Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5469–5473, July 1990
Biochemistry

Site-directed mutagenesis reveals a liver transcription factor essential for the albumin transcriptional enhancer

(polymerase chain reaction/albumin promoter/liver-specific transcription/*in vitro* mutagenesis)

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Communicated by Fred Sherman, May 16, 1990

ABSTRACT **A method based on the polymerase chain reaction is described for constructing a clustered set of base-pair changes, deletions, or insertions at any site on a DNA fragment. Advantages of the procedure are that virtually every product has the desired sequence alteration and that only a single round of polymerase chain reaction is required. We used this method to demonstrate that the binding of a specific liver nuclear protein, which we call eH-TF, is essential for the function of the enhancer element of the mouse albumin gene. The eH-TF binding activity is hepatocyte-specific; it binds to a functional region of the albumin promoter and is distinct from other albumin promoter factors, and part of the eH-TF binding sequence, TGTTTGC, occurs in functional regulatory sites of other liver-specific genes.**

Molecular genetic analysis has been advanced tremendously by the development of techniques that create specific base-pair changes at particular sites in DNA. For example, site-directed mutagenesis has been used to systematically analyze genetic regulatory sequences, functions of RNA and protein molecules, and complex processes such as cell division, growth, and development (1, 2). Two fundamentally different approaches were originally developed to create clustered base-pair changes of a defined sequence: oligonucleotide-directed (3, 4) and linker-scanning (5) mutagenesis. The discovery by Higuchi *et al.* (6) that novel sequences could be introduced into DNA by using the polymerase chain reaction (PCR) (7) has led to the development of several PCR-based procedures for site-directed mutagenesis (8–11). Here we present a variant of PCR-based mutagenesis that employs an *in vitro* selection for fragments containing the desired mutations, so that essentially all end products contain the desired sequences.

We demonstrated the utility of this approach by introducing clustered base-pair changes at nuclear protein binding sites distributed over an 830-base-pair (bp) fragment of the mouse albumin transcriptional enhancer. Using transgenic mice, Pinkert *et al.* (12) showed that the albumin enhancer stimulated transcription from the albumin promoter only in the liver. We (13) and others (14) have shown that the enhancer is active when transfected into simian virus 40-transformed hepatocytes cultured in hormonally defined, serum-free medium (15, 16). The hepatocyte-derived cell line we used, H2.35, was created with a temperature-sensitive mutant of simian virus 40. The albumin enhancer was active when transfected into H2.35 cells cultured at the restrictive temperature for large tumor (T) antigen function and under conditions that promote hepatocyte differentiation (13). However, the transfected enhancer was inactive in H2.35 cells grown at the permissive temperature and under dedifferentiating conditions. Thus, the activity of the albumin enhancer is a sensitive indicator of the differentiated state of hepatocytes. To better understand mechanisms that control hepatocyte differentiation, we wanted to identify the enhancer's essential protein binding sites.

In this report, we use PCR-based mutagenesis to show that the binding of a liver nuclear protein, which we call eH-TF, is critical for the activity of the albumin enhancer in differentiated H2.35 cells. PCR-based mutagenesis was particularly useful for studying the mutant eH binding site in the context of different enhancer subfragments. A functional binding site for the eH transcription factor also occurs in the albumin promoter and apparently other liver-specific genes, suggesting a general role for the factor in liver-specific transcription.

MATERIALS AND METHODS

Oligonucleotides and PCRs. Oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer and full-length products were purified by electrophoresis in denaturing 20% polyacrylamide gels. The sequences of the eH mutant oligonucleotides are shown in Fig. 1*A*; the sequences of the flanking oligonucleotides were as follows: F1, AGCGGATAACAATTTCACACAGGA; F2, GCGGAATTCGCAAGCATAGCACAGAG; F3, GCGCCATGGAATTCGTAGACAAGTTGGCCT; F4, GCGCCATGGAATCGGTCCCCGTGTACTCAT. PCRs were performed with *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer) in a Perkin–Elmer thermocycler as suggested by the manufacturer and ref. 7. PCR products were passed through 1.5-ml spin columns containing Bio-Gel P-30 (Bio-Rad) preequilibrated with 10 mM Tris, pH 8/1 mM EDTA (TE) and were added to a subsequent wash of the columns with 100 μl of distilled water. DNA fragment yields were quantitated by electrophoresis and staining with ethidium bromide.

Recombination of PCR Products. PCR products were digested with the appropriate restriction enzyme (Boehringer Mannheim or New England Biolabs), extracted twice with chloroform, precipitated, and resuspended in 0.1× TE. Occasional PCR reaction products that contained spurious DNA fragments were subjected to gel electrophoresis and fragment purification with GeneClean (Bio 101; ref. 17). Equimolar amounts of each PCR fragment (10–200 ng) were combined in a 20-μl reaction volume with 1 μl of T4 DNA ligase (New England Biolabs) in a buffer recommended by the manufacturer; the reaction mixtures were incubated at 16°C for 1–4 hr and then at 68°C for 15 min. One microliter of 1 M Tris (pH 7.5), 1 μl of 1 M NaCl, and 5–10 units of *Eco*RI were added and the mixtures were incubated at 37°C for 30 min. This postligation restriction digest was necessary to eliminate concatemeric forms of the desired fragment. The desired recombinant products were purified from 1.6% agarose minigels with GeneClean and suspended in 20 μl of 0.1× TE.

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Abbreviation: PCR, polymerase chain reaction.

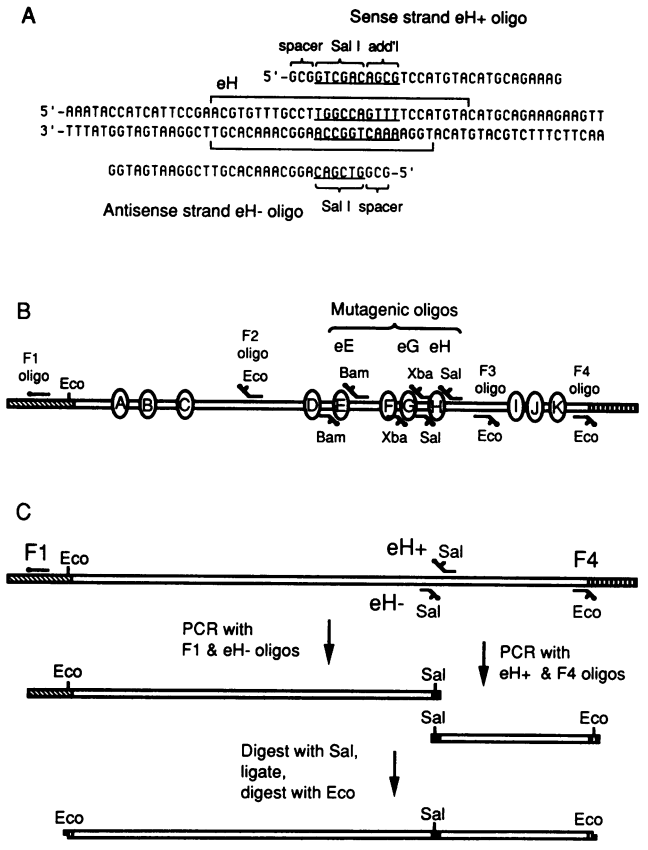

FIG. 1. PCR-based mutagenesis of the albumin enhancer element. (*A*) The double-stranded DNA sequence of a portion of the albumin enhancer is shown; the eH DNase I footprint is outlined by brackets. Oligonucleotides used to mutagenize the eH site are shown above and below the enhancer sequence. PCR-based mutagenesis converts the underlined sequences in the enhancer to the underlined sequences in the oligonucleotides. (*B*) The double line is a schematic representation of the 830-bp albumin enhancer fragment (open lines) in the plasmid pAT2-NA (hatched lines, pUC DNA; vertical lines, albumin promoter). Ovals with letters indicate the relative positions of footprint sites with liver nuclear extracts. Oligonucleotides ("oligo") are shown as short lines with dots representing the 5′ ends; bent lines indicate oligonucleotides with 5′ sequences different from the template. These 5′ sequences introduce the designated restriction sites into PCR fragments. (*C*) Strategy to construct a mutation of the eH footprint by PCR. The first level shows the template sequence used in the PCRs, the next level shows the PCR products, and the bottom level shows the final recombinant product; black segment indicates the site of the mutation.

One-quarter to one-half of the recovered DNA was ligated to 2 ng of *Eco*RI-digested, phosphatase-treated pAT2 vector (13). The final recombinant plasmids were recovered from transformed *Escherichia coli* JM83 cells (18) by standard procedures and screened for those with the normal orientation of the insert. Dideoxy sequencing confirmed that only the desired sequence changes were found in the amplified regions.

DNase I Footprinting Analysis. Nuclear extracts were prepared from tissues as described previously (19); H2.35 cell nuclear extracts were prepared by a modification of the procedure of Dignam *et al.* (20). Nuclear extracts were preincubated in 30-μl reaction mixtures containing nonspecific competitor DNA as described (19). After 10 min at 23°C, ^{32}P-end-labeled DNA probes were added and the incubations continued for another 30 min. DNase I digestion and purification of the DNA were adapted from the methods of Galas and Schmitz (21); the products were applied to a 6% polyacrylamide sequencing gel, along with wild-type and mutant sequence ladders, and autoradiographed with intensifying screens.

For competition footprinting experiments, competitor DNAs were added after the preincubation step and incubated for 10 min before addition of probe. Double-stranded oligonucleotide competitors were composed of the following albumin sequences: pB site, TGGTTAATGATCTACAGTTA; pD site, ATGATTTTGTAATGGGGTAG; pF site, GGGATTTAGTCAAACAACTTTTTGGCAAAGAT; eH site, CCGAACGTGTTTGCCTTGGCCAGTTTTCCATGTACATGCA.

Transfection and Cell Culture. Preparation of differentiated H2.35 cell cultures and transfection analysis were as described (13), except that 10^6 cells were transfected on 60-mm dishes with 6 μg of pAT2-derived plasmid and 0.2 μg of the pRT1 control plasmid.

RESULTS

DNase I footprint analysis of an 830-bp *Nhe* I–*Ava* II subfragment of the albumin enhancer revealed 11 liver nuclear protein binding sites, designated eA to eK (Fig. 1*B*). Some of the binding sites have been reported by Herbst *et al.* (14); a detailed analysis of our findings will be presented in a separate report. To assess the importance of individual sites, we focused our initial mutagenesis on three footprints, designated eE, eG, and eH, that occur in the middle of the enhancer fragment and bind nuclear proteins present in differentiated H2.35 cells (see Fig. 2 for eG and eH). In this report, we describe the effect of an eH-site mutation on protein binding and enhancer function.

PCR-Based Mutagenesis of the Albumin Enhancer. Our mutations were 10-bp substitutions near the middle of each footprint and were composed of 6 bp of a restriction site and four additional base changes. We synthesized sense- and antisense-strand mutagenic oligonucleotides for each site (see Fig. 1*A* for eH-site example). Each sense-strand oligonucleotide contained (5′ to 3′) the sequence GCG as a spacer, a 6-bp cohesive end restriction site, 4 bp of additional mutations, and 17 to 24 bp of downstream wild-type sequence that flanked the mutated site. Each corresponding antisense-strand oligonucleotide contained the same GCG spacer and restriction site, but these were followed directly by upstream wild-type flanking sequence. The spacer sequences facilitated restriction enzyme cleavage later in the procedure. We chose restriction sites that maximized the number of transversions and that were absent in the wild-type enhancer fragment. Different restriction-site substitutions were introduced at different footprint sites, to facilitate the subsequent construction of double-site mutations.

To permit us to mutate different sites in the context of the same *Nhe* I–*Ava* II enhancer fragment, we synthesized a "flanking" sense-strand oligonucleotide (F1, Fig. 1*B*) that annealed to pAT2 vector sequences (10) upstream of the wild-type enhancer in the plasmid pAT2-NA. We also synthesized a flanking antisense-strand oligonucleotide (F4) that annealed to the terminal 17 bp of the enhancer; an *Eco*RI recognition sequence was added to the 5′ end of this oligonucleotide.

To create a mutation of the eH binding site, we used a combination of the oligonucleotide F1 and the antisense eH− oligonucleotide for PCR with the parent vector pAT2-NA as the template (see Fig. 1*C*); the reaction incorporated an *Eco*RI site from the pAT2-NA template into 5′ end of the resulting fragment. In a separate PCR with the same template, we used the sense-strand eH+ oligonucleotide and oligonucleotide F4. The PCR products were digested with *Sal* I and then ligated together. We then digested the ligation products with *Eco*RI, which liberated the full-length, mutagenized enhancer fragment from concatemeric forms, and

purified the desired product by electrophoresis in an agarose gel; this step provided an important size selection for the full-length mutant fragment. The fragment was ligated into the *Eco*RI site upstream of the albumin promoter in the plasmid pAT2; upon transformation of *E. coli*, all recombinant plasmids contained the desired insert with the *Sal* I site at the location of the eH footprint.

A similar approach was taken to mutagenize the eE and eG footprint sites of the albumin enhancer, except that the mutagenic oligonucleotides introduced *Bam*HI and *Xba* I sites, respectively (Fig. 1*B*). As a positive control for the integrity of the PCR, we synthesized the wild-type enhancer fragment with the F1 and F4 oligonucleotides and the pAT2-NA template; the full-length fragment was cleaved with *Eco*RI and inserted into the pAT2 vector as above.

The eH-Site Mutation Prevents the Binding of a Liver-Specific Nuclear Protein Complex. Nuclear protein extracts were prepared from liver, differentiated H2.35 cells, and the albumin-negative tissues kidney and spleen and used in a DNase I footprinting assay with a subfragment of the albumin enhancer. As seen in Fig. 2*A*, nuclear protein from both liver and differentiated H2.35 cells protected the eH region on the antisense strand from DNase I digestion and created a strong hypersensitive site within the footprint (denoted by asterisk). Both spleen and kidney extracts protected regions that overlapped the liver eH footprint, but they spanned different sequences and lacked the hypersensitive site. DNase I footprinting of the sense strand of the DNA in this region confirmed these findings (data not shown). The eG footprint, including a hypersensitive site at one edge, was observed solely with the liver and H2.35 cell extracts. Proteins that protected the eF site were abundant in liver, less so in differentiated H2.35 cells and kidney, and absent in spleen. Thus, in this limited survey, the eG and eH footprinting activities were hepatocyte-specific.

We found that the 10-bp PCR-generated substitution of the eH site completely prevented the binding of the eH factor, even at the highest concentration of protein tested (Fig. 2*B*; bar indicates site of mutation). The eH-site mutation had no effect on the formation of the eG footprint, suggesting that cooperative interactions are not required for eG factor binding.

The eH-Site Binding Activity Is Essential for Albumin Enhancer Function. The PCR-generated wild-type and eH-site mutant fragments were tested for their ability to enhance transcription from the albumin promoter after transient transfection into differentiated H2.35 cells. We also cotransfected a control plasmid containing the Rous sarcoma virus promoter fused to the same thymidine kinase reporter gene (13). Total cellular RNA was prepared after 2 days, and expression from the test and control plasmids was assayed simultaneously with a primer extension assay. Using this approach, we previously showed that a 1.2-kb *Nhe* I–*Sca* I enhancer fragment (13) and an 830-bp *Nhe* I–*Ava* II subfragment (data not shown), each cloned by conventional means, were active in H2.35 cells; Fig. 3 (lanes 2 and 3) shows that the PCR-generated *Nhe* I–*Ava* II fragment enhanced expression from the albumin promoter to a comparable extent. However, the

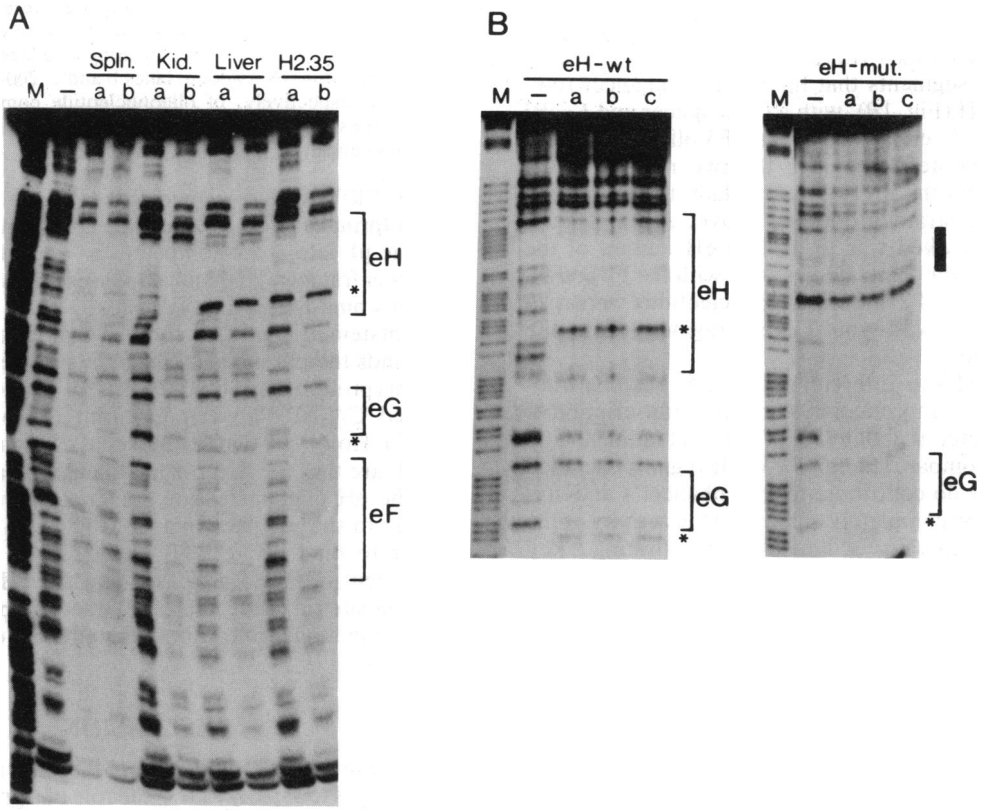

FIG. 2. DNase I footprint analysis of the eF, eG, and eH sites of the albumin enhancer. Nuclear extracts were incubated with a *Dde* I–*Sau*3A1 enhancer fragment labeled on the antisense strand and then were treated with DNase I. The digest products were separated in a sequencing gel and autoradiographed with an intensifying screen. (*A*) Footprinting with extracts from spleen (Spln.), kidney (Kid.), liver, and differentiated H2.35 cells. Lane M, purine cleavage ladder; lane –, no extract; lanes a and b, 20 and 50 μg of nuclear extract protein respectively. Brackets show positions of footprints from liver nuclear extracts; asterisks indicate the positions of DNase I-hypersensitive sites induced by protein binding. The protections above the eH site extend from flanking pUC18 sequences that are absent in the probes in *B*. (*B*) Footprinting of the wild-type (eH-wt) and mutant eH site (eH-mut.) enhancer fragments with differentiated H2.35 cell extracts. Lanes a, b, and c: 15, 30, and 60 μg of nuclear extract protein, respectively. The black bar indicates the position of the eH-site mutation, which is evident from the purine cleavage ladder.

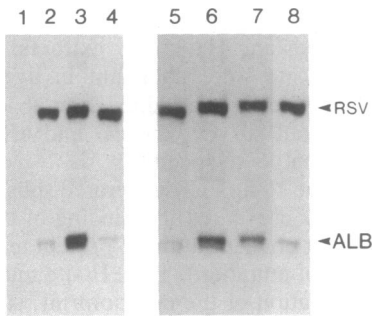

FIG. 3. Mutation of the eH site inactivates the albumin enhancer. Plasmid DNAs were transfected into H2.35 cells cultured under conditions that promote hepatocyte differentiation (13). Two days later, RNAs were isolated and analyzed by quantitative primer extension (13). ALB, reaction products due to properly initiated transcripts from the albumin test promoter; RSV, transcripts from the control plasmid pRT1. Lane 1, reaction products from mock-transfected cells. Lanes 2, 3, and 4, reaction products from pAT2, pAT2-NA, and pAT2-NA with the mutation at the eH site, respectively. Lanes 5–8 are from a separate transfection experiment with the plasmids pAT2, pAT2-NA, pAT2 with a 333-bp enhancer subfragment, and pAT2 with the 333-bp subfragment mutated at the eH site, respectively.

enhancer fragment containing the eH-site mutation was virtually inactive (Fig. 3, lane 4). We conclude that the eH site binds a transcription factor, designated eH-TF, that is essential for the enhancer's function.

To ask whether regions of the enhancer that bind other factors were essential, we used PCR to construct a subfragment of the enhancer. In this case, the flanking sense and antisense oligonucleotides (F2 and F3, respectively) were identical to 17-bp segments that flanked the enhancer footprint sites eD to eH (Fig. 1*B*), with a GCG spacer and *Eco*RI site added to their 5′ ends. The F2 and F3 oligonucleotides were used in separate PCRs with the two mutagenic eH oligonucleotides and the pAT2-NA template, to generate an eH mutant by the strategy outlined above. A simpler approach that also worked was to use the eH mutant of the 830-bp enhancer as the template in a PCR with the F2 and F3 oligonucleotides. The F2 and F3 oligonucleotides were also used to generate a wild-type 333-bp fragment from the pAT2-NA template.

When transfected into differentiated H2.35 cells, the wild-type 333-bp fragment reproducibly enhanced transcription of the albumin promoter at about 75% of the level of the 830-bp fragment (Fig. 3, compare lanes 5–7). While regions excluded from this fragment do contribute to the enhancer's activity, the eH-site mutation completely eliminated the activity of the enhancer subfragment (Fig. 3, lane 8), demonstrating a key role for the eH-TF binding site in this context.

The eH-TF Transcription Factor Binds to a Functional Site of the Albumin Promoter. To ask whether eH-TF corresponded to previously identified liver-specific transcription factors, we used oligonucleotide binding sites from the albumin promoter (22, 23) as competitors in a DNase I footprinting assay with liver extracts. No competition for the eH footprint was observed with binding sites pB, for HNF1 (24), or pD, for C/EBP (25) (data not shown), although these oligonucleotides effectively self-competed for the factors binding to the albumin promoter (Fig. 4). However, the eH-site oligonucleotide was an efficient competitor for the distal footprint site, pF, on the albumin promoter; the pF binding site is necessary for full promoter activity *in vitro* (26) and *in vivo* (27). The pF footprint spans the sequences TGTTTGACT and TGTTTGCCA on the antisense strand (26), and the nearly identical sequence TGTTTGCCT occurs in the eH footprint (Fig. 1). An oligonucleotide that spanned

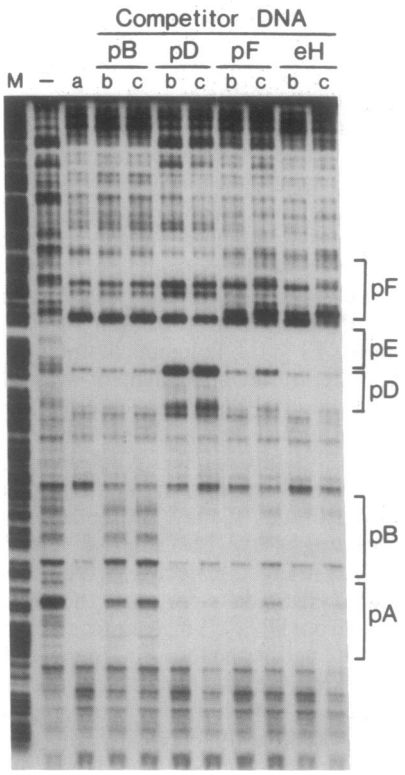

FIG. 4. The eH factor binds to a functional site of the albumin promoter. Liver nuclear extracts (40 μg) were incubated with an albumin promoter fragment labeled on the sense strand and then were analyzed by DNase I footprinting (see legend to Fig. 2). Lane a, no competitor DNA added; lanes b and c, 200- and 400-fold molar excesses, respectively, of oligonucleotide competitors containing binding sites as indicated, for the footprinting activities marked at right (nomenclature adapted from ref. 23).

the TGTTTGACT sequence of the pF site competed for the pF footprint as effectively as the eH oligonucleotide (Fig. 4), and the pF site cross-competed for the eH footprint on the enhancer fragment (data not shown), suggesting that the same proteins bind the pF and eH sites.

Lichtsteiner *et al.* (23) showed that a heat-stable protein that binds the pD site also binds the pF site. They suggested that this protein might be C/EBP (25), and we have found that purified C/EBP (28) can footprint the pF site (data not shown). However, two features that distinguish C/EBP from eH-TF are that the latter is heat-labile (data not shown) and that the eH oligonucleotide does not prevent C/EBP from binding to the pD site (Fig. 4). Thus, two different factors appear to bind pF: eH-TF and C/EBP. The competition experiment in Fig. 4 indicated that the eH binding activity predominates in crude nuclear extracts, implying that eH-TF plays a functional role at both the albumin enhancer and the promoter.

DISCUSSION

We developed a strategy for site-directed mutagenesis that permitted us to rapidly assess the role of a protein binding site in the albumin transcriptional enhancer. Of the existing PCR-based mutagenesis protocols, each has advantages and disadvantages. While all of the protocols can be performed rapidly and some of them obviate the need for enzymatic manipulation *in vitro* (6, 9, 11), one protocol is limited by the need for a natural restriction site close to the mutation (8), another requires the synthesis of four oligonucleotides corresponding to sequences in the immediate vicinity of the mutation (11), and some require two sequential PCRs (6, 9,

10), which could increase the potential for error in the procedure (29–31). Other differences with our procedure are that we could monitor all mutagenesis and reconstruction steps *in vitro* and that every recombinant product assayed had the desired sequence.

The protocol presented here should also be useful for altering RNA and protein structures. For example, adjacent codon changes can be made by creating 6-bp restriction sites at the points of recombination by DNA ligase. Smaller perturbations, such as single codon changes, can be made by incorporating different 4- or 6-bp restriction sites that leave complementary extensions on each fragment. Larger perturbations can be made by simply incorporating substitutions, deletions, or insertions adjacent to the recombination site (Fig. 1*A*). A final benefit is that the flanking and mutagenic primers can be used in PCR to generate DNA fragments directly suitable for DNA binding assays.

There are two ways our procedure can be used to combine separate mutations without intervening cloning steps. First, three PCR-generated fragments can be ligated together at once; for example, three PCR products generated by oligonucleotide combinations F1/eE−, eE+/eH−, and eH+/F4 (see Fig. 1*B*) can be ligated simultaneously at *Bam*HI and *Sal* I sites, generating mutations of the eE and eH footprints. Alternatively, a gel-purified, recombinant fragment containing the first mutation can be used as the template for a second round of PCR, with a different set of oligonucleotides, to generate the double mutant.

Using PCR-based mutagenesis, we found that the eH binding site was essential for the function of the albumin enhancer. Herbst *et al.* (14) recently identified an eH-site binding protein, called NLS1, which did not appear to be cell-type-specific in their gel-shift assays. Our DNase I footprinting assays of the eH site revealed binding proteins in different tissues with clear, reproducible differences in the protection patterns (Fig. 2), demonstrating an eH binding activity specific to liver cells. These findings are consistent with previous results showing that the eH region plays a role in determining the cell specificity of the albumin enhancer (14); similarly, the eH-TF binding site at the albumin promoter (pF) contributes to cell-type-specific transcriptional activation (27).

Sequences closely related to the eH sequence TGTTTGCCT occur in control elements of other liver-specific genes, suggesting that eH-TF plays a role in hepatocyte differentiation (32). For example, the sequence TGTTTGCTC occurs within activating sites of both the α-fetoprotein gene promoter (33) and one of its enhancers (34) and within the binding site for the liver-specific factor LF-A1, which activates the α_1-antitrypsin promoter (35). Finally, the sequence TGTTTGCT is part of the E-protein binding site required for the hepatitis B virus enhancer (32). As we found for the albumin eH site, the HBV E site bound proteins in different cell extracts but gave rise to a distinct DNase I footprint pattern with hepatic extracts (32). Further studies with fractionated extracts have demonstrated conclusively that several factors can bind the HBV E site (36). We point out that in all of the above DNA-binding studies, the TGTTTGC-containing sequences occur as part of extended footprints (Fig. 1*A*) and the remaining footprint sequences have no obvious similarities. We suggest that eH-related sequences confer cell-specific activation by binding different factors in different cell types, as is the case for the octamer sequence of immunoglobulin genes (37).

We thank Peter Shank, David Jackson, and Patrice Milos for comments on the manuscript, Bill Landschulz and Steve McKnight for generously providing purified C/EBP protein, and Charles Setterlund for synthesizing oligonucleotides. The research was supported by grants to K.S.Z. from the National Institutes of Health (GM36477) and the Searle Scholars Program/Chicago Community Trust.

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