Characterization of the factors binding to a PEPCK gene upstream hypersensitive site with LCR activity

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ABSTRACT

A previously described upstream hypersensitive site (HS) in the PEPCK gene at –4800 bp, termed HS A (1), has been characterized and determined to bind at least two factors. One of these is a member of the ubiquitous CREB/ATF family, and the second is a novel tissue specific protein, pep A. A construct carrying HS A and the PEPCK proximal promoter was tested in transgenic mice and its CAT activity compared to the proximal promoter alone. The HS A was shown to drive tissue-specific, position-independent transcription of the CAT reporter gene 2–3 fold more effectively than the proximal promoter alone, with a concomitant 4–5 fold higher expression of CAT. Protein binding activity has been localized to a 33 bp region. This region contains a CRE (2) which is shown to bind a member of the CREB/ATF family through competition assays with an oligo containing a CRE from the proximal promoter and by the appearance of a supershift when the factor/oligo complex was exposed to CREB polyclonal antibody. Through restriction enzyme digests and competition of protein binding with an oligonucleotide homologous to HS A with a mutated CRE we have characterized a putative binding site for a liver-specific factor. *In vitro* and *'in vivo'* footprinting studies complement each other, as well as, mobility shift assay data in designating the binding site of the proteins. The CREB/ATF factor and Pep A bind independently of each other during short term incubations, however, both factors can be accommodated on the DNA substrate as a function of extended time of incubation. Preliminary biochemical analysis defines the subunit molecular mass of the CREB/ATF like proteins at 55, 42, and 35 kD, while the tissue specific material exists as a single homogeneous subunit polypeptide in SDS of molecular mass = 49 kD.

INTRODUCTION

In higher eucaryotes when a gene is expressed in a given tissue, regardless of the level of expression, the promoter is seen to bind various factors necessary for transcriptional competence (for

review see ref. 3). However, in a tissue where the gene is totally quiescent and cannot be activated, the promoter appears to be nucleosomal in nature. Even ubiquitous factors do not have the capacity to bind to their cognate sequences in these promoters (for review see ref. 4). Such an exclusion of binding factors provide at least one mechanism for gene control in differentiated cells. Exclusion of binding factors (closing of chromatin) may be regulated by locus control regions (LCRs), usually found upstream of the promoter. The concept of an LCR was introduced by Grosfeld and his colleagues (5) and is defined as a DNA sequence which imparts chromatin position-independent activity to genes introduced into transgenic animals. Thus far LCRs seem to correlate well with upstream hypersensitive sites, suggesting that their effect is mediated through the activity of DNA binding proteins. Indeed tandem AP-1 binding sites have been identified with one of the control regions of the human β -globin gene which possesses both ubiquitous and tissue-specific binding sites (6–10). At the present time, the mechanism whereby such sequences and their putative binding proteins might affect the availability of promoter sequences, often several kilobases distant, is not understood. However, it appears that the multiple sites upstream of the human β -globin gene act in concert with each other, while individual sites show partial LCR activity.

We have previously reported that there are at least two far upstream HS sites in the PEPCK gene environment (1). These are found at –4.8 and –6.8 kbp. We have reported that the site at –4.8 kbp has tissue-specific enhancer activity when assayed in transient transfection assays. The site at –4.8 kbp is detected only in tissues in which the gene is expressed and the enhancing activity has been localized to a 220 bp DNA sequence (2). At least one of the factors which bind this sequence appears to be liver-specific. In order to further extend our understanding of the biological role of upstream HS sites we have asked if the DNA sequence responsible for factor binding shows any LCR activity as assayed in transgenic mice. We will argue that this region of the PEPCK gene does indeed show properties characteristic of an LCR. Further study of the proteins binding this region indicates that the liver-specific protein is different from any identified thus far, and that it interacts closely with a second, ubiquitous factor which appears to be a member of the CREB/ATF-like family of proteins.

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MATERIALS AND METHODS

Tissue Culture

All tissue culture lines, with the exception of the FF515 cell line, were grown in a 1:1(v:v) mixture of Dulbecco's Modified Eagles Medium and Ham's F-12, and supplemented with 10% calf serum. Cells were incubated at 37°C and 5% CO₂. The FF515 line was grown as described above, however, the medium was also supplemented with hypoxanthine-aminopterin-thymidine (HAT) to final concentrations of 100 μM, 0.4 μM, and 16 μM respectively. All tissue culture reagents were supplied by GIBCO, with the exception of HAT, which was supplied by Sigma.

Nuclear extracts and mobility shift assays

Isolation of nuclei from rat livers was done as previously described by Gorski et al (1986). Nuclear extracts from rat livers and tissue culture was done as previously described by Ip et al. (1990) except that the supernatant was neither dialyzed nor precipitated, but kept in the 0.5 M NaCl Buffer B. On occasion, the extract was boiled for 5 minutes before storing at 70°C. Gel shift and footprinting results were unaffected by this treatment.

Mobility shift assays were performed as described by Ip et al. (1990) with the following adjustments. Probes were labeled using polynucleotide kinase and α-³²P-ATP. Oligo probes were purified by polyacrylamide gel electrophoresis and electroelution of the excised band. Three fmol of probe was used per DNA binding reaction, unless otherwise indicated. Binding reaction buffer was the same as used by Ip et al. (1990) with an addition of Ca₂Cl₂ to a final concentration of 1 mM. The time of binding reactions were carried out as specified in text. The reaction mix was loaded directly onto a 6% polyacrylamide TGE gel (1 × TGE is 25 mM Tris base; 200 mM glycine; and 1 mM disodium EDTA, pH 8.0). After electrophoresis, the gel was dried and exposed to X-ray film.

Mobility shift assay using CREB antibody was done as described above with the following adjustments. CREB antibody was preincubated with dIdC for 5 minutes then nuclear extract was added to the antibody dIdC mixture for ten minutes of preincubation before adding binding buffer, probe, and competitor. Polyclonal CREB antibody was a generous gift from Dr. M. Montminy.

Oligonucleotides

The sequence of oligonucleotides used, oligo A (2), CREB consensus site oligonucleotide (12) and the pepA oligonucleotide were as follows:

oligo A: 5'-TCCACAGTCTCTGTCTACGTCATCATTATCGATCCTGG-3'
TGTCAGAGACAGATGCAGTAGTAATAGCTAGGACCAGGG-5'

CREB: 5'-AGCTCGAGGGCCCTTACGTCAGAGGCGAGA-3
GCTCCGGGGAATGCAGTCTCCGCTCTTCGA-5'

pepA: 5'-TCCACAGTCTCTGTAGTCTAATCATTATCGATCCTGG-3'
TGTCAGAGACATCGAGATTAGTAATAGCTAGGACCAGGG-5'

Restriction Enzyme Digests

Labeled oligo A was digested with either AccI, Taq I or BstNI, according to manufacturer's specification. Labeled fragments were then run on a 20% acrylamide gel to separate out the smaller fragments. Slower mobility fragments were then excised from the gel and purified by electroelution. All enzymes and buffers were supplied by NEB.

Footprinting

In vitro footprinting was done as described by Ip et al. (1990). The DP23 plasmid that contains the PEPCK A site from HinfI-to-HinfI, bps -4902 to 4667, (2) was used to obtain DNA restriction fragments. For footprinting of the coding strand a radioactive probe was made using restriction enzymes EcoR I and Sac I for digestion of the DP23 plasmid, and the EcoR I end was filled in with ³²P-α-dATP using pol I. Similarly a probe for the non-coding strand was obtained by digesting the plasmid with Hind III and Xba I, and then filling in the Hind III site with both ³²P-α-dATP and ³²P-α-dGTP. Samples were loaded onto a 6% polyacrylamide-7M urea sequencing gel using 5 μl of loading buffer containing 5M Urea, 0.5M NaOH and 0.5 mM EDTA final.

In vivo footprinting was done as described by Faber et al. (1991). Oligonucleotide primers used contained sequences just 5' of the -4902 HinfI site. A 33mer, of nucleotide sequence 5'-GAGTCTTTTAAACCCAGGTTCCCTGAGCCTCCCA, was used as a template for making the radioactive probe to be used in later in the PCR reactions. A complementary 9mer, of nucleotide sequence 5'-GCTCAGGAA-3', was then used for making the radioactive primer.

Molecular Weights

Molecular weight analysis was done using the electrophoretic transfer of proteins to Immobilon-PVDF as described by Szewczyk and Summers (1988). Briefly, 30 μl (approximately 210 μg total protein) of nuclear extracts from H4IIE cells were boiled for 3 minutes in a 2.5% SDS loading buffer, for a final concentration of 0.5%, before loading onto a 9% polyacrylamide-1%SDS gel. The gel was electrophoresed at 200V for 1 hr and then electrophoretically transferred to

Figure 1. At Least Two Factors bind the PEPCK Upstream Hypersensitive Site. A. ³²P-labeled oligo A was incubated with H4IIE extracts for 30 minutes. Competitors were used as noted in the figure, by adding a 5 or 50 fold molar excess of either the CREB/ATF-like oligo or the Pep A oligo as described in the Methods Materials. B. Effect of anti-CREB antibodies on mobility shifts of PEPCK HS A. ³²P-labeled oligo A was incubated with H4IIE extracts for 30 minutes at 22°, lane 2 and 3 extracts were treated identically except that each was preincubated for 10 min in the presence of 1 μl of CREB Ab. Lane 2, no competitor; lane 3, a 50 fold molar excess of the Pep A oligo was added to the binding reaction along with the labeled oligo A probe.

Immobilon-PVDF. Membrane strips were then dissected into eighteen consecutive slices and placed individually in Ependorf tubes. Proteins were then eluted from the membrane strips by shaking vigorously in 40 μ l of elution buffer containing 50 mM Tris (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 100 mM NaCl, 10% Glycerol, 0.1mg/ml BSA and 1% Triton X-100, for 3 hours at 4°C. Fractions were then tested for DNA binding activity using the gel mobility shift assay.

Generation and identification of transgenic mice

Two constructs were used for the generation of transgenic mice. The control (pPL9-CAT) consists of a CAT gene driven by the PEPCK promoter (from -600 to +57 bp). The test DNA (pPL9DP23⁺-CAT), is the control construct into which 220 bp of DNA encompassing the Upstream HS-A has been inserted (2). DNA was injected into pronuclei of one-cell mouse embryos, which were then implanted into pseudopregnant foster mothers (15). Transgenic offspring were identified by PCR analysis of tail DNA. The level of expression in the livers of these animals was assayed by Northern blots of parallel RNA isolations, using defined concentrations of plasmid CAT DNA to define a standard curve for quantitation purposes. The copy number of the transgene was determined using liver DNA in quantitative Southern blots employing appropriate concentration standards. Transgene integrity was checked by size determination of PCR products encompassing most of the insert. Expression of the transgene in different tissues was determined by assaying CAT activity of whole cell homogenates.

RESULTS

PEPCK gene upstream sequences have LCR-like properties

In order to assay possible LCR properties of HS A, which is -4.8 kbp upstream of the PEPCK gene, 220 bp of sequence which includes both the protein binding domain and the previously described enhancing activity, were assayed for efficiency in facilitating the expression of the CAT gene driven by the complete PEPCK gene proximal promoter in transgenic mice. This construct (as well as a control lacking the upstream sequences) was injected into embryos and introduced into pseudopregnant female mice. The offspring were assayed for the presence of the construct by PCR, for the number of inserts in liver DNA by quantitative Southern analysis, as well as for its expression by quantitative Northern analysis. The results are shown in table I. In the case of the construct containing the PEPCK gene proximal promoter alone, we find approximately 30% of the transgenic animals are able to express the CAT gene (primarily in the liver and kidney as reported by previous workers (16-18)). In contrast the construct containing the additional sequences from the upstream region shows expression in 70% of the transgenic animals. In addition, the extent of expression is 4 fold higher, though the tissue distribution of activity is analogous to that seen in the absence of the upstream sequences, as reported previously (16-18).

Both tissue-specific and ubiquitous factors bind the upstream region

Previous studies had indicated that within this 220 bp sequence essentially all of the protein-binding activity is localized to a 33 bp region. This sequence was used to synthesize an oligonucleotide which we call oligo A (2). There is a perfect match to the CGTCA motif of the consensus CREB/ATF binding

Table 1. Effect of PEPCK gene upstream HS-A sequences on frequency and extent of expression in liver of transgenic mice.

Mouse	CAT Expression/Copy of Insert
A. DP23 PL9 CAT	
3-11	not detected
4-1	16
4-3	18
13-1	60
20-12	22
23-5	20
23-6	not detected
Average (of expressors) 27 ± 15	
Expressing mice - 70%	
B. PL9 CAT	
15-2	not detected
15-3	"
16-1	"
17-2	"
17-3	"
19-7	2
19-8	7
19-19	10
20-6	not detected
20-8	"
20-9	"
20-10	14
20-25	not detected
Average (of expressors) 8 ± 4	
Expressing mice - 30%	

Transgenic mice were generated as described in the Materials and Methods Section. DNAs used for injection were pPL9CAT (PEPCK promoter driving CAT gene expression) and pPL9DP23CAT (same as previous except this construct contains 215 bp from the Upstream HS-A). Data are reported for all constructs with less than 10 inserts per genome. One data point for pPL9CAT was anomalous (20 $\times$ above the mean), and probably reflects insertion into an unusual chromatin environment and has not been included.

domain within this upstream sequence. We have therefore assayed for the presence of CREB/ATF-like factors in H4 nuclear extracts involved in binding to oligo A. H4 nuclear extracts show a complex pattern of binding to oligo A as shown in figure 1A. We see the formation of at least three complexes (bands a-c): two major bands (a and b), and a minor slower moving band (c). Competition with an oligonucleotide containing the CREB/ATF-like binding sequence (from position -78 to -99 in the PEPCK proximal promoter (12)), leads to the loss of the slower moving bands, leaving only complex (a) intact, indicating that some of the binding activity most likely does indeed involve these factors or a factor with a closely related binding domain. Further, using a labelled CREB/ATF-like oligo we obtain a pattern of shifted complexes which is essentially identical to bands (b) and (c), namely those bands which are competed away from oligo A by the CREB/ATF-like oligo. This provides additional strong evidence indicating that this sequence in the upstream region is indeed binding to CREB-like factors.

Confirmation for this interpretation comes from the use of an anti-CREB antibody generously provided by Dr M. Montminy. As shown in figure 1B, complexes 'b' and 'c' are supershifted in the presence of the antibody, whereas the third complex 'a' is unaffected.

We then designed an oligonucleotide identical to oligo A, except that the CREB/ATF binding domain has been replaced by a random nucleotide sequence, called pep A. Competition of protein binding to oligo A with the pepA oligonucleotide leads

to the loss of the fastest moving complex (a), while the complexes attributed to CREB-like factors remain clearly visible (figure 1A). This argues that the fastest migrating band (complex a) is indeed a specific binding complex. We surmised that the binding site for this factor (which we call Pep A), is therefore at a different position to that of the CREB/ATF binding sequence. Since this region of the overall PEPCK gene sequence possesses several strategically placed restriction sites it was possible to digest with different enzymes and to assay for factor binding. The results of such an analysis are shown in figure 2. Digestion with *AccI* (which cuts within the CREB/ATF-like domain) leads to a loss of binding of the material which is competent with the CREB/ATF-like oligonucleotide. Digestion with *BstNI* preserves all binding, suggesting that the other factor binds between the CREB/ATF domain and this restriction site. This conclusion was confirmed by the loss of the other binding site after cleavage with *TaqI*. These results suggested that the binding domain for the other factor might encompass the sequence 5'-ATCATTATCGAT-3'. Indeed the Pep A oligonucleotide, which contained these sequences was able to effectively compete for the binding to the non-CREB binding domain as shown in figure 2.

The factor producing complex (a) appears to be liver-specific, since the PEPCK gene upstream sequences can form such a complex only with nuclear extracts from liver cells. In figure 3 we see mobility shifts with extracts from H4 cells (which are liver in origin and still express the PEPCK gene), HTC cells which are also liver cells which no longer express significant levels of the PEPCK gene, FF515 cells which are a non-expressing 'extinguished' hybrid line derived from the H4 cells and mouse fibroblasts, MDCK cells which are kidney cells which do not express this gene, and also XC cells, which are of fibroblast origin and do not express the PEPCK gene. In addition we have tested kidney cells, which do express the PEPCK gene (data not shown). Only liver cells which can express the PEPCK gene possess the Pep A binding activity.

Both CREB/ATF-like factor and Pep A can bind the upstream sequence together to form a ternary complex

Since the CREB/ATF-like factor and the liver-specific Pep A protein bind to contiguous regions of DNA we wondered if both factors could bind to the DNA at the same time, or if binding to one factor was exclusive of the other. We have studied binding as a function of both concentration of protein and as a function of time of reaction. As is shown in figure 4 both increased concentration and, particularly, increased reaction time, lead to increased formation of material comigrating with the slower moving complex (c), at the expense of the individual CREB/ATF-like factor and the liver-specific binding protein complexes. The ability to form increased amounts of the slower moving complex as a function of time or extract concentration is not observed in the presence of excess competing oligonucleotides against either the CREB/ATF or the Pep A factors (figure 4B). We interpret this observation as providing strong support for the hypothesis that both factors bind the same DNA molecule at the same time. We stress that although the mobility of this ternary complex overlaps that of complex 'c' it represents a separate DNA/protein complex from the minor ATF/CREB complex which also migrates in this position. Since this reaction is time dependent (figure 4A), we surmise that these factors need to rearrange themselves in order to accommodate both components on the DNA. This interpretation is supported by experiments which show that either the Pep A or the CREB/ATF

oligonucleotide alone form primarily single complexes in liver cell nuclear extracts and do not show any additional shift over time (data not shown).

In vitro protein binding to the PEPCK gene upstream sequences appears to reflect that seen *in vivo*

Because of the biological properties of the upstream sequences we have assayed for factor binding by both *in vitro* footprinting (figures 5 and 6) using rat liver and H4 nuclear extracts and *in vivo* footprinting in H4 cells. The results of such a footprinting analysis are shown in figures 5 and 6. An *in vivo* footprint (figure 6) is detected over the region from -4768 bp to -4802 bp. This encompasses the domain previously shown to have enhancer activity when multimerized in *in vitro* assays (2). When

Figure 2. Identification of the sequences governing the binding of factors to PEPCK Upstream HS A. 32 P-labeled oligo A or labelled restriction fragments of oligo A were incubated with H4IIE extracts as described above, in either the absence or presence of a 50 fold excess of competitor as indicated.

Figure 3. A tissue-specific protein from liver derived cells binds the PEPCK upstream HS A. 32 P-labeled oligo A was incubated for 30 minutes with 1 or 2 μ l of nuclear extract (approximately 7 mg of total protein) from H4IIE, XC, HTC, MDCK or FF515 cells, as specified in the figure.

a restriction fragment containing this region was incubated with H4 cell nuclear extracts an *in vitro* footprint was obtained which covers essentially the same sequence (figure 6).

The region of the footprints is quite large extending over 25 bp as shown in figure 5A and B. This is consistent with the results presented above showing that more than one factor is involved in binding this domain. However, it is clear that the CREB/ATF footprint is much more clearly defined than that of the other component seen in the mobility shift assays. A more clearly defined footprint of the pepA component will await partial

purification of this factor. The sequence of the footprinted region is shown in figure 5C. At the 5' end of the sequence is the match to the CREB/ATF-like protein binding domain and this forms the major protected domain on DNA which is contiguous with the PEPCK non-coding strand. The other strand shows a comparable extent of protection. Both *in vitro* footprints are somewhat smaller than the *in vivo* footprint. The sequences of the footprint outside the CREB/ATF recognition site do not show extensive identity to any known binding domain, though some similarity to HNF3 has been previously noted (2). However,

Figure 4. Formation of a multimeric protein complex at the PEPCK upstream HS is both time and concentration dependent, and requires the presence of both the tissue specific (Pep A) and CREB/ATF factors. **A.** 32 P-oligo A was incubated with H4IIE extracts over a range of time points for 0, 30, 60 and 120 minutes, lanes 1–4 respectively, left panel; or with increasing concentrations of H4IIE extract, 0.5, 1.0, 2.0, 3.0 μ l (3.5–21 μ g total protein), lanes 1–4 respectively, right panel. **B.** The mobility shift assay was done as described for the experiment in part A of this figure with the following changes: the incubation was done in the presence of a 50 fold molar excess of either the CREB/ATF-like family oligo in lanes 1–4, or the Pep A oligo in lanes 5–8, as noted in the figure.

Figure 5. *In vitro* DNase I footprinting of the PEPCK HS. **A.** End-labeled DP23 (a restriction fragment containing the oligo A sequence) was incubated with rat liver extracts over a range of time points (0, 60 and 180 minutes), competitor included was 200 $\times$ molar excess of CREB oligonucleotide (lanes 4–6), 200 $\times$ molar excess of Pep A oligonucleotide (lanes 7–9), or 200 $\times$ of both oligonucleotides. **A.** The *in vitro* footprint on the right is from the 'noncoding strand'. **B.** The footprint on the left is from the 'coding strand'. Lane C, for either footprint is a control probe alone treated with DNase I. **C.** Nucleotide sequence of the protected region. The sequence is numbered with respect to the transcription start site (+1) of the PEPCK gene. The solid brackets indicate the region protected *in vitro* while striped brackets indicate the protected region *in vivo* shown in figure 6.

competition with HNF3-binding oligonucleotides does not show any significant HNF3 binding (data not shown) and the modest sequence similarity is most likely coincidental.

The molecular weight of the liver-specific factor Pep A

Nuclear extracts from H4 cells were run on SDS gels and blotted to immobilon membranes. The size-fractionated DNA binding factors were eluted and renatured, prior to analysis on a mobility shift gel. The results of such an analysis are shown in figure 7. Samples from each Molecular Weight region were also assayed

Figure 6. *In vivo* DNase I footprinting of the PEPCK HS A. The left side of the panel is an *in vitro* control using labeled DP23 incubated with H4IIE extracts over a range of time points as described in the legend for figures 5a and b, lanes C and 1–3. On the right side of the panel *In vivo* DNase I cleavage of the endogenous upstream hypersensitive site DNA in H4IIE nuclei was visualized as described in Materials and Methods. Lane 1: cloned DNA containing the upstream hypersensitive site was cleaved at guanine residues; Lanes 2 and 3: purified H4IIE DNA was treated with DNase I at 0.1 or 0.4 U/ml, respectively; Lanes 4–6: H4IIE nuclei were treated with 8, 16, or 32 U/ml of DNase I, respectively.

Figure 7. Molecular Weights of Gel purified proteins which bind to the PEPCK upstream HS A. H4IIE nuclear protein extract was treated with SDS (90°/5min) and fractionated on an SDS gel to resolve fractions with Molecular Weights from 10 kD to 200 kD. The SDS gel was blotted to immobilon and fractions 5 – 18, eluted from the filter were incubated for 30 minutes with ³²P-labeled oligo A. Lane C is a control, where the probe was incubated with untreated H4IIE extract in 10 μ l binding buffer plus 5 μ l elution buffer (both as described in methods and materials) and 5 μ l glycerol. Molecular weights were determined from a control protein size marker lane on the SDS gel.

for competition with appropriate oligonucleotides to test for the presence of the CREB/ATF family or the Pep A binding material. The presence of CREB/ATF binding activity at 55, 42, and 35kD was detected in H4 extracts (as well as in extracts from all tissues examined). However, in contrast, the Pep A competent material is found only in cells of liver origin expressing the PEPCK gene. It has a subunit molecular mass of ca. 49kD.

DISCUSSION

Experiments studying the insertion of the PEPCK gene far upstream elements into transgenic mice has revealed that a single copy of 220 bp of DNA from the –4800 bp hypersensitive site can function to increase both extent and frequency of tissue-specific expression of a reporter gene. As such it appears to be showing many of the characteristics of a locus control region. That this sequence does not generate 100% independence of position effect is not surprising given similar results from the globin system (8,10,19–22). The upstream sequences of the PEPCK gene resemble those of the globin system in that there are several hypersensitive domains which cumulatively exert the full LCR effect. Thus the use of a single domain as in this report would not have been expected to produce the full effect.

In order to understand the mode of action whereby this sequence exerts its effect, we have studied in some detail the nature of the proteins to which it binds. Using *in vivo* footprinting we find that approximately 25 bp of DNA near the hypersensitive site are protected from DNase I attack. This region is coincident with that which is protected during *in vitro* binding of nuclear factors. Previous studies had indicated that the surrounding 200 bp of DNA contain no additional binding domains (2). Two protein factors bind to this sequence. One is tissue-specific and the other is ubiquitous. The liver-specific factor generates a single homogeneous complex, whereas the ubiquitous factor which is CREB/ATF-like in sequence recognition, is more heterogeneous and seems to be made up of at least three subcomponents.

Evidently both the tissue-specific and the ubiquitous factor can be accommodated on the DNA together, though the formation of such an arrangement is time dependent. The rate of reorganization to form the multimeric complex is quite slow (time scale of tens of minutes at the concentrations we employ *in vitro*). Thus the formation of such a complex *in vivo* probably does not reflect the relative rates of association of the separate factors, but rather may indicate that some kind of reorganization is required for complex formation.

We note that the tissue-specific factor is absent not only from non-hepatic tissues but also from extinguished hepatic lines such as the FF515 cells. This cell line shows a phenotype of complete PEPCK extinction, which is reflected in the absence of hypersensitive sites from all points in the PEPCK gene environment, including the far upstream 'A' site examined in this report. The FF515 cells contain an apparently normal complement of CREB/ATF-binding factors (as do all other non-PEPCK expressing cells examined which can bind CREB/ATF *in vitro*, but as judged by absence of HS sites cannot bind *in vivo*). Thus it would appear to be likely that the binding of the CREB/ATF-like factors to the upstream site may depend upon the presence of the liver-specific factor. One possible mechanism which suggests itself is that in non-expressing cells the CREB/ATF factor is inhibited from binding to the upstream region by methylation of the CpG residue at the heart of the cognate recognition sequence, though we recognize that

demethylation may be a necessary but not sufficient prerequisite to permit factor binding as shown by Weih et al. (1990). The appearance of the Pep A factor may stimulate demethylation (by inhibiting approach of the methylase) or may permit binding of the CREB/ATF-factor even to a methylated domain of interaction. Implicit in these considerations is the likelihood that the CREB/ATF-like factor or a combination of both factors is able to generate a LCR position effect. Clearly testing these ideas, which is a necessary prerequisite to understanding the molecular mechanisms of position effect, will require the generation of additional transgenic mice, a process which is currently underway.

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