C/EBP binding site completely inactivates transgene expression in the liver but has no negative influence on the activity of the transgene in the brain of the same animal.

Figure 7 depicts a Northern blot of liver and brain RNA

TABLE 3. Tissue-specific expression of the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene^a

Animal	Sex ^b	Gene copy ^c	mRNA (molecules/cell) ^d		
			Brain	Kidney	Thymus
2089-1-1	F	15	1,758	<1	ND
2089-1-2	M	15	775	<1	ND
2089-6	M	3	116	ND	<1
2091-3-5	F	12	1,301	<1	ND
2091-3-6	M	12	778	<1	ND
2091-6	F	5	452	<1	<1

^a In all animals, levels were <1 molecule per cell in liver and heart.

^b F, female; M, male.

^c Copies of the transgene per diploid mouse genome.

^d Determined by solution hybridization. ND, not determined.

from various transgenic lines hybridized to probes specific for the endogenous Tf mRNA and the transgene transcript. In all cases, 1 μg of liver RNA is compared with 5 μg of brain RNA from each animal. Lanes 1 and 2 show expression of the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene in both liver and brain, in agreement with previous results (25). Expression of the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$ transgene containing the mutated C/EBP binding site is absent in liver (lane 3) but remains high in brain (lane 4). Deletion of the Tf promoter to either -139 or -98 continues to give expression of transgene in liver (lanes 5 and 7), but the expression in brain is absent (lanes 6 and 8). The construct containing the -560 to -44 region of the Tf promoter inserted in reverse orientation upstream of hGH [$(-44$ to $-560) \text{ Tf}_{\text{mut}}\text{hGH}$] gave expression in both liver (lane 9) and brain (lane 10). These results also demonstrate that in all cases, the transgene mRNA being measured is correctly processed to the expected 1.2-kb transcript.

A more precise determination of correct initiation in the Tf promoter for the various transgenic mice was obtained by S1 mapping (Fig. 8). Protection of the expected 76-bp fragment was observed with animals expressing the $-3000 \text{ Tf}_{\text{mut}}\text{hGX}$

FIG. 7. Northern blot analysis of Tf and transgene mRNA in livers and brains of transgenic mice. RNA was prepared and analyzed by hybridization with a mixture of a nick-translated *Pvu*II-*Pst*I Tf DNA fragment and an *Ssp*I-*Bgl*II hGH gene fragment as described in Materials and Methods. One microgram of liver RNA or 5 μg of brain RNA from each transgenic animal was loaded on the gel. Lanes: 1 and 2, RNA from liver and brain, respectively, from mouse 680-6-3 ($-3000 \text{ Tf}_{\text{mut}}\text{hGX}$); 3 and 4, liver and brain RNA from 2091-6 ($-3000 \text{ Tf}_{\text{mut}}\text{hGX}$); 5 and 6, liver and brain RNA from 1122-5 ($-139 \text{ Tf}_{\text{mut}}\text{hGX}$); 7 and 8, liver and brain RNA from 1562-1-4 ($-98 \text{ Tf}_{\text{mut}}\text{hGX}$); 9 and 10, liver and brain RNA from 1707-8 [$(-44$ to $-560) \text{ Tf}_{\text{mut}}\text{hGH}$]. Positions of the Tf mRNA (Tf) and the transgene mRNA (GH) are indicated.

FIG. 8. S1 mapping analysis of transgene mRNA. (A) Diagram of the promoter region of the Tf-hGH fusion gene. The *Dde*I restriction sites that were used for probe isolation are indicated. The 228-nucleotide probe and the predicted S1-resistant 76-nucleotide fragment are shown. (B) Total RNA was hybridized with an excess of radioactive probe and digested with S1 nuclease as described in Materials and Methods. Lanes: P, undigested radioactive probe; M, radioactively labeled 1-kb ladder (Bethesda Research Laboratories) as size markers; 1, no RNA; 2, 1 μ g of liver RNA from mouse 680-6-3 ($-3000\text{Tf}_{\text{mut}}\text{hGX}$); 3, 5 μ g of brain RNA from 680-6-3; 4, 5 μ g of liver RNA from 2091-6 ($-3000\text{Tf}_{\text{mut}}\text{hGX}$); 5, 5 μ g of brain RNA from 2091-6; 6, 1 μ g of liver RNA from mouse 1122-5 (-139Tf hGH); 7, 5 μ g of brain RNA from 1122-5; 8, 1 μ g of liver RNA from 1562-1-4 (-98Tf hGH); 9, 5 μ g of brain RNA from 1562-1-4. The position of the 76-nucleotide (76NT) fragment derived from correctly initiated transcripts is indicated by an arrow on the right.

construct in liver (lane 2) and brain (lane 3). The $-3000\text{Tf}_{\text{mut}}\text{hGX}$ transcripts in brain initiate at the correct transcriptional start site (lane 5), whereas no protected fragment is seen with liver RNA from the same animal, as expected (lane 4). In lanes 6 and 8, specific initiation is observed with liver transcripts from -139Tf hGH and -98Tf hGH , and no expression is detected in brain RNA from these same animals (lanes 7 and 9).

Colocalization of transgene and endogenous Tf expression in brain. Previous studies on the expression of the endogenous Tf gene have indicated that the mRNA and protein localize to oligodendrocytes and the choroid plexus in rats (3, 45). We used in situ hybridization with riboprobes specific for either mouse Tf mRNA or hGH mRNA to localize expression in mouse brain and determine whether mutations in the C/EBP site altered expression patterns in the brain. Figure 9 illustrates the identical colocalization of endogenous Tf

FIG. 9. Colocalization of Tf and transgene mRNAs in mouse brain. In situ hybridization with riboprobes was used to specifically localize Tf mRNA (A) or hGH (B) sequences in a $-3000\text{Tf}_{\text{mut}}\text{hGX}$ transgenic mouse. Ten-micrometer transverse sections from adult mouse brain were fixed and hybridized to ^{35}S -labeled riboprobes. The slides were then exposed directly to Ultrofilm ^3H (LKB Instruments) for 7 days to obtain the images shown on the left. Structures: c.c., corpus callosum; H., hippocampus; f.h., fimbria hippocampus; i.c., internal capsule. A more detailed view of the boxed region of the hippocampus was obtained by coating the slides with emulsion and viewing the developed slides by bright field (top right of each panel) or dark field (bottom right of each panel). Structures labeled in the magnified views are the corpus callosum (c.c.) and the pyramidal cell layer CA1 (CA1) of the hippocampus.

sequences (Fig. 9A) and $-3000\text{Tf}_{\text{mut}}\text{hGX}$ transcripts (Fig. 9B) in the oligodendrocyte-rich fiber tracts of transgenic mouse brain. Expression is specifically localized in the oligodendrocyte-rich corpus callosum, with no expression in the adjacent pyramidal cell layers of the hippocampus. Other fiber tract areas rich in oligodendrocytes, including the fimbria hippocampus and the internal capsule, are also heavily labeled. The -3000Tf hGX transgene containing a functional C/EBP binding site gave a similar pattern (data not shown). These results demonstrate that the C/EBP binding site does not participate in the regulation of the Tf promoter in brain despite its crucial role in liver-specific expression.

DISCUSSION

We have used a combination of in vitro DNA-protein interaction studies and in vivo expression studies to inves-

tigate the tissue-specific regulation of the Tf gene. Stepwise deletions of the 5' upstream region of a Tf-CAT fusion gene were tested in a transient transfection assay, and the results identified a positive element between -98 and -83 that was essential for promoter activity in hepatoma cell cultures. A high-affinity protein-DNA interaction was characterized by gel retardation and DNase footprinting that centered around bp -91, and this sequence was shown to contain a binding site for C/EBP. This site is in a region of high sequence identity with the human and chicken promoters, and the human counterpart has also been shown to bind C/EBP (7). We conclude from these transient assays that C/EBP binding (or binding of a related CCAAT-binding factor) is essential for mouse Tf promoter activity in cultured hepatoma cells, and these results are in good agreement with those reported by Brunel et al. (7) for the human Tf promoter. Previous work with the human promoter has also demonstrated a binding site for another liver-enriched transcription factor, Tf-LF1 (HNF-4), downstream of the C/EBP site which has also been shown to be essential for hepatocyte-specific expression (40, 49). Since the mouse Tf gene is highly homologous in this promoter-proximal region, we conclude that C/EBP, HNF-4, and basal transcription factors are all likely to be essential for liver-specific expression in the mouse.

C/EBP is a transcription factor that was first identified in rat liver (27, 29). Closely related isoforms of C/EBP have been more recently identified; these include C/EBP- β and C/EBP- δ (9), immunoglobulin enhancer-binding protein (Ig/EBP-1) (38), and CRP-1 (47). An inhibitory modulator of C/EBP activity was recently described; this factor (named CHOP-10) also has a high degree of sequence similarity with C/EBP (39). These transcription factors are members of the larger bZIP family of proteins that interact to form homodimers and heterodimers via a hydrophobic α -helical surface and interact with DNA through an adjacent basic region (9, 47). C/EBP binding sites have been identified in the control elements of many liver-specific genes (13, 31, 32, 50), and direct evidence implicating C/EBP as a transactivator of gene expression has been obtained by cotransfection experiments with the albumin promoter and a C/EBP expression vector (18, 19). Although C/EBP is highly expressed in the liver, it is not a liver-specific factor, and expression of the gene can be found in other tissues. It has been shown that C/EBP activates transcription of adipocyte-specific genes (11) and is a component of a differentiation switch in adipogenesis (16, 46). Expression of C/EBP or one of the closely related isoforms has also been observed in a variety of other terminally differentiated tissues, including lung, intestine, and skin (2, 48).

Does the C/EBP binding site in the Tf promoter function as an essential tissue-specific element in normal tissues as suggested by the transient transfection data? To answer this question, experiments using transgenic mice as the assay system were designed. Several examples are now available to suggest that results from transient transfection must be treated with caution and may not always yield an accurate picture of the role of promoter elements in stable transformants or in transgenic animals. Certain enhancer elements may not function unless integrated into an appropriate chromosomal structure (43), and elements that appear essential in transient assay may not be required in transgenic animals (42).

One advantage of using the Tf promoter for these studies is the active expression of Tf in several tissues other than liver. Using transgenic mice, we could test whether the

C/EBP binding site participates only in liver expression or whether it also plays a role in expression of the promoter in another tissue such as brain. A 5-bp mutation that prevented the high-affinity binding seen with mouse liver nuclear extracts was introduced into the C/EBP binding site. This mutation rendered the Tf promoter completely inactive in transient transfection assays using cultured hepatocytes. We then introduced the C/EBP mutation into the -3000Tf_hGX construct and produced several lines of transgenic mice. Expression from this mutated construct was below detection in transgenic mouse liver. However, the -3000Tf_{mut}hGX transgene continued to be actively expressed in the brain and was localized to the oligodendrocyte-rich fiber tracts in the brain coincident with endogenous Tf mRNA expression. Thus, despite the essential role of the CCAAT sequence element in allowing expression from the Tf promoter in liver, there appears to be no dramatic positive or negative influences of this sequence on the initiation of Tf transcription in brain oligodendrocytes.

As illustrated by these experiments, specific manipulation of regulatory sequences within transcriptional control elements such as the Tf promoter can limit expression to a subset of cell types that normally express the endogenous gene. A similar result has been observed with use of specific alterations of the dopa decarboxylase gene promoter and expression in *Drosophila melanogaster* (28). In addition to demonstrating the *in vivo* role of identified transcription factor binding sites in tissue-specific expression, this approach can be used to develop novel promoters for targeting the expression of foreign genes in transgenic organisms.

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REFERENCES

1. Adrian, G. S., B. H. Bowman, D. C. Herbert, F. J. Weaker, E. K. Adrian, L. K. Robinson, L. A. Walter, C. A. Eddy, R. Riehl, C. J. Pauerstein, and F. Yang. 1990. Human transferrin. *J. Biol. Chem.* 265:13344-13350.
2. Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight. 1989. Tissue-specific expression, developmental regulation and genetic mapping of the gene encoding C/EBP. *Genes Dev.* 3:1146-1156.
3. Bloch, B., T. Popovici, M. J. Levin, D. Tuil, and A. Kahn. 1985. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using *in situ* hybridization and immunohistochemistry. *Proc. Natl. Acad. Sci. USA* 82:6706-6710.
4. Boissier, F. C., C. Augé-Gouillou, E. Schaeffer, and M. M. Zakin. 1991. The enhancer for the human transferrin gene is organized in two structural and functional domains. *J. Biol. Chem.* 266:9822-9828.
5. Borgmeyer, U., J. Nowock, and A. E. Sippel. 1984. The TGGCA-binding protein: a eukaryotic nuclear protein recognizing a symmetrical sequence on double-stranded linear DNA. *Nucleic Acids Res.* 12:4295-4331.
6. Brinster, R. L., H. Y. Chen, M. E. Trumbauer, A. W. Seneear, R. Warren, and R. D. Palmiter. 1981. Somatic expression of herpes

- thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27:223-231.
7. Brunel, F., A. Ochoa, E. Schaeffer, F. Boissier, Y. Guillou, S. Cereghini, G. N. Cohen, and M. M. Zakin. 1988. Interaction of DNA-binding proteins with the 5' region of the human transferrin gene. *J. Biol. Chem.* 263:10180-10185.
 8. Cadd, G. G., and G. S. McKnight. 1989. Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain. *Neuron* 3:71-79.
 9. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* 5:1538-1552.
 10. Chen, L., and M. J. Bissel. 1987. Transferrin mRNA level in the mouse mammary gland is regulated by pregnancy and extracellular matrix. *J. Biol. Chem.* 262:17247-17250.
 11. Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte specific genes. *Genes Dev.* 3:1323-1335.
 12. Cochet, M., F. Gannon, R. Hen, L. Maroteaux, F. Perrin, and P. Chambon. 1979. Organization and sequence studies of the 17-piece chicken conalbumin gene. *Nature (London)* 282:567-574.
 13. Crossley, M., and G. G. Brownlee. 1990. Disruption of a C/EBP binding site in the factor IX promoter is associated with hemophilia B. *Nature (London)* 345:444-446.
 14. Deschatrette, J., E. E. Moore, M. Dubois, D. Cassio, and M. Weiss. 1979. Dedifferentiated variants of a rat hepatoma: analysis by cell hybridization. *Somatic Cell Genet.* 5:697-718.
 15. Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcriptional initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475-1489.
 16. Freytag, S. O., and T. J. Geddes. 1992. Reciprocal regulation of adipogenesis by Myc and C/EBP. *Science* 256:379-382.
 17. Fried, M. G., and D. M. Crothers. 1984. Equilibrium studies of the cyclic AMP receptor protein-DNA interaction. *J. Mol. Biol.* 172:241-262.
 18. Friedman, A. D., W. H. Landschulz, and S. L. McKnight. 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* 3:1314-1322.
 19. Friedman, A. D., and S. L. McKnight. 1990. Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. *Genes Dev.* 4:1416-1426.
 20. Graves, B. J., P. F. Johnson, and S. L. McKnight. 1986. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* 44:565-576.
 21. Guillou, F., M. M. Zakin, D. Part, F. Boissier, and E. Schaeffer. 1991. Sertoli cell-specific expression of the human transferrin gene: comparison with the liver-specific expression. *J. Biol. Chem.* 266:9876-9884.
 22. Hammer, R. E., G. H. Swift, D. M. Ornitz, C. J. Quaife, R. D. Palmiter, R. L. Brinster, and R. J. McDonald. 1987. The rat elastase I regulatory element is an enhancer that directs correct cell-specific and developmental onset of expression in transgenic mice. *Mol. Cell. Biol.* 7:2956-2967.
 23. Hoffman, L. M., M. K. Fritsch, and J. Gorski. 1981. Probable nuclear precursors of preprolactin mRNA in rat pituitary cells. *J. Biol. Chem.* 256:2597-2600.
 24. Huggenvik, J., R. L. Idzerda, L. Haywood, D. C. Lee, G. S. McKnight, and M. D. Griswold. 1987. Transferrin mRNA: molecular cloning and hormonal regulation in rat Sertoli cells. *Endocrinology* 120:332-340.
 25. Idzerda, R. L., R. R. Behringer, M. Theisen, J. T. Huggenvik, G. S. McKnight, and R. L. Brinster. 1989. Expression from the transferrin gene promoter in transgenic mice. *Mol. Cell. Biol.* 9:5154-5162.
 26. Idzerda, R. L., H. Huebers, C. A. Finch, and G. S. McKnight. 1986. Rat transferrin gene expression: tissue-specific regulation by iron deficiency. *Proc. Natl. Acad. Sci. USA* 83:3723-3727.
 27. Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight. 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* 1:133-146.
 28. Johnson, W. A., C. A. McCormick, S. J. Bray, and J. Hirsh. 1989. A neuron-specific enhancer of the *Drosophila* dopa decarboxylase gene. *Genes Dev.* 3:676-686.
 29. 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.
 30. Lucero, M., E. Schaeffer, G. N. Cohen, and M. M. Zakin. 1986. The 5' region of the human transferrin gene: structure and potential regulatory sites. *Nucleic Acids Res.* 14:8692.
 31. Maire, P., J. Wuarin, and U. Schibler. 1989. The role of cis-acting elements in tissue-specific albumin gene transcription. *Science* 244:343-346.
 32. Metzger, S., T. Leff, and J. L. Breslow. 1990. Nuclear factors AF-1 and C/EBP bind to the human Apo B gene promoter and modulate its transcriptional activity in hepatic cells. *J. Biol. Chem.* 265:9978-9983.
 33. Mikscek, R., A. Heber, W. Schmid, U. Danesch, G. Poseckert, M. Beato, and G. Schutz. 1986. Glucocorticoid responsiveness of the transcriptional enhancer of Molony murine sarcoma virus. *Cell* 46:283-290.
 34. Morgan, E. H. 1983. Synthesis and secretion of transferrin, p. 331-355. In H. Glaumann, T. Peters, Jr., and C. Redman (ed.), *plasma protein secretion by the liver*. Academic Press, New York.
 35. Nye, J. A., and B. J. Graves. 1990. Alkylation interference identifies essential DNA contacts for sequence-specific binding of the eukaryotic transcription factor C/EBP. *Proc. Natl. Acad. Sci. USA* 87:3992-3996.
 36. Palmiter, R. D., H. Y. Chen, and R. L. Brinster. 1982. Differential regulation of methallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* 29:701-710.
 37. Petropoulos, I., C. Aug'e-Gouillou, and M. M. Zakin. 1991. Characterization of the active part of the human transferrin gene enhancer and purification of two liver nuclear factors interacting with the TGTTTGC motif present in this region. *J. Biol. Chem.* 266:24220-24225.
 38. Roman, C., J. S. Platero, J. Shuman, and K. Calame. 1990. Ig/EBP-1: A ubiquitously expressed immunoglobulin enhancer binding protein that is similar to and heterodimerizes with C/EBP. *Genes Dev.* 4:1404-1415.
 39. 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.
 40. Schaeffer, E. F., F. Boissier, M. C. Py, G. N. Cohen, and M. M. Zakin. 1989. Cell type-specific expression of the human transferrin gene. Role of promoter, negative, and enhancer elements. *J. Biol. Chem.* 264:7153-7160.
 41. Schmitz, A., and D. J. Galas. 1979. The interaction of RNA polymerase and lac repressor with the lac control region. *Nucleic Acids Res.* 6:111-137.
 42. Swift, G. H., F. Kruse, R. J. MacDonald, and R. E. Hammer. 1989. Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. *Genes Dev.* 3:687-696.
 43. Tapscott, S. J., A. B. Lassar, and H. Weintraub. 1992. A novel myoblast enhancer element mediates MyoD transcription. *Mol. Cell. Biol.* 12:4994-5003.
 44. Theisen, M., A. Stief, and A. E. Sippel. 1986. The lysozyme enhancer: cell-specific activation of the chicken lysozyme gene by a far upstream DNA element. *EMBO J.* 5:719-724.
 45. Tsutsumi, M., M. K. Skinner, and E. Sanders-Bush. 1989. Transferrin gene expression and synthesis by cultured choroid plexus epithelial cells. *J. Biol. Chem.* 264:9626-9631.
 46. Umek, R. M., A. D. Friedman, and S. L. McKnight. 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. *Science* 251:288-292.

47. Williams, S. C., C. A. Cantwell, and P. F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev.* **5**:1553–1567.
48. Xanthopoulos, K. G., V. R. Prezioso, W. S. Chen, F. M. Slodek, R. Cortcáo, and J. E. Darnell, Jr. 1991. The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3 and HNF-4, protein factors that govern liver specific transcription. *Proc. Natl. Acad. Sci. USA* **88**:3807–3811.
49. Zakin, M. M. 1992. Regulation of transferrin gene expression. *FASEB J.* **6**:3253–3258.
50. Zhang, D. E., J. P. Rabek, C. C. Hsieh, R. C. Torres, and J. Papaconstantinou. 1992. Functional analysis of the mouse alpha-fetoprotein enhancers and their subfragments in primary mouse hepatocyte cultures. *J. Biol. Chem.* **267**:10676–10682.