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Interaction of a Liver-Specific Factor with an Enhancer 4.8 Kilobases Upstream of the Phosphoenolpyruvate Carboxykinase Gene

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We have previously identified a series of five DNase-I hypersensitive (HS) sites within and around the rat phosphoenolpyruvate carboxykinase (PEPCK) gene. The far upstream region has now been sequenced, and the tissue-specific HS site has been mapped more precisely at 4,800 base pairs upstream of the transcription start site of the PEPCK gene. DNA fragments that include the HS site were cloned upstream of various promoters to test whether these regions modulate transcription of the chloramphenicol acetyltransferase reporter gene. Chloramphenicol acetyltransferase activity was enhanced when the DNA fragment encompassing the upstream HS site was linked to various lengths of the PEPCK promoter or to the heterologous simian virus 40 promoter. This upstream region in conjunction with the proximal promoter, which may contain a tissue-specific element, conferred maximum activation in H4IIE hepatoma cells, which express the endogenous PEPCK gene. When these experiments were performed in XC cells, in which the gene is not expressed, transcriptional activation by the upstream element was still significant. Evidence of a specific protein-DNA interaction, using DNA mobility shift and DNase I footprinting assays, was obtained only when using H4IIE cell nuclear extracts. Competition assay showed that the interacting factor may be similar or identical to the liver-specific factor HNF3. We suggest that this protein factor binds to DNA within the HS site and interacts with the proximal promoter region to control tissue-specific high-level expression of the PEPCK gene.

Eucaryotic DNA is packed efficiently in the nucleus wrapped around core histone octamers to form nucleosomes (32). This continuous structure is occasionally interrupted in the genome, and the interruption can be detected as nuclease-hypersensitive (nuclease HS) sites (7, 8). Nuclease HS sites often correspond to a range of regulatory elements, including promoters, enhancers, silencers, replication origins, topoisomerase-binding sites, and nuclear matrix association regions (8, 18, 37). In addition, much evidence supports the notion that non-histone proteins bind to these specific sequences and comprise part of the mechanism by which genes are regulated.

Many studies on eucaryotic transcriptional control mechanisms have focused on the *cis*-acting DNA elements and the *trans*-acting factors that interact with these elements. Transcription initiation is regulated by proximal promoter sequences such as TATA, CCAAT, and G+C-rich regions, which control the basal transcription of the gene (31, 34). Different protein factors that bind to these sequences and are responsible for the transcriptional effects have been identified and in some cases purified (11, 26, 27). Additional regulatory elements are often located further upstream of many genes. These include enhancer elements which modulate transcription in a distance- and orientation-independent manner (6, 41). Such elements, on occasion, regulate tissue-specific transcription (15, 36, 46). Enhancer elements in many cases consist of cassettes of sequences which form the basis for protein-DNA interactions and which in turn control the tissue-specific expression of the gene (9, 20).

An additional class of upstream functional elements has recently been described. This class encompasses what is defined as a dominant control region (DCR). In experiments with transgenic mice, the DCR upstream regions from several genes have been shown to facilitate chromatin position-independent high-level expression of both homologous and heterologous promoters in a tissue-specific manner (17, 19). It has been shown that DCR regions generally contain chromatin HS sites, though an exact identity between HS sites and DCR function has not yet been established.

We have previously identified a series of five DNase I HS sites around the rat phosphoenolpyruvate carboxykinase (PEPCK) gene (24). One of these sites is located far upstream of the transcription start site and is tissue specific. Studies from several laboratories have shown that some liver-specific genes contain tissue-specific enhancer elements located far upstream of the transcription start site (5, 39, 40, 45). The possibility that the tissue-specific, far upstream HS site found in the PEPCK gene reflects binding of a non-histone protein, which in turn regulates some aspect of transcription, was tested in this study.

Using indirect end labeling, this HS site has been mapped more precisely, and this region of the PEPCK gene has now been sequenced. Various DNA fragments, either spanning or proximal to the HS region, were placed upstream from vectors containing different amounts of the PEPCK gene 5′-flanking region. Activity of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene was assayed after transient transfection. We show that this upstream region is able to enhance CAT activity with all the promoters tested. The enhancement is seen in H4IIE hepatoma cells and to a modest degree in XC fibroblasts, even though the upstream HS site is not detectable in these cells. These transfection results also indicate the presence of a tissue-specific element in the PEPCK gene promoter. The in vivo function of the upstream region may be to enhance the tissue-specific transcriptional activity of the proximal PEPCK gene promoter.

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The domain responsible for such activity is localized in about 230 base pairs (bp) of DNA spanning the HS site. DNA gel mobility shift assays show that at least two factors (or different forms of the same factor) are able to interact with this region. One of these factors is detectable only in H4IIE nuclear extracts and binds specifically to this region with high affinity. There are at least two closely clustered binding sites available for this protein factor. Mobility shift competition assay shows that this factor is also able to bind an oligonucleotide containing a sequence recognized by the previously identified liver-specific factor HNF3 (3). We propose that this protein factor probably plays a role in the formation of the HS site and in enhancer activity.

MATERIALS AND METHODS

Tissue culture. H4IIE and rat hepatoma tissue culture (HTC) cells were maintained as monolayers in Swim 77 medium supplemented with a final concentration of 2.5% each newborn calf and supplemented calf serum (Hyclone). XC cells were grown in Dulbecco modified Eagle medium with a 10% final concentration of supplemented calf serum.

Molecular cloning and sequencing. For sequencing the entire far upstream region, a 4.8-kilobase-pair (kb) *Eco*RI fragment from the clone pPC112.R2 (1) was subcloned into the *Eco*RI site of pGem7Zf(+) (Promega Biotec). Either M13 universal primer or synthetic oligonucleotides were used for double-stranded plasmid DNA sequencing with a Sequenase kit (United States Biochemical Corp.). The sequences were further confirmed by subcloning of smaller fragments into plasmid vectors, followed by sequencing using the M13 universal or reverse primer.

DNA fragments used for the construction of CAT plasmids were obtained by appropriate restriction digestion, polyacrylamide gel purification, and finally blunt-end ligation to the upstream sites of various CAT vectors. The cloning site in pSV1-CAT (16), pPL49-CAT, and pPL7-CAT was the *Nde*I site which is 50 bp upstream of the promoter; in pPL9-CAT (29) it was *Hin*dIII which is immediately upstream of the promoter. The orientation of the inserts in the constructs was confirmed by restriction mapping and DNA sequencing.

DNase I sensitivity study. Tissue culture cells were collected after washing the monolayers with phosphate-buffered saline supplemented with 5 mM EDTA. The cells were washed two times with the same buffer. Nuclei were prepared by lysing the cells in ice-cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 8], 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 0.25 M sucrose), followed by centrifugation at 500 × *g*. After the nuclear pellet was washed once in the same buffer and then twice in digestion buffer (10 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 M sucrose), the nuclear pellet was resuspended in the digestion buffer at a concentration of 25 A_{260}/ml. DNase I was added at 0 to 25 U/ml (usually 0, 2, 8, 15, and 25 U/ml for a set of five). Digestions were initiated by the incubation of samples at 25°C. After 5 min, the reaction was stopped by adding sodium dodecyl sulfate (SDS) to 1%, EDTA to 25 mM, and proteinase K to 200 μg/ml, and the DNA was purified by standard methods (30).

Purified DNA, digested with appropriate restriction enzymes where indicated, was resolved by electrophoresis in agarose gels. The gel was treated with alkali and set up for capillary transfer in 0.4 N NaOH to a Zeta-probe membrane (Bio-Rad Laboratories) as described by Reed and Mann (42). DNA fragments used as probes for hybridization were obtained from restriction enzyme digestion of cloned plasmids containing PEPCK gene sequences. The restriction fragments were separated by polyacrylamide gel electrophoresis, and the appropriate fragments were recovered by electroelution. The polyacrylamide gel-purified DNA fragments were used as templates for oligonucleotide-primed labeling with [α-^{32}P]dATP (3,000 Ci/mmol, Amersham Corp.) (10). A specific activity of 10^9 cpm/μg was consistently obtained. Prehybridization and hybridization were carried out at 65°C for 3 and 16 h, respectively, in a buffer containing 1.5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 1% Carnation nonfat dry milk, 2% SDS, 10% dextran sulfate, and 200 μg of denatured salmon sperm DNA per ml. The filters were washed as described by the supplier, and the final wash was carried out at 65°C in 0.2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. The optimum exposure was for 1 to 3 days, using Kodak X-omat AR film and a Dupont Lightning-Plus intensifying screen placed at −70°C.

Isolation of nuclear extracts. Cells grown to confluence (about 4 × 10^8) were harvested and washed in 1× phosphate-buffered saline. They were suspended in buffer A (10 mM HEPES-NaOH [pH 8], 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100), vortexed gently, and centrifuged at 750 × *g* for 5 min. The nuclear pellet was washed once in buffer A and suspended in 3 ml of buffer B (buffer A plus 25% glycerol) supplemented with NaCl to a final concentration of 0.5 M. The sample was placed on ice for 30 min and centrifuged at 12,000 × *g*, and the supernatant was collected. The supernatant was dialyzed directly or precipitated in 70% ammonium sulfate, and the resuspended pellet was dialyzed against a buffer containing 10 mM HEPES (pH 8), 1 mM EDTA, 50 mM NaCl, 7 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 50% glycerol.

Mobility shift assay. Plasmid DNA was digested with appropriate restriction enzymes and end labeled with Klenow DNA polymerase and α-^{32}P-deoxyribonucleotides, and the DNA fragments were purified through polyacrymide gel electrophoresis and electroelution of the excised bands. Usually, 1 fmol of end-labeled fragment was used in a binding reaction. The DNA was incubated with 1 μl of nuclear extract (~4 μg of total protein) in 25 μl of a buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 1.4 mM β-mercaptoethanol, 80 μg of poly (dI-dC) per ml, and 10% glycerol. The binding reaction was carried out for 10 min at room temperature. 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). After electrophoresis, the gel was dried and exposed to X-ray film.

DNase I footprinting. The end-labeled DNA fragment was incubated with nuclear extract under the conditions described above. After 10 min, DNase I was added to a concentration of 1.6 U/ml, and the sample was incubated at 25°C for 90 s. The reaction was stopped by adding 4 volumes of stop buffer to a final concentration of 1% SDS–10 mM EDTA–100 μg of proteinase K per ml–10 μg of sonicated salmon sperm DNA per ml. The mixture was incubated at 65°C for 10 min, extracted once with chloroform-phenol and once with chloroform, and then precipitated with ethanol. The pellet was suspended in loading buffer containing 95% formamide, and the DNA was resolved in an 8% polyacrylamide–7 M urea sequencing gel.

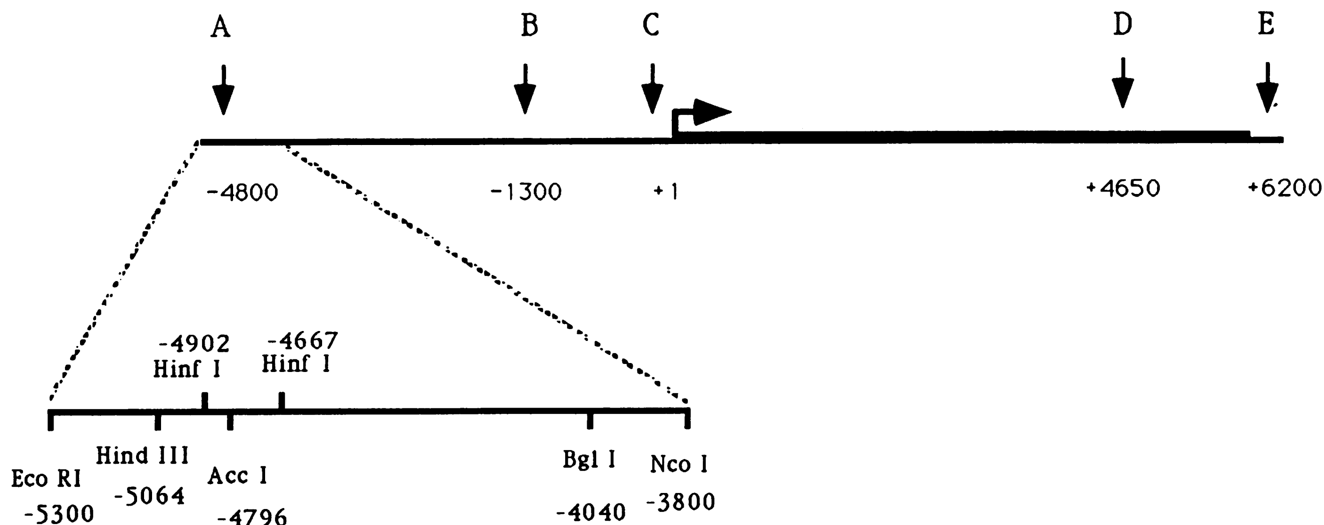

FIG. 1. Chromatin structure of the rat PEPCK gene. The thick line represents the coding sequence of the gene. The five previously identified DNase I HS sites are shown. Arrows indicate the HS sites designated A to E. The enlarged lower portion represents the upstream region that has been sequenced as shown in Fig. 2; a partial restriction map is shown.

Transfection and CAT assays. H4IIE cells were grown to 60 to 80% confluency in Corning T-150 flasks. The cells were trypsinized and transfected in suspension by incubation for 30 min with 2 ml of a calcium phosphate-DNA coprecipitate containing 50 μg of plasmid DNA and 50 μM chloroquine (38). They were divided into two T-75 flasks. After 6 to 8 h, the cells were shocked with 20% dimethyl sulfoxide for 5 min and then returned to fresh medium for 24 h. Cells were harvested and suspended in 150 μl of 0.25 M Tris (pH 7.8). Cell lysates were prepared by sonication. An 80-μl sample of the lysates was incubated at 37°C in a final volume of 150 μl of buffer consisting of 400 mM Tris (pH 7.8), 4 mM acetyl coenzyme A, and 0.5 μCi of [^{14}C] chloramphenicol (16). After 30 min, an additional 4 mM acetyl coenzyme A was added and incubated for an additional 30 min. The acetylated derivatives were separated from chloramphenicol by thin-layer chromatography (95% chloroform–5% methanol) on silica gel-impregnated glass fiber paper (Gelman Sciences, Inc.). Spots identified by autoradiography were cut out and quantitated by liquid scintillation counting.

Transfection of XC cells was performed essentially as described above, with the following modifications. Cells were grown to 50% confluency in T-75 flasks. A 1.5-ml sample of calcium phosphate-DNA coprecipitate containing 15 μg of plasmid was added to the monolayer and incubated for 30 min at room temperature; 15 ml of medium was then added to the flasks and incubated at 37°C for 4 h. The cells were then shocked with 20% dimethyl sulfoxide for 1.5 min, returned to fresh medium for 24 h, and assayed for CAT activity (20 μl of extract was used for the assays).

RESULTS

Sequence analysis and fine mapping of the upstream HS site. Results presented previously (24) located a tissue-specific DNase I HS site at about 4800 bp upstream of the PEPCK gene transcription start site. To further characterize the functional relationship of this upstream region and its effect on the transcriptional activity of the PEPCK gene, we first determined the DNA sequence of this region. Figure 1 shows a partial restriction map of this region relative to the rest of the PEPCK gene. The region sequenced starts at an *Eco*RI site (−5300 bp) and ends at a *Bgl*I site (−4040 bp). The position of the *Eco*RI site relative to the PEPCK gene transcription start site is not known because about 2 kb of intervening DNA has not yet been sequenced. For convenience (based on restriction mapping), the *Eco*RI site is designated as −5300 bp and the rest of the sequence shown is numbered relative to this site.

The sequence of this region is largely unremarkable except for a 140-bp region consisting of 48 CT dinucleotide repeats followed by 26 CA dinucleotide repeats (Fig. 2). In vitro studies have shown that under superhelical torsion, extended arrays of CT dinucleotide repeats can form triple-stranded H-DNA (21, 47), and arrays of CA dinucleotides can form Z-DNA (35). We have found that this sequence is highly repeated in the rat genome and that there is no evidence that this region is other than nucleosomal in organization in vivo (data not shown).

To map the position of the HS site more precisely, an *Eco*RI-*Hin*dIII DNA fragment (−5300 to −5064 bp) close to the HS site was subcloned and used as a probe for indirect end labeling. The relative position of the probe is indicated in Fig. 3. Nuclei were isolated from H4IIE cells and digested with DNase I as described in Materials and Methods. The DNA purified from the nuclease-treated nuclei was digested with *Eco*RI, resolved in an agarose gel, and transferred to a Zeta-probe membrane for subsequent hybridization with the *Eco*RI-*Hin*dIII probe. The HS region was actually composed of two distinct sites located at −4875 and −4800 bp (Fig. 3A). The region between the sites appeared to be protected even at late stages of digestion, implying the presence of extensive non-histone protein binding to this 75-bp stretch of DNA. Furthermore, the precision of this mapping allows us to state with some assurance that the HS sites are at least 200 bp from the nearest dinucleotide repeats, which are therefore unlikely to play a role in the disruption of the chromatin structure.

A region further upstream was analyzed by using *Hin*dIII-digested genomic DNA and the same probe. An additional, previously undetected HS site was found at −6200 bp (Fig.

Eco RI

GAATTCACCA TGAAGTCCAG GGTGTTTCTT ATGTTTACCT GGTCTGTAGC CTCTGTCTGT 60

GTCCACCAAC CCATCCATGT ATGACAACTC TTGCATCATG ATGAGGCCTG GCAGGGCTAG 120

GTGGCCTTTT TCGGGAGGTA GGGACTTGAG CTGATCTGTC TGACCTTGAA TTTATTATGT 180

AGCCAAGGAT GACCTTGAAA GTCTAATCCT CCTGTTTCCA CCTCCTAATC TAGGAAGCTT 240 **Hind III**

CCTATCTGCT CTATTCTGTT TGGGGTTCCT AGAGCCCTGG AGACAGCCTA TCTGAAGAGA 300

CAATCGGGCT GCTGCGTTAC CAGTACTCTT CCGTCTTCTG CTCAGGAACA TCAGGAGAGC 360

CTCCTGGCTG GGAGGCTCAG GAACCTGGGT TTAAAAGACT CCTTAAATGT GTGGCTCATC 420 **Hinf I**

CCTGTCTCTT TGTCCCGTGG CTTCTGAGGA CAAGTGTAAA CATGTCTGGG AAGGAACTTG 480

CTGACCGTAG AACCCACAGT CTCTGTCTAC GTCATCATTA TCGATCCTGG TCTGTATGTA 540 **Acc I**

AACACAGGTG TCGATGTCCT CACAGGACTT GAGCTATGGG CATGGGGGAT AGACCAGGTG 600

TGTGTAGCGT CTTTCTATAG GACCACTGAA TGAGTCAAAT TCACTATCCT CCTTTCTCTG 660 **Hinf I**

CCTCCTCGTT GCTAGATTAC ACGATACCTG GCAGGGAT*TC* *TCTCTTTCTC* *TCTCTCTCTC* *720*

TCTCTCTCTC *TCTCTCTCTC* *TCTCTCTCTC* *CCCCTCTGTC* *TCCCTCTCTC* *TCTCTCTCTC* *780*

TCTCTCTCTC *TCTCACACAC* *ACACACACAC* *ACACACACAC* *ACACACACAC* *ACACACACAC* *840*

*ACACAC*CTCT CTTTTTTAAG ACAAGGTTTC TCTGTTTAGC CCTGGCTGCC CTGGAACTTA 900

CTCTGTAAAC CAGGCTGGCC TCGAACTCAC AGAAATCCAC CTGCCTCTGC CTCCTGAGTG 960

CTTAAAGGCA TAAGGCTAAA GGTGTGTGCC ACCACCACCC AGTTCAGGGA TTTTCTTAAA 1020

ACATCCAGCA CCACATGTAC GGGCTGGCTG TGTGATTTGC AGGGTCAAGT ACAGAACAAA 1080

AGTAAAGGAC CCTTCCTTTG CTTTCTCCAC GTGCATGGCG CTCACAACTT GCTAGTTAAA 1140

TGTCACACTT CTTCAGGCTC TTGGATACTG ACAGACATTG GAAGATGCCC AAAGATGCCT 1200

GGACTCAACC TACCTCTCTC TGAAGCAAGG TCCTTTTCAG GGATAGAGTG TGTTGGTCAC 1260

TCGGTAGGGT AGCCACAAAG GCCCAA*

Bgl I

FIG. 2. Sequence of the DNA in the region of the PEPCK gene upstream HS site A. The *Eco*RI site (shown as nucleotide 1) is at −5300 bp from the transcription start site of the PEPCK gene. Some of the restriction sites referred to frequently in this report are underlined. The dinucleotide repeats are in italics. Arrows indicate the positions of the DNase I HS sites as measured in Fig. 3.

3B). Other than this new site, the DNA as far as −9000 bp was devoid of any other interruption in the nucleosomal structure. The DNA beyond the *Eco*RI site at −5300 bp has not been cloned, and the additional HS site has not been analyzed further.

Identification of an enhancer element. The possibility that the DNA in the region of the upstream HS site possesses transcriptional regulatory activity was tested in two different cell lines with various plasmid vector constructs containing the CAT reporter gene. DNA fragments from the HS region were cloned into a site (see Materials and Methods) upstream of the promoters of the CAT vectors. The vectors used were pSV1-CAT (which contains a simian virus 40 promoter) and a range of CAT constructs containing different lengths of sequence from the PEPCK gene promoter (27; Fig. 4A). These constructs include pPL49-CAT (which contains a minimal PEPCK promoter from −112 to +60 bp), pPL7-CAT (which contains PEPCK gene sequences from −207 to +60 bp), and pPL9-CAT (which contains a complete PEPCK gene promoter from −600 to +60 bp). Transient transfection and subsequent transcriptional activity were studied in two cell lines: H4IIE cells, which are liver derived and possess the capacity to transcribe the PEPCK gene, and XC cells, which are of fibroblast origin and have never possessed the capacity to transcribe the PEPCK gene.

The various promoters alone (i.e., parent vectors without the upstream sequences) showed different responses in the two cell lines (Fig. 4B). The relative activities of the simian virus 40 promoter and the PEPCK gene minimal promoter were comparable in the cell lines studied (pSV1- and pPL49-CAT in Fig. 4B). In contrast, the complete PEPCK gene promoter appears to contain a tissue-specific stimulatory element that increases the transcriptional activity in H4IIE cells but not in XC cells (compare basal activities of pPL49- and pPL7-CAT with that of pPL9-CAT in the two cell lines). This observation is consistent with that reported recently by Benvenisty et al. (2).

Fragment A, a 1,500-bp *Eco*RI-to-*Nco*I fragment (sites at −5300 and −3800 bp, respectively) that encompasses the HS site and flanking sequences (including the dinucleotide repeats), was initially used to study the effects of upstream sequences on the various promoters described above. The

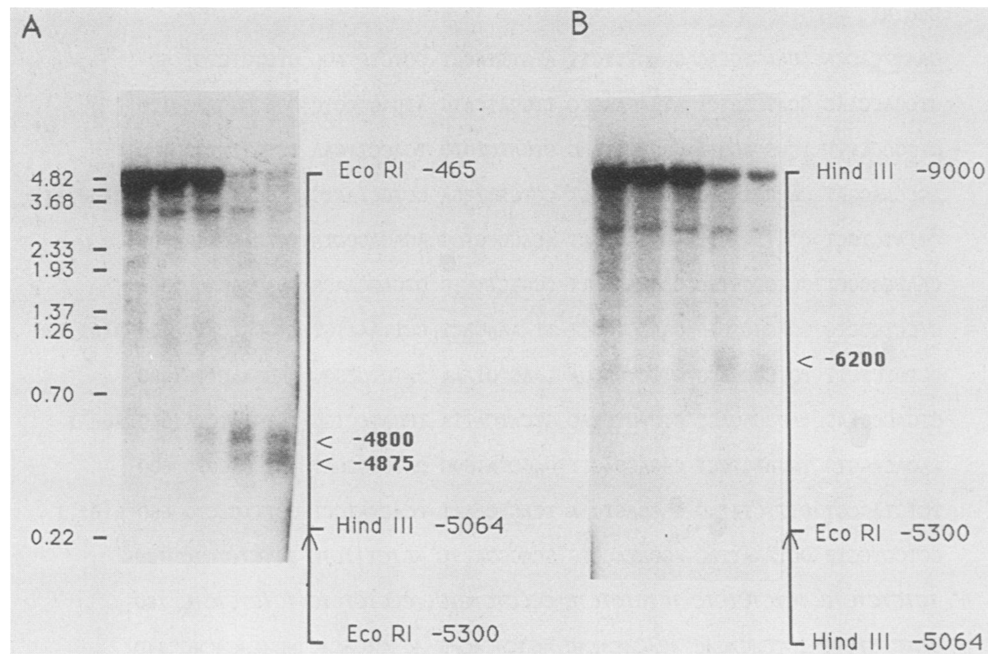

FIG. 3. Fine mapping of the upstream HS site A. Purified H4IIE genomic DNA from DNase I (increasing amount in a set of five)-treated nuclei was digested with either *Eco*RI (A) or *Hin*dIII (B) and resolved in a 1.6% agarose gel. The DNA was then transferred and probed with a short *Eco*RI-*Hin*dIII fragment (see also restriction sites in Fig. 2). Autoradiographs of the Southern blots are shown.

upstream sequences were clearly able to enhance transcription over that of the parent vectors in the H4IIE hepatoma cells (Fig. 4B). There was also a significant activation in the XC fibroblast line (see Discussion). The transcriptional activation seen in the H4IIE cells appeared to be independent of the type or length of the promoter, although it seemed to be highly orientation dependent. Thus, the transcriptional activation was seen most clearly when the inserted DNA fragment was in the orientation opposite that which occurred in the liver cell chromosome (this is defined as a negative orientation in Fig. 4). However, a smaller upstream fragment, DP8 (from −4796 to -4040 bp), which contained primarily the dinucleotide repeats and excluded some of the DNA sequences encompassing the HS sites, had a strong negative effect on the transcriptional ability of the basal promoter in both cell lines (pPL9DP8-CAT in Fig. 4). These observations suggest that decreased transcriptional activity is found when the dinucleotide repeats are situated close to the promoter (as in the positive orientation of fragment A discussed above).

To further investigate this phenomenon, a series of constructs was generated by using different, smaller DNA fragments from within the 1,500-bp upstream sequences which lacked the dinucleotide repeat sequences. These fragments were cloned immediately upstream of the complete PEPCK promoter in the pPL9-CAT vector in order to define more precisely the element responsible for the enhancer activity. DP23, a 235-bp fragment (*Hin*fI to *Hin*fI; sites at −4902 to −4667 bp) that contains the HS site, had enhancer activity similar to that of the intact upstream fragment in the negative orientation. Moreover, this construct, which lacks the repeat sequences, showed activation regardless of its orientation. Interestingly, if the complete 1,500-bp fragment A is inserted 4,000 bp upstream of the PEPCK gene promoter, one observes an orientation-independent enhancement of transcription, and at this distance from the promoter, the dinucleotide repeat sequences seem to no longer exert a selective inhibitory effect (data not shown). Thus, the biological activity of the upstream sequence seems to reflect an ability to activate transcription and is centered around an *Acc*I site at −4796, close to the HS site.

Mobility shift assays reveal binding of liver-specific factors to the enhancer element. Nuclear extracts obtained from H4IIE cells were tested for the presence of a protein factor(s) that could bind to the upstream region. Different DNA fragments encompassing the HS region were end labeled and used as substrates in gel mobility shift assays (13). Four different fragments around the *Acc*I site bp were used (Fig. 5A). The DP2A fragment (−5064 to failed to show any binding in this assay. The DP2B fragment (−4902 to −4796), just 5′ to the *Acc*I site, formed a single complex (indicated by an arrow). This shift was specific, since an excess of unlabeled DP2B competed efficiently for binding whereas another oligonucleotide, TTR (for transthyretin) did not. The TTR oligonucleotide contains a specific binding domain for the protein C/EBP, which interacts with the enhancers of liver-specific genes such as transthyretin, α_1-antitrypsin, and albumin (3). Interestingly, the DP3 fragment (−4796 to −4667), immediately 3′ to the *Acc*I site, formed a complex of identical mobility, along with additional slower-migrating DNA-protein complexes. This finding provided a first indication that the same protein factor binds to the two fragments. Cross-competition assays showed that this is indeed very likely the case. Binding of DP2B was very efficiently competed for by the DP3 fragment, whereas binding to the DP3 fragment was competed for only about 25% as efficiently by DP2B (Fig. 5A; compare the competition by DP2B and the lane without competitor). This result shows that the 3′ binding site is the stronger one.

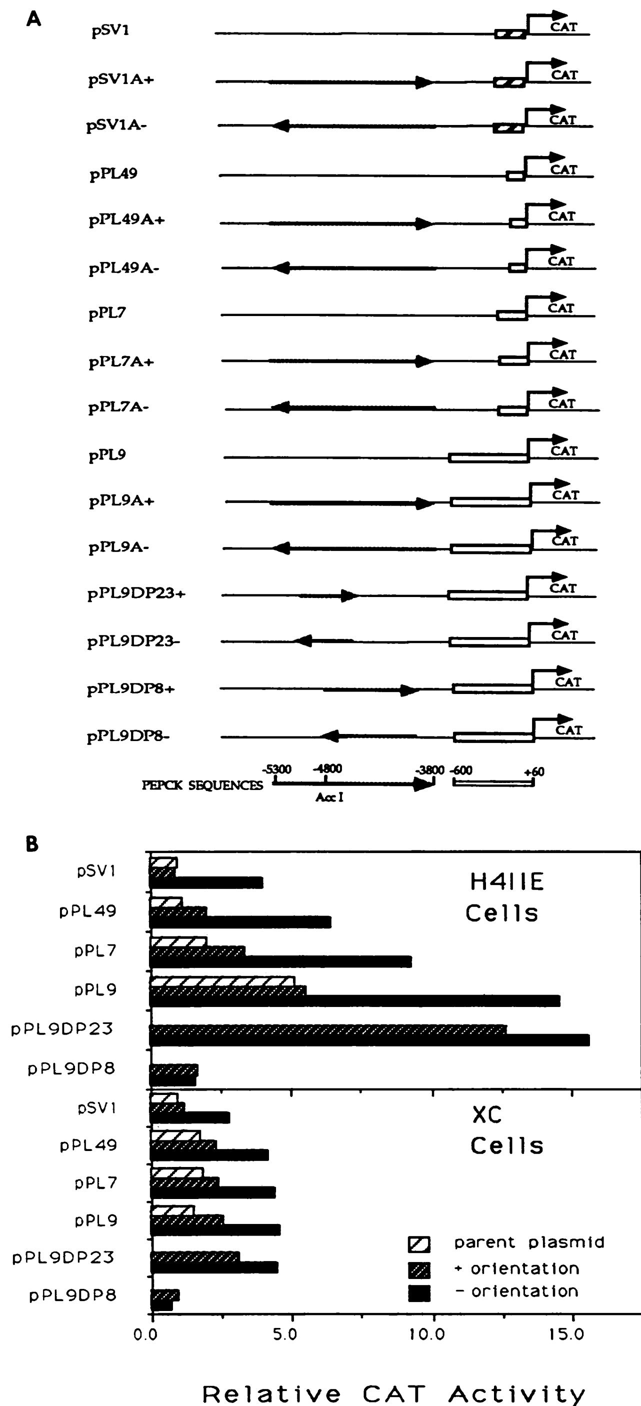

FIG. 4. Transcriptional analysis of the upstream enhancer. Molecular cloning of the various plasmids and details of the transfection assays are described in Materials and Methods. (A) Constructs used. Hatched boxes represent the simian virus 40 promoter; open boxes represent the PEPCK promoter sequences. The scale is indicated at the bottom. Solid arrows represent the upstream sequences used, and the scale is indicated. In all cases, the upstream sequences were inserted within 50 bp upstream from the 5′ end of the promoter fragment; for convenience of comparison among the upstream sequences, the DNA between upstream and promoter sequences is not drawn to scale. (B) Results of transient transfection in the H4IIE and XC cell lines. Orientations refer to the A fragment (or DP23 or DP8, as indicated) cloned in the parent vector. CAT activity was calculated as the percent conversion to acetylated compounds after thin-layer chromatography and liquid scintillation quantitation (the percent conversion of pSV1-CAT in our standard assay averaged 1.8 and 4.6 in H4IIE and XC cells, respectively). The relative CAT activity was then determined by normalizing values relative to that of plasmid pSV1-CAT in a given cell line. The results shown are the averages of at least four independent experiments done in duplicate on separate occasions, with the plasmids transfected in parallel for each cell line. The values varied within 20% among different experiments. On occasion, we have assayed by dot blot hybridization the amount of plasmid DNA in the nuclei of the transfected cells to ensure that overall transfection efficiency was comparable between experiments in a given cell line.

Longer exposure of the autoradiograph shown in Fig. 5A indicated the presence of a slower-migrating complex (not shown), possibly reflecting additional binding to the DP3 fragment. When the DP23 fragment, which includes the two binding sites (DP2B and DP3), was used in the binding assay, a major shift from the more rapidly migrating complex I to the slower complex II was seen as a function of extract concentration (Fig. 5B). The addition of increasing amounts of extract shifted all of the DP23 probe to the higher complex, whereas the DP2B probe remained as complex I under these conditions. A competition assay (DP23 binding versus DP3 binding) showed that all of the binding was competable with the same efficiency (data not shown; also see Fig. 6B).

Since the HS site detected around this region is tissue specific, we tested whether the binding activity is also tissue specific. Different tissues were used as sources for preparing nuclear extracts and subjected to mobility shift assays (Fig. 5C). Binding activity toward either DP2B or DP3 was totally absent in XC fibroblast and rat testis extracts. Recent experiments showed that rat liver nuclear extracts contain a binding activity indistinguishable from that of H4IIE nuclear extract except that the liver factor is usually obtained as a highly proteolyzed form (unpublished observation). Nuclear extracts obtained from rat kidney were also used to test for the presence of this binding factor. The major binding activity identified in previous experiments was not present in the kidney extract (data not shown).

DNase I footprinting identifies the binding sequence. DNase I footprinting was used to identify the specific binding site within the DP23 fragment. The subcloned *Hin*fI-to-*Hin*fI fragment (sites at −4902 to −4667 bp) was labeled at the upstream end relative to the binding site and footprinted with H4IIE nuclear extract (Fig. 6A). A strongly protected region was obtained immediately 3′ to the *Acc*I site. Although the mobility shift assays strongly suggested the presence of two independent but related binding sites on both sides of the *Acc*I site, it has not yet been possible to footprint the 5′ site by using a crude nuclear extract. In fact, detection of the 3′ but not the 5′ binding site in this assay is consistent with the observation from the mobility shift experiments that the factor binds to the DP3 fragment more strongly than to the DP2B fragment.

On the basis of these results, two 39-nucleotide-long complementary oligonucleotides were synthesized and annealed to regenerate the binding region (oligo-A). This synthetic fragment was end labeled and used in mobility shift assays. Oligo-A bound to material in the H4IIE nuclear extract to give a complex pattern, including a major shift (arrow in Fig. 6B). This binding activity was not competed for by the TTR oligonucleotide but was efficiently competed for by both oligo-A itself and the DP3 fragment. Since the double-stranded oligo-A has a 4-bp overhang, the possibility

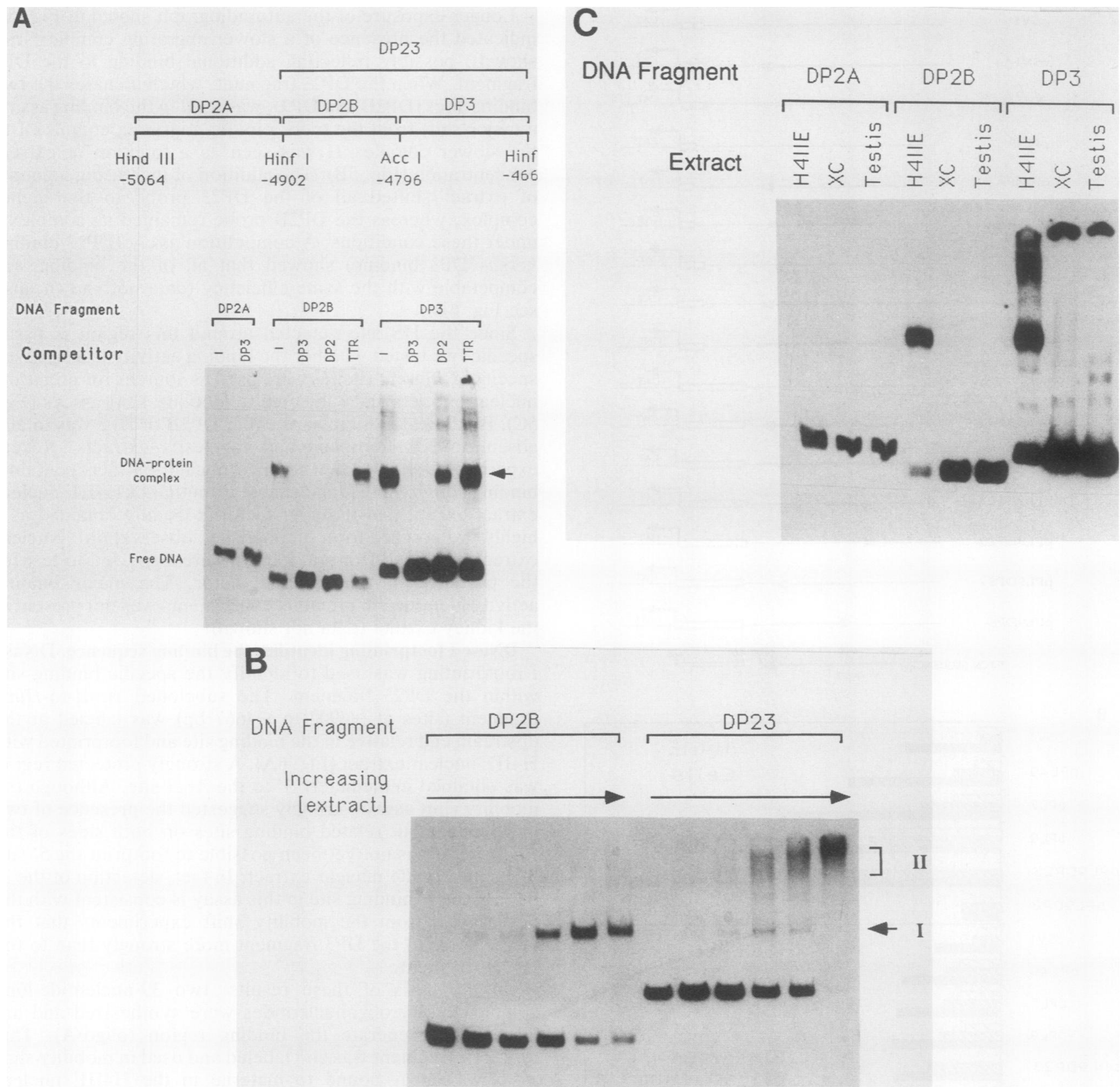

FIG. 5. DNA gel mobility shift assay. (A) Specific interaction of a nuclear factor with the upstream sequences. The DNA fragments used are illustrated at the top. Polyacrymide gel-purified end-labeled DNA fragments were incubated with H4IIE nuclear extract (1 μl; about 4 μg of total protein), and the mixture was resolved in a 6% polyacrylamide TBE gel. The autoradiograph of the dried gel is shown. The competitors used were in 150-fold molar excess of the labeled fragment. TTR is an oligonucleotide corresponding to the protein-binding site (oligomer 2) of the transthyretin gene enhancer (4). (B) Binding as a function of nuclear extract concentration. Increasing amounts of H4IIE nuclear extracts were added to the reaction mixture containing the indicated DNA fragments. The amounts of extract used were from 0.2 to 3 μl and were the same in both sets of experiments. (C) Tissue-specific binding to the DNA fragments. Approximately equal amounts (4 μg of total protein) of nuclear extracts from different tissues were incubated with the indicated end-labeled DNA fragments and analyzed as for panel A. The radioactivity appearing at the top of the gel in DP3 samples does not represent shifted bands but rather materials that stuck to the wells and did not migrate into the gel.

that a single-stranded-DNA-binding protein may give rise to the shift was also tested. The major shift could not be competed for by any single-stranded oligonucleotide (Fig. 6B). Nonetheless, one of the complexes detected in this assay (arrowhead in Fig. 6B) was the result of single-stranded DNA binding. With respect to binding of DP23 (Fig. 6B), the mobility shifts were specifically competable by both DP3 and oligo-A. It is therefore likely that this oligonucleotide does indeed contain the bulk of the binding domain, as previously indicated by the binding behavior of DP23.

The binding activity is heat stable. Initial characterization of the binding protein (Fig. 7) revealed that the factor is heat stable under certain conditions. Heating the H4IIE nuclear

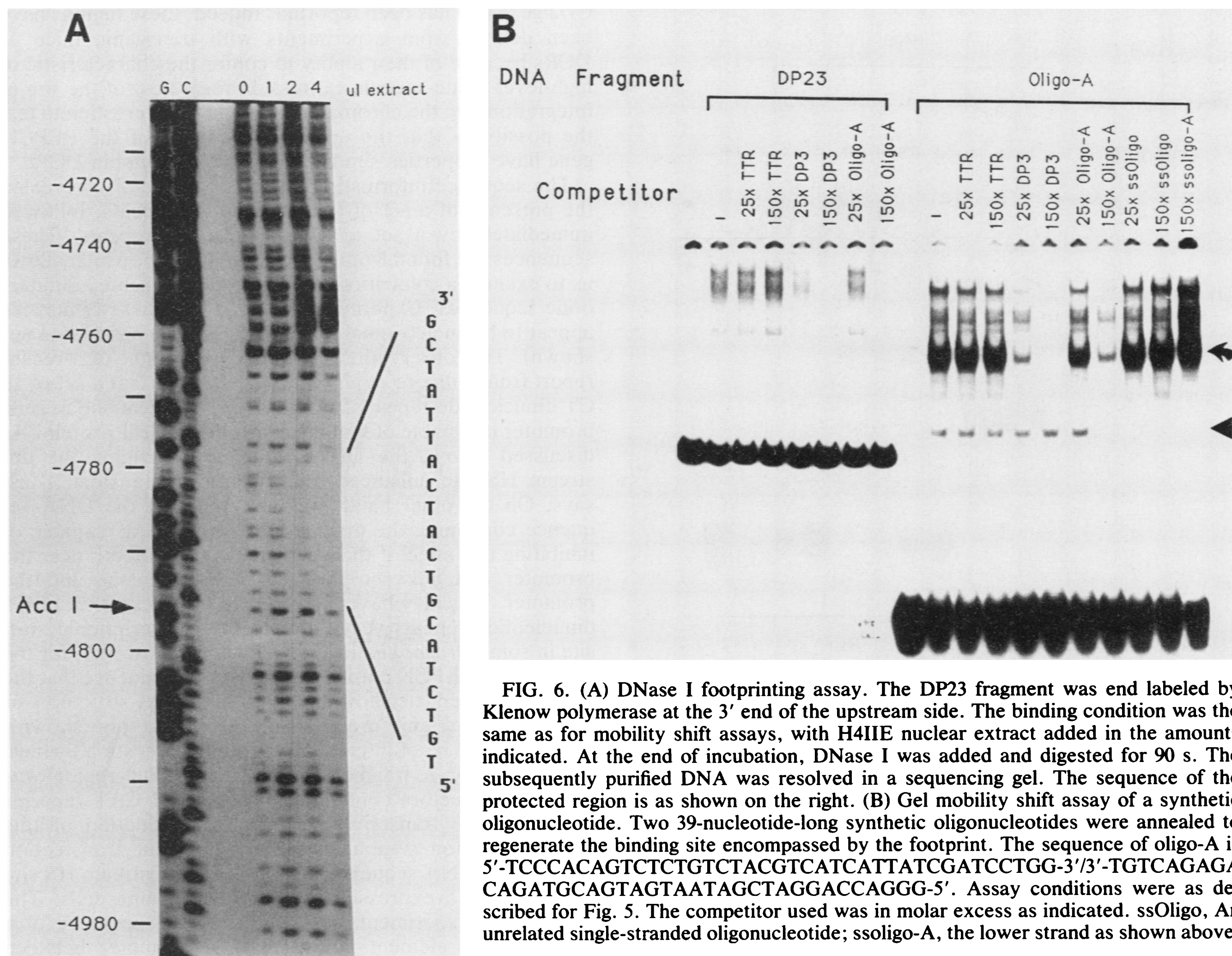

FIG. 6. (A) DNase I footprinting assay. The DP23 fragment was end labeled by Klenow polymerase at the 3' end of the upstream side. The binding condition was the same as for mobility shift assays, with H4IIE nuclear extract added in the amounts indicated. At the end of incubation, DNase I was added and digested for 90 s. The subsequently purified DNA was resolved in a sequencing gel. The sequence of the protected region is as shown on the right. (B) Gel mobility shift assay of a synthetic oligonucleotide. Two 39-nucleotide-long synthetic oligonucleotides were annealed to regenerate the binding site encompassed by the footprint. The sequence of oligo-A is 5'-TCCCACAGTCTCTGTCTACGTCATCATTATCGATCCTGG-3'/3'-TGTCAGAGA CAGATGCAGTAGTAATAGCTAGGACCAGGG-5'. Assay conditions were as described for Fig. 5. The competitor used was in molar excess as indicated. ssOligo, An unrelated single-stranded oligonucleotide; ssoligo-A, the lower strand as shown above.

extract to boiling precipitated 90% of the total protein, whereas the binding activity toward the DP2B fragment was recovered in the supernatant. Heating the extract under the same conditions at 65°C, however, destroyed the activity. This result may be explained by the possibility that some proteases which might be present in the crude extract are also stable at this temperature and that the incubation of the extract under such conditions (5 min of heating plus 10 min of centrifugation) caused extensive proteolysis.

Various lines of evidence suggest that there are at least two factors interacting with the upstream region. Using oligo-A, it was found that increasing the amount of added extract shifted the complex I to I' (Fig. 7), suggesting that there might be two binding sites for this factor. This view is in agreement with the previous results of DP2B and DP3 binding experiments. This pattern of binding activity was recovered after boiling. In contrast, after heating at 65°C, the characteristic binding to oligo-A disappeared and a second type of binding behavior remained (indicated as complex II). Complex II was less easily seen when complexes I and I' were the major shifts. Whether there is protein-protein interaction or the formation of complex I excludes that of complex II needs further investigation. More interestingly, the rat HTC cell extracts contained only the binding activity that gave rise to complex II. The absence of a major binding activity (complex I) in HTC cells within the enhancer domain might be responsible for the inability of the endogenous PEPCK gene to be transcribed in this cell line.

The enhancer-binding protein C/EBP is also heat stable and is also present in liver extracts (25). C/EBP binds to various enhancers and CCAAT sequences. The binding activity we have identified, involved in interaction with the upstream element of PEPCK gene, is, however, not C/EBP, as evidenced by a direct test that purified C/EBP (a generous gift from Steven McKnight) did not show any significant binding toward oligo-A, whereas positive controls showed that the sample of C/EBP was active and could bind to a cognate binding oligonucleotide (data not shown). In addition, the TTR oligonucleotide, which contains a C/EBP-binding site, did not compete to any extent in our experiments (Fig. 5 and 6).

The liver-specific factor interacts with an HNF3-binding site. To further investigate whether the major binding factor identified in this study is novel and specific to the PEPCK gene, an extensive competition assay was performed, using DNA fragments or synthetic oligonucleotides corresponding to binding sites for both liver-specific and ubiquitous factors. The liver-specific factor-binding sites used included HNF1, HNF3, and HNF4 (3, 12); ubiquitous factor-binding sites included the cyclic AMP response element (CRE) (44),

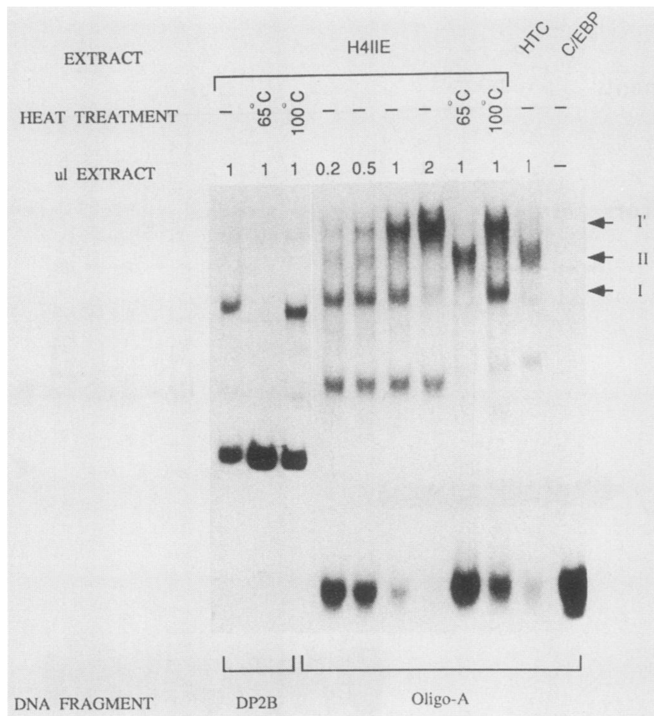

FIG. 7. Heat stability of the binding activity. A 20-μl sample of H4IIE nuclear extract was incubated in an Eppendorf microcentrifuge tube at the indicated temperature for 5 min and then spun for 10 min at room temperature. The supernatant was collected and used for the binding assay as described for previous figures. Lane C/EBP was a binding assay using a sample of C/EBP protein obtained from S. McKnight.

NLS1 (22), and nuclear factor 1 (28). We also used a DNA fragment containing one of the upstream enhancers of the mouse α-fetoprotein gene, MER-I. This enhancer element may contain three or more putative sites for protein binding (15). The liver-specific protein we have identified also bound to the oligonucleotide corresponding to the HNF3-binding site but not to any other elements tested (Fig. 8). The competition was as efficient as when the unlabeled oligo-A was used.

Close scrutiny of the PEPCK gene upstream binding sequences reveals a region highly homologous to the HNF3 consensus sequence (3) (Fig. 8B). There is an 8-of-11 match in the sequences, and the three mismatches are all pyrimidines.

DISCUSSION

The chromatin structure and function of a far upstream region of the rat PEPCK gene have been studied. Approximately 1.3 kb of DNA was sequenced around the general region that was previously identified as containing a tissue-specific HS site. Using this sequence information, an indirect end-labeling probe was employed to map more precisely the location of the upstream HS site. It was found to be composed of two closely situated sites at about −4875 and −4800 bp relative to the transcription initiation site. A novel HS site was also found at −6200 bp by using the same strategy. This latter region has not been cloned yet, and further analysis awaits the availability of the genomic clone. The presence of similar multiple HS regions around the coding region of the β-globin gene (19, 43) and T-cell-specific CD2 gene (17) has been reported. Indeed, these regions have been defined from experiments with transgenic mice as DCRs because of their ability to confer the characteristic of high-level tissue-specific expression regardless of the site of integration into the chromatin. It would be interesting to test the possibility that the upstream elements of the PEPCK gene have properties similar to those of the globin DCR.

The sequence information described above also revealed the presence of a set of CT dinucleotide repeats, followed immediately by a set of CA dinucleotide repeats. These sequences are found from −4600 to −4455 bp, which allows us to exclude a colocation of HS sites and repeated dinucleotide sequences. Experiments showed that these sequences appear to be nucleosomal in the PEPCK chromatin (data not shown). This observation is interesting in light of a recent report from Gilmour et al. (14), who showed that a set of 10 CT dinucleotide repeats from a *Drosophila* heat shock gene promoter is capable of binding a specific nuclear protein. As discussed below, the DNA sequence containing the upstream HS site enhanced transcription in transfection assays. On the other hand, we observed that the DNA sequence containing the dinucleotide repeats was capable of inhibiting this effect if this sequence is placed both near the promoter and between the activating sequence and the promoter. This may have relevance to the occurrence of CT dinucleotide repeats very close to the transcriptional start site in some *Drosophila* heat shock genes. However, for the endogenous PEPCK chromatin we have no evidence that the dinucleotide repeats show any abnormal DNA structures or that they bind any proteins in vitro (data not shown). Clearly, it will be of interest to pursue this question further.

A 1.5-kb DNA fragment around the −4800 region was cloned into vectors containing the bacterial CAT reporter gene, and the transcriptional regulatory function of this upstream region was analyzed by transient transfection assays. The DNA sequence containing the upstream HS site enhanced CAT expression in H4IIE hepatoma cells. The transfection experiments also indicated the presence of a tissue-specific element in the PEPCK promoter between −600 and −207 bp, in agreement with previous studies using transgenic mice (33) as well as transient transfection assays (2). This conclusion was deduced from the increased transcription of pPL9-CAT (which contains the full promoter) in H4IIE cells as opposed to XC cells, relative to the transcriptional level seen in the minimal promoter (pPL49-CAT) in that cell line. Thus, use of the upstream element in conjunction with the complete PEPCK gene promoter generated a 15- to 20-fold overall enhancement of activity relative to the minimal PEPCK gene promoter. Therefore, the in vivo function of the upstream element may involve possible collaboration with the proximal element to control high-level, tissue-specific expression of PEPCK gene.

By using transient transfection assays, the enhancer effect of the upstream region is also seen to a significant degree in XC cells. It is probable that in fibroblasts the transiently transfected DNA template is able to bind activating proteins (other than the one we have identified in H4IIE cells), which are not normally able to interact with the endogenous, genomic PEPCK sequences. Certainly, DNA binding assays show that this region is capable of interacting with more than one factor, and some of them may be ubiquitous (Fig. 7). Nonetheless, we have not been able to detect any binding activity in vitro toward this upstream region by using XC extract. Furthermore, there is a strong indication that no protein is bound to these regions of the endogenous PEPCK gene in XC cells, as evidenced by a total lack of any HS over

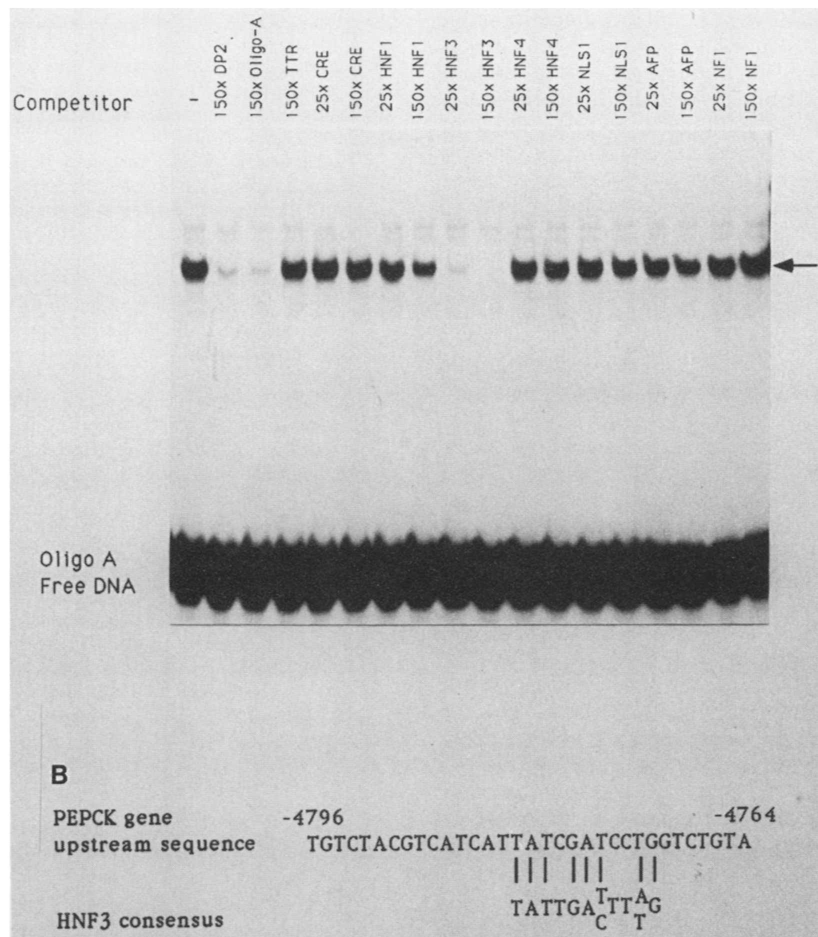

FIG. 8. Interaction of the major binding factor with the HNF3-binding site. (A) Gel mobility shift assay done as in previous experiments except that only 0.3 μl of nuclear extract was used in order to show clearly the binding of complex I (complexes I′ and II are not as pronounced). The competitor DNA as indicated was included in molar excess of the labeled oligo-A DNA. CRE is an oligonucleotide containing the binding site for the CREB; HNF1 oligonucleotide is from −103 to −74 of the α-fibrinogen gene promoter; HNF3 and HNF4 are from −115 to −85 and −151 to −130, respectively, of the transthyretin gene promoter; NLS1 is from −10015 to −9900 of the mouse albumin gene far upstream enhancer; AFP is the α-fetoprotein gene enhancer containing 300 bp of DNA at −2500 (MER-I); NF1 contains the binding site for nuclear factor 1. (B) Relevant structures.

the PEPCK gene region in these cells (24). It seems likely that this finding reflects a limitation in the transient transfection approach and argues that these studies should be extended by using stable transfection assays and transgenic mice.

The presence of transcriptional activity (albeit at a modest level) of transfected promoters in a tissue in which the endogenous gene is not expressed (i.e., in XC cells) raises the interesting question of why the transcription factors are able to bind to the exogenous promoter as introduced by transfection, yet the endogenous promoter is evidently unable to bind any of these factors. Possible explanations are that the endogenous promoter is methylated and that this specifically inhibits binding of any protein factors; alternatively, the chromatin environment may be different, though how this could exclude factor binding during replication is not at all clear. We have previously compared the methylation levels of the PEPCK gene promoter in a range of cell types, including H4IIE and XC cells (24). This analysis was limited to CCGG sequences which are differentially sensitive to *Msp*I and *Hpa*II, depending on the state of methylation. Only very minor differences were found between the PEPCK gene promoter in the two cell lines used for the experiments described in this report. We are currently assaying all other CpG dinucleotide pairs in the promoter by genomic sequencing.

The coincidence of a nuclease HS site and an attendant biological activity is a strong indication of the presence of non-histone protein binding to a functional genetic element. The nuclease cleavage pattern around the −4800-bp region shown in Fig. 3A revealed two sites of sensitivity separated by a 75-bp region which was strongly protected. Such an extended zone suggested that this DNA may well be a binding domain for more than one protein. DNA mobility shift assays showed that in H4IIE cells, at least two different nuclear proteins (or different forms of the same protein) bind to this region. Multiple binding of one of these proteins is also evident from the cross-competition assay of the two DNA fragments around the *Acc*I site. The DNA fragment DP23, containing both binding sites, showed a complex shift pattern of much slower mobility rather than a discrete second complex, indicative of the binding of multiple proteins within this domain. Whether a single protein is binding many times or whether two or more proteins are involved is

still under investigation. However, since competition by different DNA fragments reduces all complexes equally, it will not be surprising to find that additional protein-protein interactions lead to the complex retardation pattern described for DP23. Since many enhancers possess reiterated binding sequences within the functional domain, and since in vitro experiments also indicate that the presence of more than one copy of a binding site is required for full activity of a transcriptional enhancer (23, 28), the presence of the multiple binding sites in the PEPCK gene upstream enhancer may be of functional importance. In fact, the presence of both *Acc*I 5′ and 3′ binding domains is important for biological function in our transfection assay system. Thus, the DP2 fragment, which contains only the 5′ binding site, did not show much enhancer activity (data not shown). Likewise, the DP8 fragment, which contains the 3′ binding site, showed no enhancer activity and in fact repressed transcriptional activity (compare parent plasmid pPL9 and pPL9DP8 in Fig. 4). The best enhancer activity was seen when the DP23 fragment, which contains all of the contiguous binding sites, was used. Furthermore, incorporation of three or six copies of oligo-A into the expression vectors shows an activity very similar to that of DP23 fragment, whereas a single copy of oligo-A is less effective in transfection experiment (unpublished observation).

Initial biochemical characterization of the binding activities showed that the protein factors are heat stable. At least two proteins are able to interact with the upstream element, and they are of somewhat different heat stability (under the heating conditions used). The details of different protein bindings to the upstream enhancer domain and their functional significance must await the purification of the factors for unambiguous proof. The binding domain and associated footprint within the DP23 fragment (Fig. 6A) show an 8-of-10 homology with the CRE (44). However, the CRE palindrome is interrupted, and this region did not respond to cyclic AMP during transfection assays (data not shown), nor did an oligonucleotide containing the CRE compete for the binding to oligo-A (Fig. 8). On the other hand, the binding activity was competed for efficiently by an oligonucleotide containing the binding site for the previously identified liver-specific factor HNF3, which binds to transthyretin, α_1-antitrypsin, and albumin gene enhancers (3). Thus, the liver-specific factor reported here may be similar or identical to HNF3. Further biochemical characterization and molecular cloning are required for definite proof. It would be interesting if the same factor or a family of such factors is responsible for controlling the tissue-specific expression of such a variety of genes which are developmentally and hormonally regulated in different manners.

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LITERATURE CITED

1. **Beale, E. G., N. B. Chrapkiewicz, H. A. Scoble, R. J. Metz, D. P. Quick, R. L. Noble, J. E. Donelson, K. Biemann, and D. K. Granner.** 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase: structure of the protein, messenger RNA and gene. J. Biol. Chem. **260**:10748–10760.
2. **Benvenisty, N., H. Nechushtan, H. Cohen, and L. Reshef.** 1989. Separate *cis*-regulatory elements confer expression of phosphoenolpyruvate carboxykinase(GTP)gene in different cell lines. Proc. Natl. Acad. Sci. USA **86**:1118–1122.
3. **Costa, R. H., D. Grayson, and J. E. Darnell.** 1989. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α1-antitrypsin genes. Mol. Cell. Biol. **9**: 1415–1425.
4. **Costa, R. H., D. R. Grayson, K. G. Xanthopoulos, and J. E. Darnell.** 1988. A liver-specific DNA-binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, α_1-antitrypsin, albumin and SV40 genes. Proc. Natl. Acad. Sci. USA **85**:3840–3844.
5. **Costa, R. H., E. Lai, and J. E. Darnell.** 1986. Transcriptional control of the mouse prealbumin (transthyretin) gene: both promoter and a distinct enhancer are cell specific. Mol. Cell. Biol. **6**:4697–4708.
6. **Cullen, B. R., K. Raymond, and G. Ju.** 1985. Transcriptional activity of avian retroviral long terminal repeats directly correlates with enhancer activity. J. Virol. **53**:515–521.
7. **Eissenberg, J. C., I. L. Cartwright, G. H. Thomas, and S. C. R. Elgin.** 1985. Selected topics in chromatin structure. Annu. Rev. Genet. **19**:485–536.
8. **Elgin, S. C. R.** 1988. The formation and function of DNase I hypersensitive sites in the process of gene activation. J. Biol. Chem. **263**:19259–19262.
9. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science **227**:134–140.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
11. **Fletcher, C., N. Heintz, and R. G. Roeder.** 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell **51**:773–781.
12. **Frain, M., G. Swart, P. Monaci, A. Nicosia, S. Stampfli, R. Frank, and R. Cortese.** 1989. The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell **59**:145–157.
13. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6507–6525.
14. **Gilmour, D. S., G. H. Graham, and S. C. R. Elgin.** 1989. *Drosophila* nuclear proteins bind to regions of alternating C and T residues in gene promoters. Science **245**:1487–1490.
15. **Godbout, R., R. Ingram, and S. M. Tilghman.** 1988. Fine-structure mapping of the three mouse α-fetoprotein gene enhancers. Mol. Cell. Biol. **8**:1169–1178.
16. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
17. **Greaves, D. R., F. D. Wilson, G. Lang, and D. Kioussis.** 1989. Human CD2 3′-flanking sequences confer high-level, Tcell-specific, position-independent gene expression in transgenic mice. Cell **56**:979–986.
18. **Gross, D. S., and W. T. Garrard.** 1988. Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. **57**:159–197.
19. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.
20. **Hammer, R. E., R. Krumlauf, S. A. Camper, R. L. Brinster, and S. M. Tilghman.** 1987. Diversity of α-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. Science **235**:53–58.
21. **Hanvey, J. C., M. Schimizu, and R. D. Well.** 1988. Intramolecular DNA triplexes in supercoiled plasmids. Proc. Natl. Acad. Sci. USA **85**:6292–6296.
22. **Herbst, R. S., N. Friedman, J. E. Darnell, and L. E. Babiss.** 1989. Positive and negative regulatory elements in the mouse albumin enhancer. Proc. Natl. Acad Sci. USA **86**:1553–1557.
23. **Herr, W., and Y. Gluzman.** 1985. Duplication of a mutated SV40 enhancer restore its activity. Nature (London) **313**:711–714.
24. **Ip, Y. T., D. K. Granner, and R. Chalkley.** 1989. Hormonal regulation of phosphenolpyruvate carboxykinase gene expression is mediated through modulation of an already disrupted

chromatin structure. Mol. Cell. Biol. **9**:1289–1297.

25. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
26. **Jones, N. C., P. W. Rigby, and E. B. Ziff.** 1988. *trans*-Acting factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
27. **Kadonaga, J. T., K. R. Carner, S. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
28. **Karnitz, L. M., D. Poon, P. A. Weil, and R. Chalkley.** 1989. Purification and properties of the Rous sarcoma virus internal enhancer binding factor. Mol. Cell. Biol. **9**:1929–1939.
29. **Magnuson, M. A., P. G. Quinn, and D. K. Granner.** 1987. Multihormonal regulation of phosphoenolpyruvate carboxykinase-chloramphenicol acetyltransferase fusion gene: insulin's effects oppose those of cAMP and dexamethasone. J. Biol. Chem. **262**:14917–14920.
30. **Maniatis, T., E. F. Fritsch, J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
32. **McGhee, J. D., and G. Felsenfeld.** 1980. Nucleosome structure. Annu. Rev. Biochem. **49**:1115–1156.
33. **McGrane, M. M., J. deVente, J. Yun, J. Bloom, E. Park, A. Wynshaw-Boris, F. M. Rottman, and R. W. Hanson.** 1988. Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic mice. J. Biol. Chem. **263**:11443–11451.
34. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46**:795–805.
35. **Noedheim, A., and A. Rich.** 1983. The sequence $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ forms left-handed Z-DNA in negatively supercoiled plasmids. Proc. Natl. Acad. Sci. USA **80**:1821–1825.
36. **Ott, M.-O., L. Sperling, P. Herbomel, M. Yaniv, and M. C. Weiss.** 1984. Tissue-specific expression is conferred by a sequence from the 5′ end of the rat albumin gene. EMBO J. **3**:2505–2510.
37. **Pederson, D. S., F. Thoma, and R. T. Simpson.** 1986. Core particles, fiber and transcriptionally active chromatin structure. Annu. Rev. Cell Biol. **2**:117–147.
38. **Petersen, D. D., M. A. Magnuson, and D. K. Granner.** 1988. Location and characterization of two widely separated glucocorticoid response elements in the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. **8**:96–104.
39. **Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter.** 1987. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev. **1**:268–276.
40. **Prowse, K., and H. Baumann.** 1988. Hepatocyte-stimulating factor, β_2 interferon, and interleukin-1 enhance expression of the rat α_1-acid glycoprotein gene via a distal upstream regulatory region. Mol. Cell. Biol. **8**:42–51.
41. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
42. **Reed, K. C., and D. E. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**: 7207–7221.
43. **Ryan, T. M., R. R. Behringer, N. C. Martin, T. M. Townes, R. D. Palmiter, and R. L. Brinster.** 1989. A single erythroid-specific DNase I super-hypersensitive site activates high levels of human β-globin gene expression in transgenic mice. Gene Dev. **3**:314–323.
44. **Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson.** 1987. Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-bp element. Proc. Natl. Acad. Sci. USA **84**:2198–2202.
45. **Swadaishi, K., T. Morinaga, and T. Tamaoki.** 1988. Interaction of a hepatoma-specific nuclear factor with transcription-regulatory sequences of the human α-fetoprotein and albumin genes. Mol. Cell. Biol. **8**:5179–5187.
46. **Theisen, M., A. Stief, and M. Sippel.** 1986. The lysozyme enhancer: cell specific activation of the chicken lysozyme gene by a far upstream DNA element. EMBO J. **5**:719–724.
47. **Well, R. D., D. A. Collier, J. C. Hanvey, M. Shimizu, and F. Wuhlrab.** 1988. The chemistry and biology of unusual DNA structure adopted by oligopurine · oligopyrimidine sequences. FASEB J. **2**:2939–2949.