Upstream region of rat serum albumin gene promoter contributes to promoter activity: presence of functional binding site for hepatocyte nuclear factor-3

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Transcription of the serum albumin gene occurs almost exclusively in the liver and is controlled in part by a strong liver-specific promoter. The upstream region of the serum albumin gene promoter is highly conserved among species and is footprinted *in vitro* by a number of nuclear proteins. However, the role of the upstream promoter region in regulating transcription and the identity of the transcription factors that bind to this region have not been established. In the present study, deletion analysis of the rat serum albumin promoter in transiently transfected HepG2 cells demonstrated that elimination of the region between −207 and −153 bp caused a two-fold decrease in promoter activity ($P < 0.05$). Additional analysis of the −207 to −124 bp promoter interval led to the identification of two potential binding sites for hepatocyte nuclear factor-3 (HNF-3) located at −168 to −157 bp (site X) and −145 to −134 bp (site Y). Electrophoretic mobility-shift assays performed with the HNF-3 X and Y sites demonstrated that both sites are capable of binding HNF-3α and HNF-3β. Placement of a single copy of the HNF-3 X site upstream from a minimal promoter increased promoter activity by about four-fold in HepG2 cells, and the reporter construct containing this site could be transactivated if co-transfected with an HNF-3 expression construct. Furthermore, inactivation of the HNF-3 X site by site-directed mutagenesis within the context of the −261 bp albumin promoter construct resulted in a 40% decrease in transcription ($P < 0.05$). These results indicate that the positive effect of the −207 to −153 bp promoter interval is attributable to the presence of the HNF-3 X site within this interval. Additional results obtained with transfected HepG2 cells suggest that the HNF-3 Y site plays a lesser role in activation of transcription than the X site.

Key words: hepatocyte nuclear factor-3, liver, serum albumin.

INTRODUCTION

Serum albumin is the most abundant plasma protein in adult vertebrates and accounts for nearly half of the protein circulating in blood [1]. The high steady-state concentration of serum albumin in plasma (35–50 g/l in humans) is generated in part by a high level of transcription of the serum albumin gene, which occurs almost exclusively in the liver [2–4]. During embryonic development, the serum albumin gene is one of the first liver-specific genes to be expressed in the hepatocyte lineage [5,6]. Transcription of the albumin gene continues at a constitutively high rate throughout the remainder of embryonic, fetal and postnatal development. Transcription of the serum albumin gene in the liver is directed by a liver-specific promoter element [7–12] and an enhancer element located at −10 kb relative to the transcription start site [6,13–18]. Three strong tissue-specific enhancer elements, termed the α-fetoprotein (AFP) gene enhancers and located between the albumin and AFP genes, may also contribute to the high level of transcription of the albumin gene [19–22]. The serum albumin gene has served as a model for understanding liver-specific gene transcription, and the serum albumin promoter in combination with the albumin or AFP enhancers has been used to target the expression of transgenes to the liver in a number of studies [23–26].

The strong liver-specific activity of the serum albumin promoter and enhancer elements results from the binding of liver-enriched as well as ubiquitously expressed transcription factors to these elements [7–22]. The serum albumin promoter contains a number of well-defined transcription factor-binding sites, which have been identified by transient transfection assays as functionally important elements in directing albumin gene transcription [7–12]. Starting with the most proximal, these include the B site (also called the proximal element), which binds hepatocyte nuclear factor-1 (HNF-1), the C site (a CCAAT element), which binds nuclear factor-Y, the D site (distal element I), which binds proteins of the CCAAT/enhancer binding protein (C/EBP) family and D-site binding protein (DBP), and the E site (distal element II), which binds nuclear factor-1. Located further upstream are additional sites, designated F–I in the mouse, which are footprinted by liver nuclear proteins [7,8,12]. The upstream promoter region, extending to −460 bp, is highly conserved among species, suggesting an important functional role in maintaining promoter activity [12]. However, to date, transient transfection analysis has not conclusively established a positive role for any of the individual upstream footprinted sites in directing transcription, nor have proteins that bind to these sites been identified.

HNF-3 is a member of the winged-helix family of transcription factors [27]. Three isoforms of HNF-3, α, β and γ, are expressed in the adult liver and activate the expression of many genes

Abbreviations used: AFP, α-fetoprotein; HNF, hepatocyte nuclear factor; C/EBP, CCAAT/enhancer binding protein; DBP, D-site binding protein; HSV-TK, herpes simplex virus thymidine kinase; EMSA, electrophoretic mobility-shift assay; TATA-luc, TATA-luciferase; DTT, dithiothreitol.

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during gut development [27–30]. Binding sites for HNF-3 play an important role in enhancer and promoter elements for a large subset of genes that are preferentially expressed in the liver [27,31–33]. The binding of HNF-3 to two sites within the albumin enhancer element occurs during early hepatocyte development and helps position a nucleosome over the eG and eH sites of the enhancer [6,15–18]. A binding site for HNF-3 has also been identified in one of the enhancer elements located between the albumin and AFP genes [21,22]. Although a role for HNF-3 in regulation of the albumin gene promoter has not been established, Zaret et al. [14] noted a resemblance between two sequences located in the upstream region of the mouse albumin promoter and a site within the eH enhancer region later identified as an HNF-3-binding site [18]. However, the possible role of the two promoter sequences in activating transcription was not established, nor was the identity of protein(s) that might bind to the sequences determined.

In the present study, we demonstrate that the upstream region of the rat serum albumin promoter contributes to albumin promoter activity. A functional HNF-3-binding site is identified within this promoter interval.

EXPERIMENTAL

Plasmids

Plasmids were gifts from the following individuals: the pHNF-3α and pHNF-3β cDNAs in pGEM1 were from Dr. R. Costa, University of Illinois College of Medicine, Chicago, IL, U.S.A. [28,29]. Rat serum albumin genomic clone JB was from T. Sargent, National Institutes of Health, Bethesda, MD, U.S.A. [34]. The herpes simplex virus thymidine kinase (HSV-TK) TATA-luciferase (TATA-luc) plasmid was from J. Tillman and S. Spindler, University of California, Riverside, CA, U.S.A. The pMT7 vector, which was used for high-level expression of HNF-3α and HNF-3β in COS-7 cells [35], and the pCMV-β-galactosidase vector were from Dr. F. Sladek, University of California, Riverside, CA, U.S.A. HNF-3 expression vectors were prepared by subcloning the rat HNF-3α and HNF-3β cDNAs from pGEM1 into pMT7 at a unique *Eco*RI site. pGL2-Basic and pGEM3-Zf(+) were purchased from Promega (Madison, WI, U.S.A.), and pBluescript (SK$^-$) was from Stratagene (La Jolla, CA, U.S.A.).

Oligonucleotides

Double-stranded blunt-end oligonucleotides were used for construction of reporter constructs with multimerized HNF-3 binding sites and for electrophoretic mobility-shift assays (EMSAs). Oligonucleotide X, which contained the distal HNF-3-binding site, extended from position −177 to position −154 in the rat albumin promoter [9–11]. Oligonucleotide Y, which contained the proximal HNF-3-binding site, extended from position −152 to position −132 bp in the rat albumin promoter [9–11]. Oligonucleotide Z extended from position −207 to position −178 bp in the rat albumin promoter [9–11]. The oligonucleotide containing the strong HNF-3 binding site of the mouse transthyretin gene corresponded to positions −111 to −85 bp of the transthyretin promoter [31]. The HNF-1 oligonucleotide was derived from the rat serum albumin gene proximal element (−61 to −42 bp) [10,11]. An oligonucleotide containing a perfect consensus C/EBP binding site was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). For use as probes for EMSAs, oligonucleotides were end-labelled with T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD, U.S.A.), using [γ-^{32}P]ATP (3000 Ci/mmol).

Construction of albumin luciferase reporter and deletion constructs

The rat albumin promoter was isolated from rat serum albumin genomic clone JB [34] by digesting out a 402 bp fragment (−386 to +16 bp) using *Hin*cII. The *Hin*cII fragment was ligated into the *Sma*I site of the pGEM3-Zf(+) plasmid. Orientation of the insert was determined by sequencing. The fragment was re-isolated from pGEM3-Zf(+) using *Sac*I and *Hin*dIII and cloned into pGL2-Basic using the same sites to create an albumin-promoter–luciferase expression construct (alb386-luc). The *Kpn*I site in pGEM3-Zf(+) was eliminated by digesting the site, filling with Klenow and religating the vector in order to avoid placing a second *Kpn*I site in the pGL2 vector when the albumin promoter was inserted.

Using Vent polymerase (New England Biolabs, Beverly, MA, U.S.A.), 5′ deletions of the albumin promoter were constructed by PCR and either the clone JB *Hin*cII fragment in pBluescript or the alb386-luc construct as template. Conditions for thermocycling were as follows: the first cycle was 45 s at 95 °C, 40 s at 30 °C and 40 s at 72 °C. This was followed by 30 cycles of 40 s at 95 °C, 40 s at 45 °C and 40 s at 72 °C using a Perkin-Elmer Cetus Thermal Cycler. In each case, the 5′ primer contained an *Xho*I site to introduce this site at the upstream end of the promoter fragment. The 3′ primer was either the M13 forward primer or a primer located within the luciferase gene. At the 3′ end of the promoter the *Hin*dIII site from the pGL2 or pBluescript polylinker was amplified along with the promoter fragment. The fragments were then cloned into pGL2 using the *Xho*I and *Hin*dIII sites.

Construction of the TATA-luc vector

A fragment containing the TATA box from the HSV-TK promoter (−39 to +52 bp) previously connected to a luciferase gene was isolated by cutting at a *Hin*dIII site (inserted at −51 bp of the TK promoter region) and at an *Xba*I site (which lies at +48 bp, upstream from the luciferase ATG start codon). This fragment was ligated into the pGL2-Basic vector digested with the same enzymes. The movement of the TK TATA box into the pGL2-Basic vector facilitated further cloning steps due to the greater number of restriction sites in the pGL2-Basic multiple cloning region.

Construction of reporter constructs with monomeric or multimeric HNF-3-binding sites

The oligonucleotides were annealed to complementary strands and phosphorylated for ligation into the *Eco*RV site of the pBluescript (SK$^-$). The orientation of the monomeric or multimeric constructs was determined by sequencing. The multimer constructs were re-isolated from pBluescript by digesting each with *Kpn*I and *Sac*I or *Sac*I and *Xho*I, and were then ligated into the same sites upstream from the TATA-luc construct. For construction of the reporter containing one copy of HNF-3-binding site Y, a Y-site oligonucleotide containing a 5′ overhang complementary to a *Sac*I site and a 3′ overhang complementary to an *Xho*I site was ligated directly into the same sites of TATA-luc.

Albumin promoter fragment W/TATA-luc and fragment W/alb70-luc constructs

A DNA fragment containing the rat albumin promoter fragment W (−207 to −150 bp) was generated using the *Hin*cII albumin promoter fragment as template and the same amplification conditions used to create the promoter deletion constructs. The forward primer for the fragment W construct was used with a reverse primer which binds at −150 bp in the albumin promoter. Following PCR amplification, fragment W was cloned into the *Eco*RV site of pBluescript (SK⁻). Orientation and correct base sequence were determined by sequencing. The fragment was isolated by digesting with *Kpn*I and *Sac*I and inserted into the TATA-luc and alb70-luc luciferase constructs cut with the same enzymes.

Mutation of the albumin promoter HNF-3 X site

Site-directed mutagenesis of the HNF-3 X site in the albumin promoter was accomplished by using Vent polymerase to amplify the albumin promoter in two separate fragments. Two oligonucleotides were designed to introduce a mutation containing a *Pst*I site within the HNF-3-binding site X. These oligonucleotides were used either with a pGL2 primer or a primer located within the luciferase gene to amplify both the 5′ and 3′ portions of the albumin promoter using the albumin promoter deletion construct alb261-luc as template. Conditions for the PCR were the same as stated above. The two PCR fragments were ligated back into pGL2-Basic using the newly introduced *Pst*I site along with *Sac*I and *Hin*dIII sites amplified from the polycloning site of the vector. Authenticity of sequence was verified by sequencing. The final sequence of the mutant HNF-3 site X was TGGCtgcaggAC, as compared with the TGGCAAACATAC for the wild-type site (lower-case letters denote bases that differ between sequences).

Cell culture and transient transfection

Human hepatoma (HepG2) cells [36] were maintained as monolayer cultures and grown in minimal essential medium (Cellgro) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA, U.S.A.)/penicillin (100 units/ml)/streptomycin (100 μg/ml) (Gibco BRL). Plasmid DNAs were purified on Qiagen Maxiprep kit columns. For the transfections, cells were plated in 60-mm dishes at a density of 1×10^6 cells per dish in 4 ml of medium and cultured for 2 days. Luciferase expression constructs (5 μg) were transfected into HepG2 cells by the calcium phosphate method [37]. To correct for variation in transfection efficiency, 1 μg of the pCMV-β-galactosidase (pCMV-βgal) DNA was co-transfected with each construct. Calcium phosphate/DNA precipitates were added directly to each culture, and cells were incubated for 24 h at 37 °C. Cultures were then washed twice with Earle's balanced salt solution and incubated with 4 ml of serum-free minimal essential medium for an additional 24 h. Cell lysates were prepared by scraping the cells into 450 μl of lysis buffer [100 mM Tris/HCl (pH 7.5)/1 mM dithiothreitol (DTT)/1% (v/v) Triton X-100] and centrifuged in a microcentrifuge to pellet the cell debris. Supernatants were then assayed for luciferase and β-galactosidase activity as described previously [38].

COS-7 cells [39] were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum/penicillin (100 units/ml)/streptomycin (100 μg/ml). For transfections, cells were plated in 150-mm dishes (2×10^6 cells/dish) in 20 ml of medium 1 day before the transfection. Transfections were performed as described in [38] with the HNF-3α/pMT7, HNF-3β/pMT7 or pMT7 constructs (18 μg). After 5 h at 37 °C, the cells were shocked with 15% (v/v) glycerol and then changed to fresh medium with serum for 24 h prior to extraction of nuclear proteins.

Nuclear extraction and EMSAs

Nuclear extracts were prepared from HepG2 cells as described elsewhere [40]. Briefly, HepG2 cell pellets were resuspended in 100 μl of ice-cold buffer A [10 mM Hepes (pH 7.9)/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM DTT] and forced through a 28 G needle to lyse cell membranes. Nuclei were pelleted in a microcentrifuge for 10 s at 4 °C. The supernatant was discarded, and the nuclei were resuspended in 60 μl of ice cold buffer C [20 mM Hepes (pH 7.9)/25% (v/v) glycerol/420 mM KCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM DTT/0.5 mM PMSF] and incubated on ice for 15 min. An equal volume of buffer D [20 mM Hepes (pH 7.9)/20% (v/v) glycerol/0.2 mM EDTA/0.5 mM PMSF/0.5 mM DTT] was added, and the suspension was mixed by pipetting. Nuclear debris was pelleted in a microcentrifuge for 10 min at 4 °C. Nuclear protein extracts were prepared from COS-7 cells as described by Jiang et al. [41]. Protein concentrations were determined using the Lowry assay [42].

EMSAs were performed with HepG2 nuclear extracts as follows: nuclear protein (5 μg) was mixed with 1 μg of poly(dI.dC), 200 ng of sheared genomic *Escherichia coli* DNA and 50000 d.p.m. of labelled probe (approximately 1 ng) in reaction buffer [20 mM Hepes (pH 7.5)/1 mM $MgCl_2$/1 mM DTT/0.018% (v/v) Nonidet P-40/75 mM KCl] in a total volume of 12 μl. In competition experiments, a 100-fold molar excess of unlabelled competitor oligonucleotide was added to the mixture prior to the addition of nuclear protein extracts. This mixture was allowed to incubate for 30 min at 23 °C. Samples were mixed with 3 μl of running buffer [50% (w/v) sucrose/0.5 × TBE (where 1 × TBE buffer is 0.09 M Tris/borate/0.002 M EDTA, pH 8.0)/0.1% (w/v) Bromophenol Blue] and then run on a non-denaturing 6% polyacrylamide gel in 0.25 × TBE buffer. EMSAs were performed with COS-7 nuclear extracts as described by Jiang et al. [41], with gels run under the same conditions as EMSAs performed with HepG2 nuclear extracts. The gels were dried and exposed to Kodak XAR-5 X-ray film. Results of some of the EMSAs were quantified by phosphorimage analysis using a Molecular Dynamics PhosphorImager.

Statistics

For comparison of differences between more than two means, data were subjected to analysis of variance followed by the Student–Newman–Keuls multiple-comparison test, with a probability value (α) of 0.05 used to evaluate significance. The significance of the difference between two means was determined by Student's *t*-test, using $P < 0.05$ as the cut-off for significance.

RESULTS

Analysis of rat albumin promoter deletions

For functional analysis of the upstream region of the rat albumin promoter, we constructed a series of 5′-deletions beginning with a promoter construct that extended from −386 to +16 bp (Figure 1, top panel). The locations of five sites known to bind nuclear proteins from mouse and rat liver (sites B, C, D, E and F) are shown over the deletion constructs. In addition to these five sites, previous footprinting studies of the −386 to −153 bp upstream promoter region with mouse liver nuclear proteins

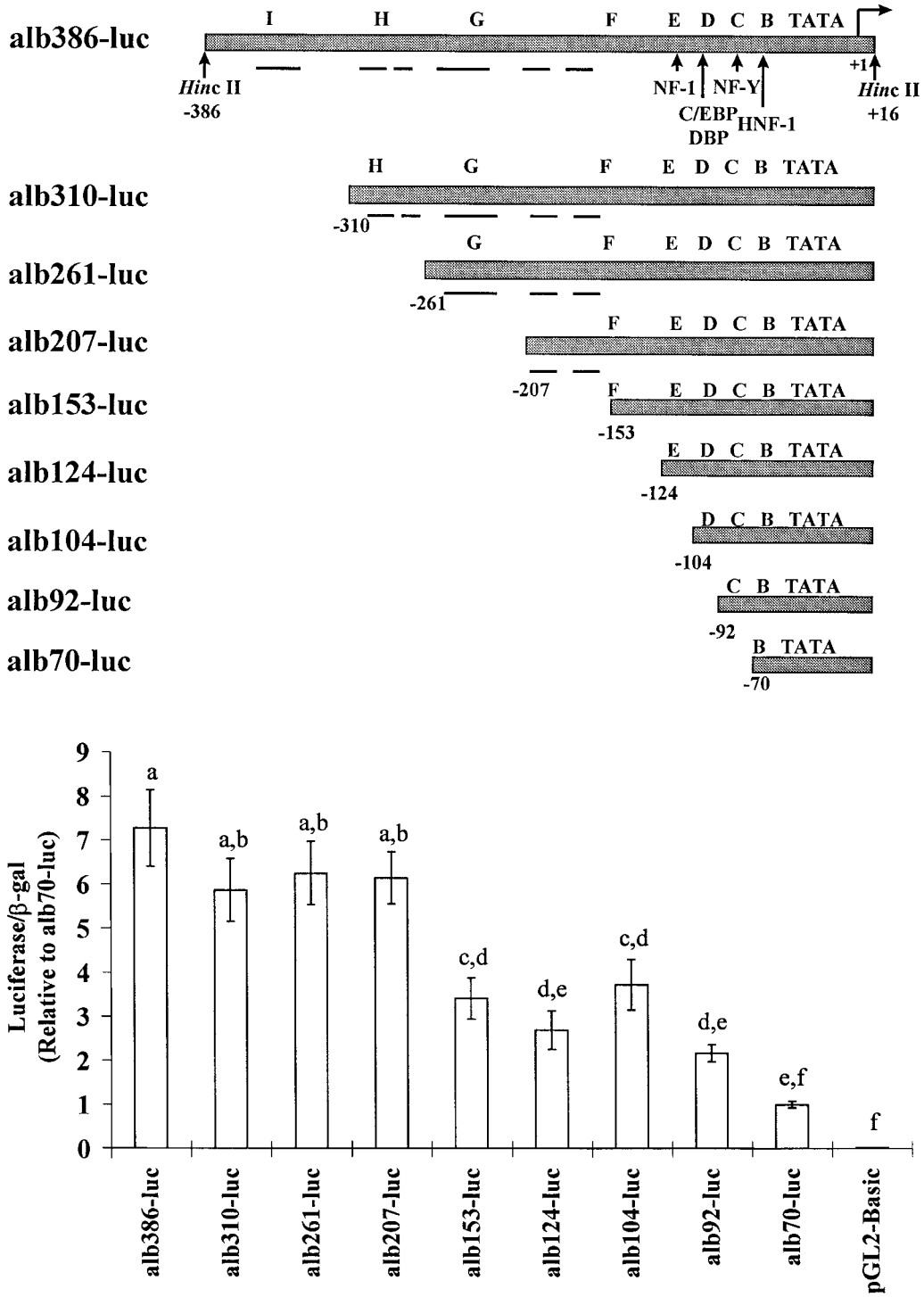


Figure 1 Albumin promoter 5′-deletion series and relative activities of promoter deletions

(Top panel) Albumin promoter 5′-deletion series. The locations of sites B–F, and the factors that bind to sites B–E, are described in [7–11]. Underlined regions show the location of several footprinted regions in the upstream mouse albumin promoter, including strong footprints G, H and I, and three weaker footprints (underlined) [12]. NF, nuclear factor. (Bottom panel) Relative activities of the deletion constructs were determined by transient transfection into HepG2 cells. Results were averaged from four experiments, each done in triplicate ($n = 12$). Luciferase activity for each construct was normalized to β-galactosidase activity. The mean for the alb70-luc group was set at 1. Error bars represent the S.E.M. for the combined values. Data were subjected to ANOVA followed by Student–Newman–Keuls multiple comparison test using $\alpha = 0.05$ as cut-off for significance. Means with different letters are significantly different.

indicated the presence of three strong footprints on both strands (G, H and I) as well as several weaker footprints (Figure 1, top panel; [12]).

Deletion of the interval extending from −386 to −207 bp had no significant effect on promoter activity (Figure 1, bottom panel). This indicated that sites G, H and I did not contribute to

albumin promoter activity in transiently transfected cells. In contrast, further deletion of the promoter down to −153 bp, resulting in loss of most of the F site, led to an almost two-fold loss of promoter activity ($P < 0.05$). The results shown in Figure 1, bottom panel, combine data from four different experiments, each performed in triplicate. A similar loss of promoter activity with deletion of the interval between −207 and −153 bp was also consistently observed in other experiments. These results indicate that sequence(s) within the 54 bp interval between −207 and −153 bp contribute to albumin promoter activity. Deletion of the interval between −153 and −124 bp did not significantly affect promoter activity. Deletion of the interval between −104 and −70 bp, which includes the C/EBP and DBP-binding site (D site) plus the CCAAT element (C site), resulted in a further loss of activity.

Additional functional analysis of the −207 to −124 bp promoter interval

The nucleotide sequence of the upstream region of the albumin promoter is shown in Figure 2, top panel. The numbering scheme in the Figure is for the rat albumin promoter sequence, according to the numbering convention used in [9–11]. The sequences of the mouse, human and bovine albumin promoters are also shown in the Figure, to illustrate regions that are conserved. Also illustrated are the locations of the E and F sites of the mouse albumin promoter and of distal elements II and III of the rat albumin promoter [7–9]. The sequence of the upstream region of the promoter contains two close matches to the HNF-3 consensus binding sequence VAWTRTTKRYTY [30]. One of the possible HNF-3-binding sites (site X) is located at −168 to −157 bp (sequence GTATGTTTGCCA on the antisense strand). This sequence matches the consensus HNF-3-binding site in 9 of 12 bp, including the entire 8 bp core region. The second possible HNF-3-binding site (site Y) is located at −145 to −134 bp (sequence AGTTGTTTAACT on the antisense strand). This sequence also matches the consensus HNF-3-binding site in 9 of 12 bp, including 7 of the 8 central bp.

Analysis of the deletion constructs indicated that promoter sequence(s) located within the interval extending from −207 to −150 bp (fragment W) had a positive effect on promoter activity. For further functional analysis of fragment W, additional constructs were prepared in which this sequence was cloned upstream from a minimal promoter (TATA-luc) or upstream from the serum albumin promoter −70 bp deletion (alb70-luc), which contains the HNF-1-binding site. These constructs were transfected into HepG2 cells, and the effect of fragment W on luciferase activity was determined. Placement of fragment W upstream from the TATA box caused about a two-fold increase in luciferase expression (Figure 2, bottom panel). Similarly, placement of fragment W upstream from alb70-luc caused a 2.1-fold increase in luciferase expression (results not shown). These results confirmed that fragment W contains sequence(s) that have a positive effect on transcription.

For further identification of sequences within the upstream promoter region that were responsible for the positive effect on promoter activity, additional constructs were prepared in which various portions of the upstream region were cloned upstream from a minimal promoter (TATA-luc). A construct in which transcription was directed by a multimer of the sequence extending from −207 to −178 bp (fragment Z) yielded luciferase expression equivalent to TATA-luc (Figure 2, bottom panel), indicating that the interval between −207 and −178 bp has little effect on transcription. In contrast, a construct containing a single copy of the distal HNF-3 site (oligo X) cloned upstream from the TATA box exhibited a four-fold increase in expression relative to TATA-luc. The activity of this construct actually exceeded the activity of the construct in which the entire −207 to −150 bp fragment was cloned upstream from the TATA box, suggesting that the entire two-fold effect of the upstream region on luciferase expression was attributable to HNF-3 site X (Figure 2, bottom panel). Other constructs in which multimers of the distal HNF-3 site were cloned upstream from the TATA box also exhibited increased luciferase expression relative to TATA-luc (Figure 2, bottom panel).

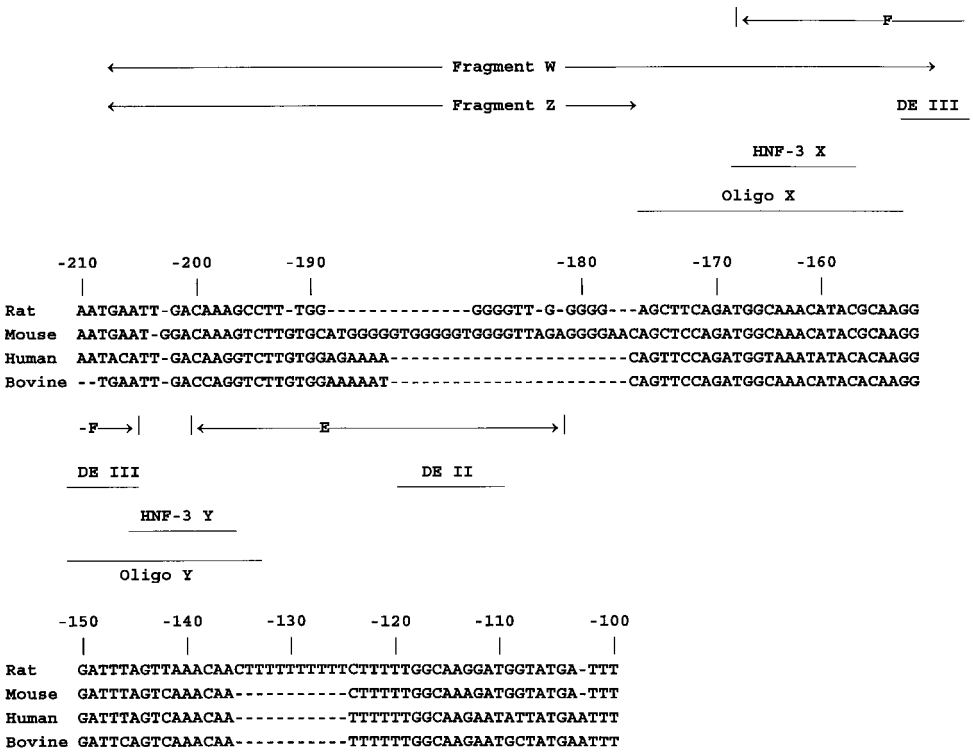
Additional constructs were also prepared to evaluate the effect of the proximal HNF-3-binding site (site Y) on promoter activity. In contrast with the results obtained with the distal HNF-3-binding site, a construct with a single copy of the proximal HNF-3-binding site (oligo Y) cloned upstream from the TATA box did not exhibit an increase in expression relative to TATA-luc (Figure 2, bottom panel). However, placement of multimerized copies of oligo Y upstream from the TATA box resulted in an increase in luciferase expression (Figure 2, bottom panel). The combined results of the functional studies described above suggested that the positive effect of the −207 to −153 bp promoter interval on transcription was due to the presence of the distal HNF-3 site within this region. Furthermore, the proximal HNF-3 binding site appeared to have a weaker positive effect on promoter activity than the distal HNF-3 binding site. In particular, deletion of this site had little effect on promoter activity (Figure 1, bottom panel), and a monomer of the site did not affect transcription directed by a minimal promoter (Figure 2, bottom panel). Therefore, subsequent studies were focused primarily on site X, which had higher functional activity.

Transactivation by HNF-3 of constructs containing site X

To investigate further the possible role of HNF-3 in activating the albumin promoter by binding to site X, transactivation experiments were performed in which an HNF-3β expression plasmid was co-transfected with the luciferase reporter construct containing a single copy of site X cloned upstream from a minimal promoter. A luciferase reporter plasmid containing the minimal promoter alone (i.e. TATA-luc) served as a control for specificity in these experiments. The results (Figure 3B) indicated that HNF-3β specifically transactivated the site X–luciferase reporter construct. Similar results were obtained with an HNF-3α expression construct, although the level of transactivation with HNF-3α was not quite as high as with HNF-3β (results not shown). In contrast to the transactivation of the site X–luciferase reporter by HNF-3, no transactivation was observed with a similar luciferase reporter construct containing one copy of the Y site (Figure 3C).

Site-directed mutagenesis of HNF-3 site X within the context of the albumin promoter

To investigate further the possible role of HNF-3-binding site X in activation of the albumin promoter, a mutation was introduced to eliminate HNF-3 binding to this site, within the context of the −261 bp albumin promoter–luciferase reporter construct. This mutation reduced promoter activity by about 40 % in comparison with the parental −261 bp albumin promoter–luciferase construct (Figure 4). The extent of reduction of promoter activity was similar to that observed with the −153 bp deletion, providing further evidence that the X site is the major functional element responsible for the positive effect of the −261 to −153 bp promoter sequence on promoter activity.



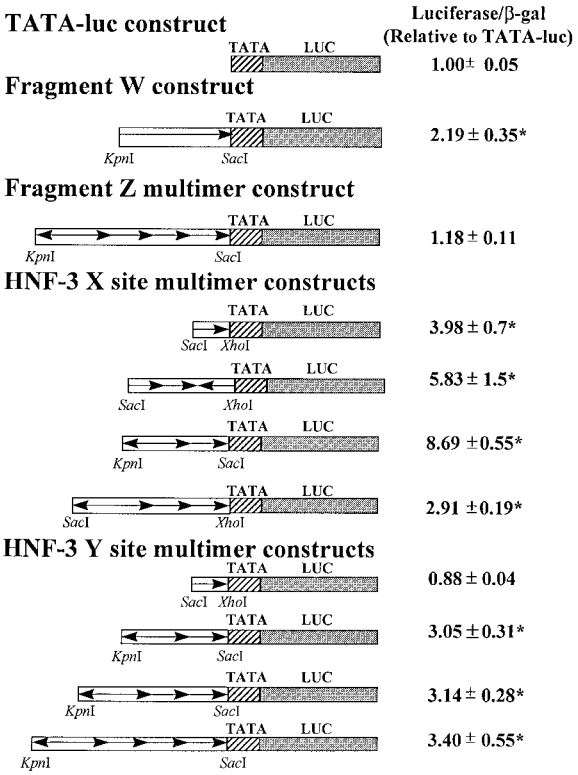


Figure 2 Sequence of the albumin promoter upstream region and activities of reporter constructs with fragments of the albumin promoter cloned upstream from TATA-luc

(Top panel) Sequence of the upstream region of the serum albumin promoter. The numbering scheme follows the convention for the rat albumin promoter [9–11]. The alignment of sequences for the four species is as suggested in [44]. The co-ordinates for footprints E and F [7,8] and of distal elements (DE) II and III [9] are shown. Positions of promoter fragments W and Z and oligonucleotides X and Y are also indicated. (Bottom panel) Structures and relative activities of reporter constructs with fragments of the albumin promoter cloned upstream from TATA-luc. Promoter fragment W and monomeric or multimerized oligonucleotides X, Y and Z were cloned into the TATA-luc vector using restriction sites as indicated. Arrows in multimer regions indicate the number and orientation of oligonucleotides. Relative activities of the constructs were determined by transient transfection into HepG2 cells. Results represent the average of at least two experiments. All data were normalized to β-galactosidase (β-gal) activity, and the mean for the TATA-luc group was then set at 1.0. Errors represent S.E.M. for the pooled data. Each mean was compared with the mean for the TATA-luc group by Student's t test. *, Significantly different from the TATA-luc group, $P < 0.05$.

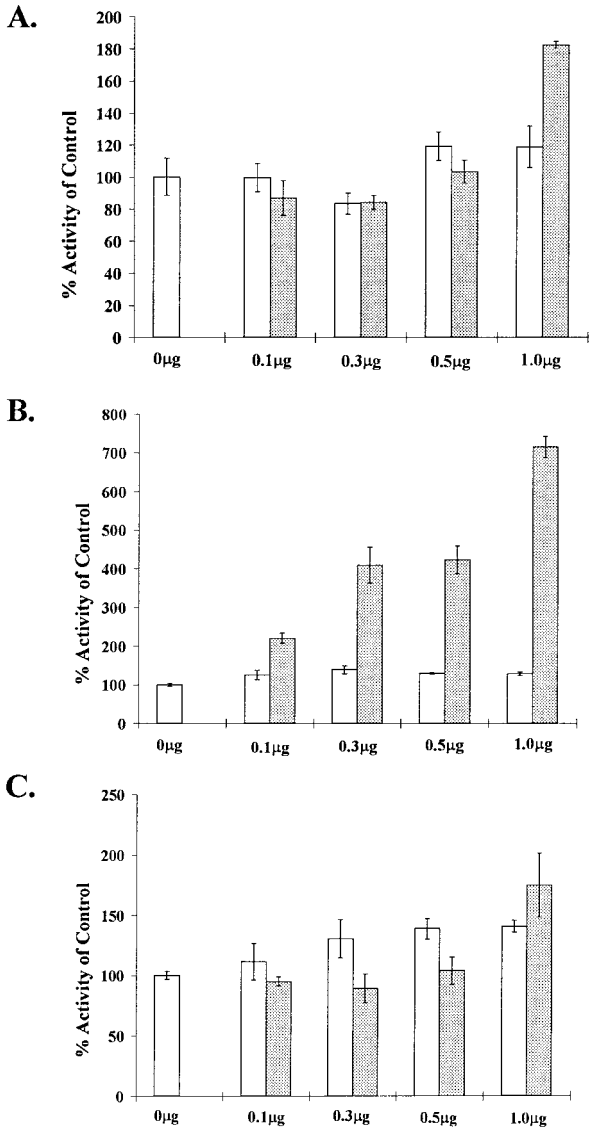


Figure 3 Transactivation by HNF-3β of constructs containing one copy of putative HNF-3-binding site X or Y cloned upstream from TATA-luc

Transactivation was determined by transient transfection of HNF-3β/pMT7 expression construct (shaded bars) or empty pMT7 vector (open bars) plus TATA-luc (**A**), oligo X–TATA-luc (**B**), or oligo Y–TATA-luc (**C**) into HepG2 cells. Results were normalized to β-galactosidase activity and represent the means $\pm$ S.E.M. of 3–6 replicates. The mean for the reporter plasmid alone was set at 100%.

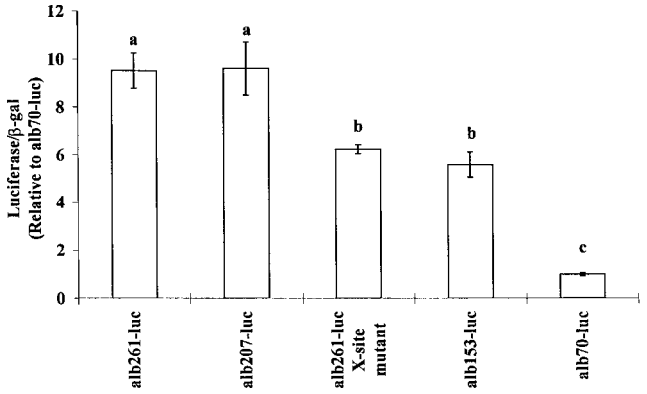


Figure 4 Effect of inactivation of HNF-3-binding site X by site-directed mutagenesis

Each construct was transiently transfected into HepG2 cells along with pCMV-βgal. The results represent the combined data from two separate experiments performed at least in triplicate ($n \geqslant 6$). All data were normalized to β-galactosidase activity (β-gal) and represent the means $\pm$ S.E.M. for the pooled data. The mean for the TATA-luc group was set at 1.0. Data were subjected to ANOVA followed by Student–Newman–Keuls multiple-comparison test using $\alpha = 0.05$ as cut-off for significance. Means with different letters are significantly different.

Binding of HNF-3 to sites X and Y

To investigate whether HNF-3 was able to bind to site X, EMSAs were performed, using oligo X as probe and recombinant HNF-3α and HNF-3β proteins prepared from transfected COS-7 cells. The results (Figure 5, left panel) indicated that HNF-3α and HNF-3β both formed specific shift complexes with the X-site probe. Unlabelled oligonucleotides corresponding to the X site, the Y site, and the transthyretin gene strong HNF-3-binding site [31], competed for this binding, whereas an unrelated oligonucleotide corresponding to the albumin promoter HNF-1-binding site did not. These results indicated that HNF-3 bound specifically to the X site.

The binding of nuclear proteins prepared from HepG2 cells to the oligo X probe is illustrated in Figure 5, middle panel. A specific shift complex was observed which had a mobility identical to a complex containing HNF-3α. (HNF-3α is larger than HNF-3β and runs more slowly than HNF-3β in EMSA gels [29,32,33].) These results indicate that HepG2 cells contain a nuclear protein that binds to oligo X and strongly suggest that this protein is HNF-3α.

As the HepG2 cells expressed primarily HNF-3α and the X site had a greater effect on promoter activity than the Y site, we next considered the possibility that the X site might bind HNF-3α more tightly than the Y site. To test this possibility, the binding of oligo X and Y probes to HNF-3α was quantified by EMSA as a function of increasing oligonucleotide concentration. The slope of the binding curve (i.e. bound probe versus total probe concentration) was approximately two-fold higher for the X than the Y oligo, indicative of tighter binding of HNF-3α to the X site than to the Y site (results not shown).

For further comparison of binding of HNF-3α and HNF-3β to the X and Y sites, an additional EMSA experiment was performed in which we examined the binding of HNF-3α and HNF-3β produced in COS-7 cells to the X and Y oligonucleotide probes. The results (Figure 5, right panel) indicated that HNF-3α and HNF-3β bound to both probes, with the ratio of α:β bound being higher for the X than for the Y site. These results indicate that HNF-3α and HNF-3β can bind to both the X and Y sites and are consistent with the higher affinity of HNF-3α for the X as compared with the Y site.

DISCUSSION

The upstream region of the albumin promoter is highly conserved among mammalian species (Figure 2, top panel), suggesting a possible role for this region in the regulation of albumin gene transcription. We now provide evidence for a positive effect of the region between -207 and -153 bp on rat albumin promoter activity. This positive effect on transcription is due primarily to the presence of the functional HNF-3-binding site X. The

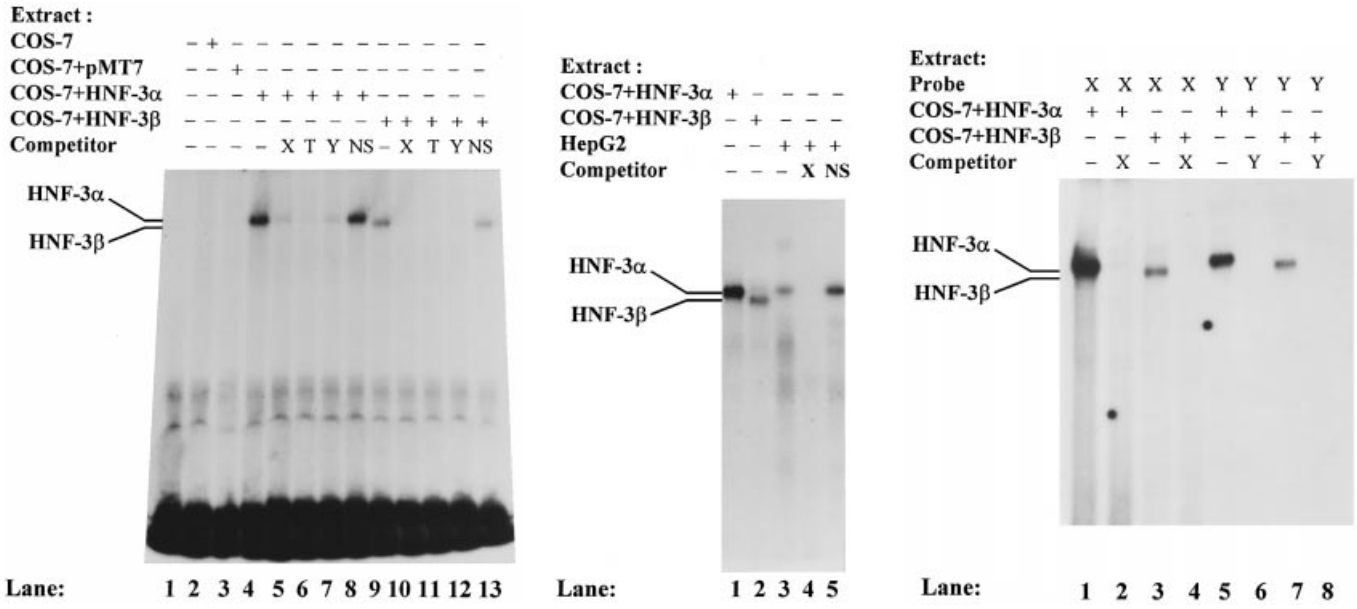


Figure 5 EMSA analysis of binding of HNF-3α, HNF-3β, and a protein from HepG2 nuclear extracts to oligo X

(Left panel) EMSAs were performed using oligo X as probe and nuclear extracts prepared from untransfected COS-7 cells (lane 2), or COS-7 cells transfected with pMT7 empty vector (lane 3), HNF-3α/pMT7 (lanes 4–8), or HNF-3β/pMT7 (lanes 9–13). Probe without nuclear extract was run in lane 1. Competitions were performed using a 100-fold molar excess of unlabelled oligo X (X, lanes 5 and 10), the strong HNF-3-binding site from the transthyretin promoter (T, lanes 6 and 11), oligo Y (Y, lanes 7 and 12), or an oligonucleotide corresponding to the HNF-1-binding site of the albumin promoter (NS, lanes 8 and 13). (Middle panel) EMSAs were performed using oligo X as probe and nuclear extracts prepared from COS-7 cells transfected with HNF-3α/pMT7 (lane 1), HNF-3β/pMT7 (lane 2), or from untransfected HepG2 cells (lanes 3–5). Competitions were performed with a 100-fold excess of oligo X (X, lane 4) or an oligonucleotide containing a perfect consensus C/EBP-binding site (NS, lane 5). (Right panel) EMSAs were performed using labelled oligo X (lanes 1–4) or an equivalent amount of labelled oligo Y (lanes 5–8) as probe and nuclear extracts prepared from COS-7 cells transfected with HNF-3α/pMT7 (lanes 1, 2, 5 and 6) or HNF-3β/pMT7 (lane 3, 4, 7 and 8). Competitions were performed with a 100-fold excess of oligo X (lanes 2 and 4) or oligo Y (lanes 6 and 8).

sequence of HNF-3 site X is highly conserved among four mammalian species (Figure 2, top panel). In the chicken serum albumin gene promoter, a similar site is also present in the same location [43]. The chicken site has the sequence TTATGTTT-TCCT, which matches the HNF-3 consensus in 8 of 12 bp, including a 6 bp core region. The HNF-3 X site is located within the F footprint, which is bound by nuclear proteins from mouse liver in *in vitro* DNase I footprinting assays [7,12]. Based on the present results, it is likely that HNF-3 is one of the nuclear proteins that binds to this site. The results presented here indicate that HNF-3 participates in activation of the serum albumin gene promoter, as it does with the promoter elements for a number of other genes that are preferentially expressed in the liver [27,31–33].

The possible role, if any, of the HNF-3 Y site in transcriptional activation of the albumin gene remains to be established. Although both the X and Y sites are clearly capable of binding both HNF-3α and HNF-3β in EMSA assays, several lines of evidence suggest that the Y site plays a lesser role in activation of transcription than the X site. First, deletion of the X site but not the Y site caused a significant loss of promoter activity (Figure 1, bottom panel). Second, the reporter construct in which luciferase expression is directed by one copy of the X site is active in the HepG2 cells, whereas a similar monomeric Y site/luciferase construct is not active (Figure 2, bottom panel). Third, HNF-3β transactivates the X site–luciferase construct but not the Y site–luciferase construct (Figures 3B and 3C). Fourth, HNF-3α binds more tightly to the X than to the Y site. Finally, the X site is located in a region previously shown to be footprinted by mouse liver nuclear proteins (the F footprint), whereas the Y site is located between the E and F footprints [7,12]. Therefore, although we cannot rule out a role for the Y site in activation of the albumin promoter in the liver *in vivo*, such a role was not evident from our functional analysis of the promoter in transfected HepG2 cells.

The results of our deletion analysis of the proximal promoter region are in general agreement with results reported previously by others [11,12]. All plasmids used in the present study were prepared in *dam*$^-$ bacteria, to avoid methylation and inactivation of the HNF-1 binding site [11]. One minor difference between the results reported here and previously published results is that we consistently observed about a two-fold positive effect of the D site, which binds proteins of the C/EBP family and DBP, on promoter activity. (This effect did not attain statistical significance at $P = 0.05$ in the results shown in Figure 1, bottom panel; however, a similar trend was consistently observed in all other experiments.) In contrast, it was reported previously that activity of the C/EBP-binding site was only evident in transient transfections if plasmids were prepared in *dam*$^+$ bacteria, to disable the HNF-1-binding site [11]. The earlier studies used H4-II-E rat hepatoma cells for the transfections whereas we used HepG2 human hepatoma cells; thus, the difference regarding activity of the D site may be due to differences in amounts or isoforms of C/EBP expressed in the two hepatoma cell lines. Power et al. [44] reported evidence suggesting that a C/EBP-binding site might also be present in the upstream region of the BSA promoter. Using the numbering scheme for the rat promoter (Figure 2, top panel), the co-ordinates for this site are −225 to −216 bp. In the deletion analysis presented in Figure 1, bottom panel, elimination of this site had no effect on promoter activity (deletion −261 compared with deletion −207). Therefore, we did not pursue the identity of proteins that might bind to this site.

Numerous recent studies have demonstrated that the native nucleosomal configuration of chromatin plays an important role in the regulation of gene expression (for reviews, see [45–47]). Effects involving changes in chromatin structure are not typically detected in transient transfection experiments such as those presented here, because nucleosomes are not thought to assemble in a physiological configuration within the transiently transfected plasmid DNA [48,49]. Zaret and co-workers [16–18] have analysed in detail the role of HNF-3 in the positioning of nucleosomes in the albumin enhancer, which is located at −10 kb relative to the transcription start site. The interesting possibility exists that either or both of the HNF-3 sites in the promoter are involved in nucleosome positioning, just as two HNF-3 sites in the enhancer are involved in nucleosome positioning [16–18]. Although the present study indicates a role for at least one of the HNF-3 sites in transcriptional activation, *in vivo* footprint analysis will be required to establish an additional role for either or both of the two HNF-3 sites in the establishment of a positioned array of nucleosomes.

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