MOLECULAR AND CELLULAR BIOLOGY, July 1992, p. 3023–3031
0270-7306/92/073023-09$02.00/0

Vol. 12, No. 7

Temporal Expression of the Human Alcohol Dehydrogenase Gene Family during Liver Development Correlates with Differential Promoter Activation by Hepatocyte Nuclear Factor 1, CCAAT/Enhancer-Binding Protein α, Liver Activator Protein, and D-Element-Binding Protein

CHRISTIAAN VAN OOIJ,[1] RICHARD C. SNYDER,[1] BRYAN W. PAEPER,[1]† AND GREGG DUESTER[2]*

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523,[1] and La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, California 92037[2]

Received 4 February 1992/Accepted 12 April 1992

The human class I alcohol dehydrogenase (ADH) gene family consists of *ADH1*, *ADH2*, and *ADH3*, which are sequentially activated in early fetal, late fetal, and postnatal liver, respectively. Analysis of ADH promoters revealed differential activation by several factors previously shown to control liver transcription. In cotransfection assays, the *ADH1* promoter, but not the *ADH2* or *ADH3* promoter, was shown to respond to hepatocyte nuclear factor 1 (HNF-1), which has previously been shown to regulate transcription in early liver development. The *ADH2* promoter, but not the *ADH1* or *ADH3* promoter, was shown to respond to CCAAT/enhancer-binding protein α (C/EBPα), a transcription factor particularly active during late fetal liver and early postnatal liver development. The *ADH1*, *ADH2*, and *ADH3* promoters all responded to the liver transcription factors liver activator protein (LAP) and D-element-binding protein (DBP), which are most active in postnatal liver. For all three promoters, the activation by LAP or DBP was higher than that seen by HNF-1 or C/EBPα, and a significant synergism between C/EBPα and LAP was noticed for the *ADH2* and *ADH3* promoters when both factors were simultaneously cotransfected. A hierarchy of ADH promoter responsiveness to C/EBPα and LAP homo- and heterodimers is suggested. In all three ADH genes, LAP bound to the same four sites previously reported for C/EBPα (i.e., −160, −120, −40, and −20 bp), but DBP bound strongly only to the site located at −40 bp relative to the transcriptional start. Mutational analysis of *ADH2* indicated that the −40 bp element accounts for most of the promoter regulation by the bZIP factors analyzed. These studies suggest that HNF-1 and C/EBPα help establish ADH gene family transcription in fetal liver and that LAP and DBP help maintain high-level ADH gene family transcription in postnatal liver.

Human class I alcohol dehydrogenase (ADH) is encoded by three genes (*ADH1*, *ADH2*, and *ADH3*) which display differential patterns of tissue-specific and developmental gene expression (12, 39). In early human fetal liver development (second trimester), *ADH1* is expressed, with the additional expression of *ADH2* during late fetal liver development (third trimester) and the additional expression of *ADH3* during postnatal liver development at about 5 months after birth (39). ADH catalyzes the oxidation of a wide variety of xenobiotic or endogenous alcohols (12, 15). In addition to the rate-limiting step in ethanol metabolism (27), human class I ADH catalyzes vitamin A (retinol) oxidation (30), which is the rate-limiting step in the conversion of retinol to retinoic acid, a hormone known to control gene expression at the transcriptional level (19, 32, 45). Retinoic acid has been implicated as a control molecule for differentiation of epithelial tissues (37, 40), and mammalian class I ADH gene expression in the adult is restricted to epithelial cells, with highest levels in liver epithelia (39). Thus, it is suspected that a major physiological function of ADH in epithelial cells is to catalyze retinoic acid synthesis needed for epithelial cell differentiation (12), with the large amount of ADH in the liver relating to the fact that this organ is also the major site of retinol storage and metabolic turnover (excretion) via conversion to retinoic acid and further oxidized forms (3, 18). Analysis of the regulation of mammalian ADH gene expression is beginning to clarify the importance of ADH in liver development and retinoic acid-regulated processes.

The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) helps establish high-level transcription in the developing liver (2). C/EBPα is a member of a family of proteins termed bZIP proteins because of the presence of a basic DNA-binding region followed by a leucine zipper which allows dimerization (26). We have shown that human *ADH2* expression, but not *ADH1* and *ADH3* expression, is controlled by C/EBPα homodimers (42, 43). In comparison with liver cells, hepatoma cells produce very low levels of C/EBPα (17), and this correlates with the low level of ADH seen in hepatoma cells (16, 39). In addition, we have determined that *ADH3* transcription, but not *ADH1* or *ADH2* transcription, is regulated by retinoic acid (14). We have hypothesized that this constitutes a positive feedback loop for establishing retinoic acid synthesis, since ADH catalyzes the rate-limiting step in retinoic acid synthesis (13, 14). We also note that low ADH levels in hepatoma cells would further predispose these cells to remain dedifferentiated as the result of a reduction in the synthesis of retinoic acid needed for liver differentiation.

We have now extended our studies of liver-specific regulation of ADH gene expression by analyzing other factors which play a role in the differential developmental expres-

* Corresponding author.
† Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

sion of the human *ADH1*, *ADH2*, and *ADH3* genes. We have determined that three additional liver transcription factors regulate ADH gene family transcription: (i) liver activator protein (LAP) (10), also known as NF-IL6 (1), IL-6DBP (33), AGP/EBP (7), or C/EBPβ (5); (ii) D-element-binding protein (DBP) (31); and (iii) hepatocyte nuclear factor 1 (HNF-1) (29). C/EBPα and LAP are members of a bZIP protein subfamily since they share 71% homology in their basic DNA-binding region and leucine zipper region, and they have been shown to form heterodimers (5, 9). DBP has a degenerate leucine zipper, and a basic DNA-binding region similar to that of C/EBPα and LAP, but falls into a separate class of bZIP proteins since it does not heterodimerize with C/EBPα or LAP (31). HNF-1 contains a homeodomain DNA-binding region and plays a major role in fetal liver gene expression (29). C/EBPα, LAP, DBP, and HNF-1 have previously been shown to play a role in transcription of the albumin gene, which is highly active only in the liver. The bZIP proteins C/EBPα, LAP, and DBP bind a similar sequence in the albumin promoter near position −100 bp relative to the transcription start site (9, 31), whereas HNF-1 binds a distinct sequence near −50 bp (8). Albumin and ADH have similar patterns of gene expression in the liver, both being turned on at a low level in late fetal development and induced tremendously after birth. We now show in transient transfection assays that C/EBPα, LAP, DBP, and HNF-1 differentially regulate *ADH1*, *ADH2*, and *ADH3* transcription, and we propose that this accounts for the sequential turn-on of these three human ADH genes during liver development.

MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis. Fusion of various lengths of the 5′-flanking sequences of the human *ADH1*, *ADH2*, and *ADH3* genes to the chloramphenicol acetyltransferase (*cat*) gene has been described elsewhere (41, 43). A human albumin-*cat* construction containing albumin DNA from −3100 bp to −17 bp was provided by D. Wilson (Baylor College of Medicine). In all constructions, the AUG translation start codon of *cat* is the first such codon to appear downstream of the transcription start point.

Most of the mutant ADH promoters used in this study have been described previously (43). Two additional oligonucleotide site-directed mutations were constructed, one in the *ADH2* TATA box and one in the *ADH3* TATA box, by the method of Kunkel et al. (23). Because of complete sequence conservation in their TATA boxes, both *ADH2* and *ADH3* have the wild-type sequence 5′-CAAAATAAA TA-3′ between −32 and −22 bp, and this was changed to 5′-GGTACCGCTCG-3′ in both mutants. The presence of the appropriate mutation was confirmed by the presence of a new *Kpn*I restriction site and by chemical DNA sequence analysis (28).

All plasmids used for transfection and DNA-binding studies were purified by preparation of an alkaline-cleared lysate (38) followed by chromatography on a Qiagen column, using a protocol specified by the manufacturer.

Cell culture and transfection analysis. Transfection assays were used to analyze the regulation of wild-type and mutant *ADH-cat* fusions by cotransfected mammalian cDNA expression vectors encoding various liver transcription factors. C/EBPα was expressed from plasmid pMSV-C/EBP (17), LAP was expressed from pSCT-LAP (9), DBP was expressed from pCMV-DBP (31), and HNF-1 was expressed from pRSV-B1 (44). The vector pSCT-LAP used for LAP production does not produce the shorter translation product termed LIP which was recently shown to inhibit LAP function (10). Transfections and chloramphenicol acetyltransferase (CAT) assays were performed by a modification of previously described methods (20, 21). HepG2 cells (22) were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Cells were seeded in 100-mm tissue culture dishes and the following day were changed to fresh medium. Transfection by the calcium phosphate coprecipitate method was performed with a mixture of DNA including 15 μg of a reporter *cat* plasmid, 20 μg of carrier herring sperm DNA, and 5 μg of an internal control plasmid, pCH110, which contains a simian virus 40 early promoter-*lacZ* fusion used to correct for plate-to-plate differences in transfection efficiency (21). For cotransfections with expression vectors for C/EBPα, LAP, DBP, and HNF-1, various amounts of these plasmids (from 10 to 20 μg) replaced an equal amount of carrier DNA to keep the total amount of DNA constant at 40 μg. The DNA precipitate (40-μg total) was added to the cells for 16 h, after which time the cells were washed and supplemented with fresh medium. Cells were harvested 48 h after DNA addition, and cell extracts were prepared. Extracts were assayed for β-galactosidase activity, and then appropriate amounts of extract normalized for β-galactosidase activity were used in the CAT assay (21). CAT assay reaction mixtures were incubated for 2 h at 37°C, and the reaction products were analyzed by thin-layer chromatography. The amount of CAT activity (percent acetylation of chloramphenicol) was quantitated by cutting out substrate and product spots from the thin-layer chromatograms and counting the radioactivity by liquid scintillation. Reported promoter induction values for the various cotransfected transcription factors were estimated as the mean of the ratio between cotransfected and control CAT activity from three or more experiments. Standard deviations were in the range of ± 10 to 25% of the reported values.

DNA footprint analysis using bacterially expressed transcription factors. Bacterial expression vectors encoding C/EBPα (26), LAP (9), and DBP (31) were used to prepare samples of these proteins for DNA footprinting studies. *Escherichia coli* extracts containing C/EBPα and LAP were prepared as previously described, including the DEAE-cellulose chromatographic step (26). *E. coli* extracts containing DBP were prepared as described previously (31) and then passed over a DEAE-cellulose column under the conditions described for bacterial C/EBPα (26) with the exception that urea was omitted.

DNA templates for footprint analysis were 5′ end labeled by using the polymerase chain reaction (PCR) as follows. Plasmids containing *ADH1*, *ADH2*, and *ADH3* promoters fused to *cat*, i.e., *ADH1-cat*(−228), *ADH2-cat*(−272), and *ADH3-cat*(−328) described previously (43), were used as templates for PCR. A 23-mer primer (5′-AGCTCCTG AAAATCTCGCCAAGC-3′) derived from the noncoding strand of the 5′ end of the *cat* gene was 5′ end labeled with ^{32}P as described previously (38) and then used as the downstream primer for PCR of all three promoters. For *ADH1-cat*(−228), the upstream PCR primer was a 23-mer (5′-CTCTGATGCCGCATAGTTAAGCC-3′) derived from a region of the vector pSVOCAT (20) just upstream of the coding strand of the *ADH1* promoter. For *ADH2-cat*(−272) and *ADH3-cat*(−328), the upstream PCR primer was a 23-mer (5′-TTAGGAAGCAGCCCAGTAGTAGG-3′) derived from a region of the vector $pA_{10}CAT_2$ (25) just upstream of the coding strand of the *ADH2* or *ADH3* promoter. PCR was performed in a 100-μl reaction mixture containing

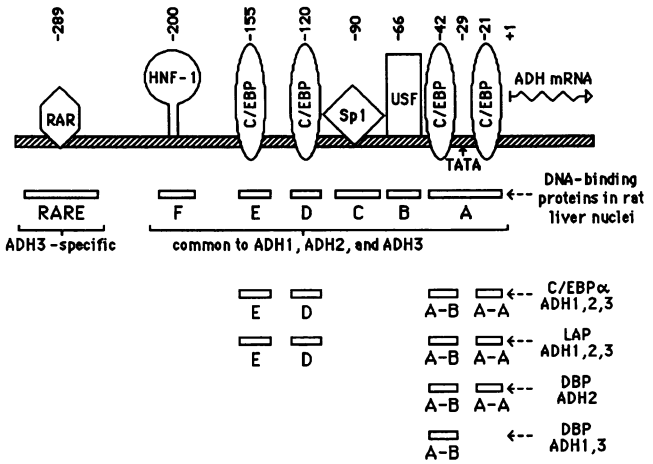


FIG. 1. Summary of DNA-binding sites in the human class I ADH promoters. The hatched bar indicates a composite of the promoter-proximal regions of the human *ADH1*, *ADH2*, and *ADH3* genes. Six regions labeled A to F common to all three genes are DNA-binding sites for rat liver nuclear proteins, whereas the site labeled RARE (retinoic acid response element) is present only in the *ADH3* promoter. The numbers above indicate the sites where binding of proteins present in a rat liver nuclear extract occurs, and the particular factors identified are shown graphically bound to the hatched bar. Binding of the bZIP proteins C/EBPα, LAP, and DBP is somewhat complex and the particular binding sites identified for *ADH1*, *ADH2*, and *ADH3* by DNA footprint analysis are indicated below. RAR, retinoic acid receptor.

0.2 μM ^{32}P-labeled downstream *cat* primer, 0.2 μM upstream primer, 0.2 ng of *cat* plasmid, 50 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, and 1 U of Hot Tub DNA polymerase (Amersham). The reaction was overlayed with 100 μl of mineral oil and then subjected to 35 cycles in a COY temperature cycler with the following sequence of settings: (i) 96°C, 20 s, 0.1 ramp rate; (ii) 94°C, 1.5 min, 0.01 ramp rate; (iii) 62.5°C, 20 s, 0.1 ramp rate, (iv) 65°C, 2 min, 0.01 ramp rate; (v) 74°C, 20 s, 0.1 ramp rate; (vi) 72°C, 2 min, 0.01 ramp rate. This procedure was followed by a final cycle at 4°C to hold the sample until further use. The sample was extracted once with chloroform to remove mineral oil and then subjected to native polyacrylamide gel electrophoresis to purify the 5'-labeled amplified DNA band (38).

The conditions for DNA footprinting were the same as those described in our previous study using bacterially expressed C/EBPα (43). Each footprint reaction mixture contained approximately 50,000 cpm of 5'-labeled template DNA and 30 μg of total protein extract from bacterially expressed C/EBPα, LAP, or DBP.

RESULTS

C/EBPα-, LAP-, and DBP-binding sites in human *ADH1*, *ADH2*, and *ADH3* promoters. The promoter-proximal regions of the human *ADH1*, *ADH2*, and *ADH3* promoters were previously analyzed by DNA footprint analysis using rat liver nuclear proteins, and several areas of protection common to all three genes termed sites A to F (Fig. 1) were described (43). Sites A, D, and E have been shown to bind C/EBPα expressed in *E. coli*, with site A containing two C/EBPα-binding sites flanking each side of the TATA box (42, 43). The rat class I ADH gene has also been shown to bind C/EBPα, but only in a region just downstream of the TATA box which is homologous to the downstream portion of human site A (35). Sites B, C, and F have been implicated in binding the transcription factors USF (6, 34), Sp1 (6), and HNF-1 (4), respectively. An *ADH3*-specific DNA-binding site located near −300 bp has been identified as a retinoic acid response element and binds the retinoic acid receptor β (14).

Recently, additional bZIP proteins with DNA-binding domains homologous to C/EBPα have been discovered (5, 9, 31, 46), and it is now clear that the regulation of liver gene expression by C/EBPα cannot be fully understood unless these other bZIP proteins are taken into account. One such bZIP protein, called LAP, has previously been shown to bind the rat albumin promoter at four sites in a manner indistinguishable from that of C/EBPα and was demonstrated to form heterodimers with C/EBPα (9). Another bZIP protein, called DBP, does not form heterodimers with either C/EBPα or LAP but was shown to bind to the rat albumin promoter specifically to only one of the four sites (near −105 bp) bound by either C/EBPα or LAP (31). All three bZIP proteins are suspected to play a role in albumin transcription, but their relative importance has not been reported. It has been proposed that there may exist liver-specific promoters designed to differentially respond to either DBP or to homo- or heterodimers of C/EBPα and LAP (9). We have decided to test this hypothesis by using the human class I ADH multigene family members as test promoters, since they have been known for some time to be differentially expressed during liver development (39).

We used LAP expressed in *E. coli* to perform DNA footprint analysis on the *ADH1*, *ADH2*, and *ADH3* promoters (data not shown) and found that it bound to sites A, D, and E in a manner indistinguishable from what we had previously observed for C/EBPα (43). We also used DBP expressed in *E. coli* to perform DNA footprint analysis on ADH promoters and found that it bound only to site A of each promoter, protecting regions which closely, but not perfectly, correspond to the C/EBPα or LAP footprints (Fig. 2). Interestingly, DBP bound to sites flanking both sides of the TATA box for *ADH2* but only to the upstream site for *ADH1* and *ADH3* (Fig. 2). Thus, much like the case for the rat albumin promoter, the human ADH promoters show more selective binding for DBP than for either C/EBPα or LAP (summarized in Fig. 1).

Response of class I ADH promoters to liver transcription factors. To help determine the relative importance of C/EBPα, LAP, and DBP on ADH liver transcription, as well as the importance of the liver-enriched transcription factor HNF-1, which has been implicated in binding site F in the human ADH promoters (Fig. 1), we turned to the use of transient transfection of HepG2 human hepatocellular carcinoma cells. Plasmids containing *ADH1-cat*, *ADH2-cat*, *ADH3-cat*, and albumin-*cat* were transfected into HepG2 cells either alone or with expression plasmids for C/EBPα, LAP, DBP, or HNF-1 (Fig. 3). Differential induction by the various factors was observed. As noted previously (42, 43), only *ADH2* and albumin responded to C/EBPα. All three ADH promoters as well as albumin responded quite well to DBP, and all except *ADH3* responded well to LAP. Only *ADH1* and albumin responded to HNF-1. Since C/EBPα and LAP produced by simultaneous cotransfection in tissue culture cells have previously been shown by others to form heterodimers intracellularly (46), we mixed C/EBPα and LAP expression vectors (1:1) and tested the effect of this combination on *ADH-cat* and albumin-*cat* expression. We observed a very significant synergistic effect for the *ADH3* promoter, with a smaller synergistic effect for *ADH2* and

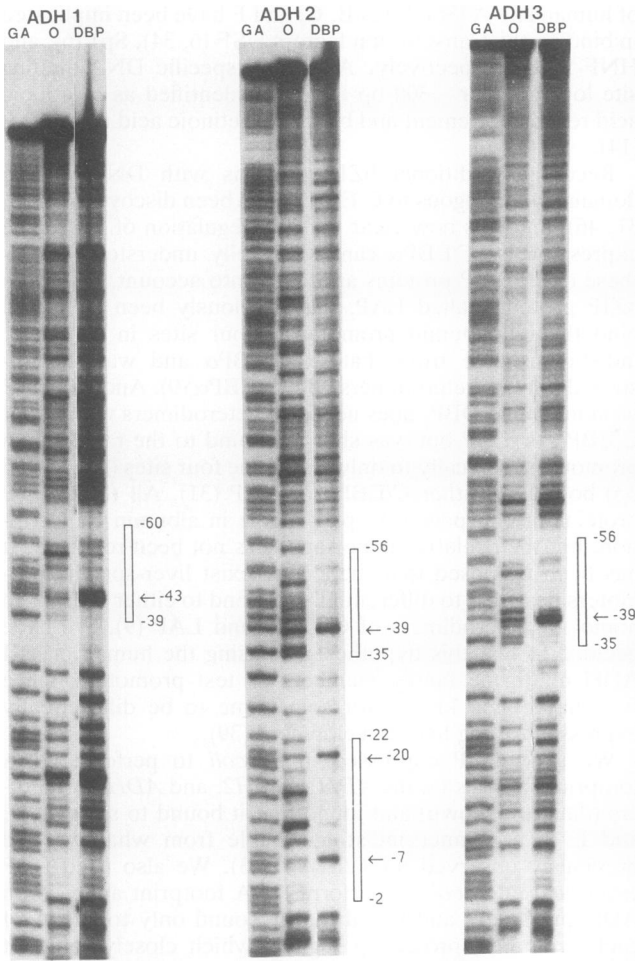


FIG. 2. DNA footprint analysis of *ADH1*, *ADH2*, and *ADH3*, using bacterially expressed DBP. The bars indicate the sites of DNA protection in a DNase I footprinting assay when DBP is incubated with the template DNA corresponding to the noncoding strand of the three promoters. The arrows indicate DNase I-hypersensitive sites. Lanes: GA, guanosine-plus-adenine chemical DNA sequence reaction; 0, DNase I reaction in the absence of added extract; DBP, DNase I reaction in the presence of 30 μg of total protein extract from *E. coli* expressing rat liver DBP.

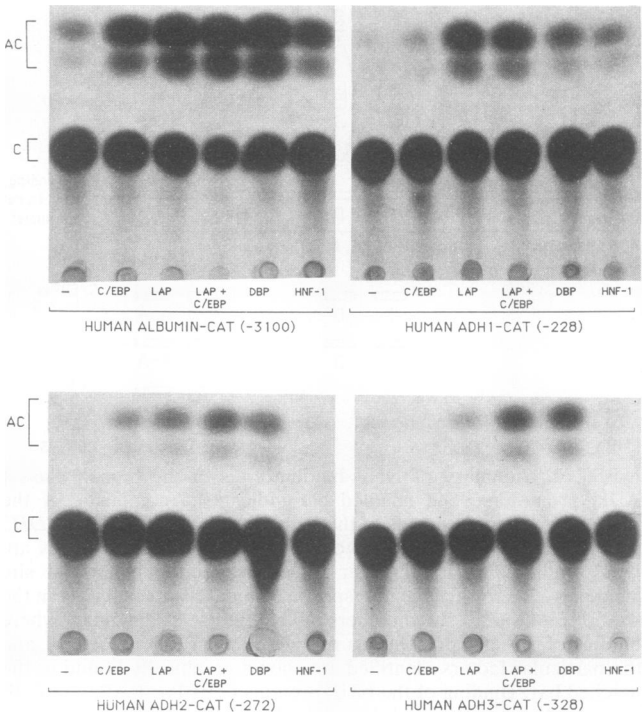


FIG. 3. CAT assay of HepG2 cells cotransfected with *ADH-cat* fusions and expression vectors for various liver transcription factors. HepG2 cells were transfected with the albumin-*cat* or *ADH-cat* constructs indicated. Control transfection reaction mixtures contained 20 μg of herring sperm carrier DNA (–), and cotransfection reaction mixtures contained the following amount of DNA: C/EBPα, 20 μg of pMSV-C/EBP; LAP, 20 μg of pSCT-LAP; LAP + C/EBPα, 10 μg of pSCT-LAP plus 10 μg of pMSV-C/EBP; DBP, 10 μg of pCMV-DBP plus 10 μg of carrier DNA; or HNF-1, 10 μg of pRSV-B1 plus 10 μg of carrier DNA. The results of a thin-layer chromatographic CAT assay are shown. C, chloramphenicol substrate; AC, acetylated chloramphenicol product.

albumin and no synergism for *ADH1*. A 1:1 mixture of DBP with either C/EBPα or LAP did not show any synergistic effects on any of the promoters tested (data not shown), which is consistent with the knowledge that DBP does not heterodimerize with either C/EBPα or LAP (31).

Cotransfection of a deletion series of *ADH-cat* fusions with C/EBPα, LAP, DBP, or HNF-1 was undertaken, and the results indicated as fold inductions are shown (Fig. 4). *ADH1* was unique in that it was the only ADH promoter able to respond to HNF-1, and this ability was lost when sequences between −228 and −76 bp containing a putative HNF-1-binding site were removed. *ADH2* responded solely to C/EBPα, and this ability was lost when sequences between −171 and −72 bp containing two C/EBPα-binding sites were removed. All three genes responded well to DBP transactivation, and the deletion mutants for *ADH2-cat* indicate that the DBP-binding site near −40 bp must be removed to severely effect DBP transactivation. *ADH1* and *ADH2* both responded well to LAP transactivation, relative to *ADH3*, and this ability was reduced greatly by removing sequences upstream of −72 bp containing two LAP-binding sites and even further reduced for *ADH2* by removing the LAP-binding site just upstream of the TATA box at −40 bp [see *ADH-cat*(−36) in Fig. 4]. Evidently, the small LAP response for *ADH3* indicates that its LAP-binding sites do not function the same as do those in the other ADH genes. This notion is further emphasized by the observation that a 1:1 mixture of C/EBPα and LAP had its greatest synergistic effect on the *ADH3* promoter. The induction levels for *ADH3* were 1.5-fold for C/EBPα alone and 3.5-fold for LAP alone (20 μg of expression plasmid in each case) but was 20-fold for the mixture (10 μg of each expression plasmid to equal 20 μg in total), indicating a clear synergistic effect (Fig. 4). *ADH1* did not display a synergistic effect of C/EBPα and LAP but displayed a high effect of LAP alone (25-fold induction for LAP alone and 26-fold for C/EBPα plus LAP). *ADH2* and albumin displayed synergistic effects of C/EBPα and LAP, reaching inductions of 32- and 39-fold, respectively, but LAP alone worked well for these promoters at 13- and 22-fold, respectively. Thus, the more significant synergistic effect on *ADH3* suggests that C/EBPα and LAP may not be able to effectively transactivate this promoter individually but instead must work together.

Effects of promoter-proximal mutations on *ADH2* response to bZIP factors. Since we had previously shown that muta-

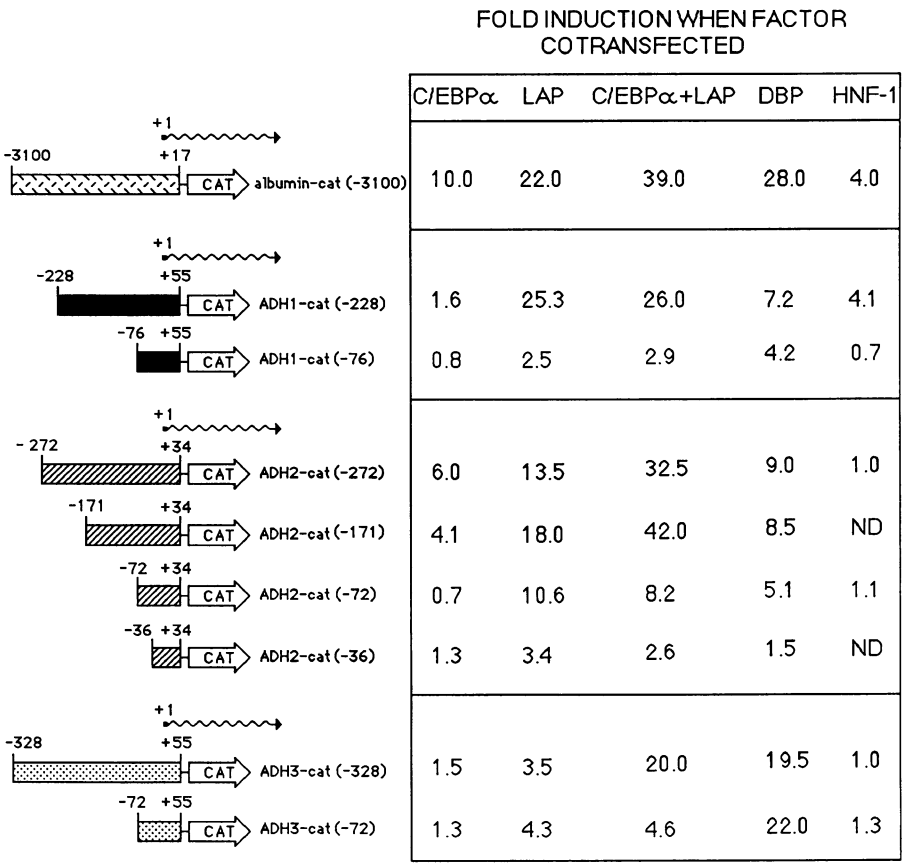


FIG. 4. Transfection analysis of a deletion series of *ADH-cat* fusions. *ADH-cat* fusions containing various lengths of promoter DNA as well as an albumin-*cat* fusion were cotransfected with expression vectors for C/EBPα, LAP, DBP, and HNF-1 as described for Fig. 3. Indicated are the fold inductions for the various transcription factors determined by the ratio of CAT activity between samples cotransfected with a transcription factor expression vector and samples treated only with carrier DNA. ND, not determined.

tions in either C/EBPα-binding site flanking each side of the *ADH2* TATA box blocked C/EBPα binding and eliminated transactivation by cotransfected C/EBPα (42, 43), we analyzed these mutants for LAP and DBP binding. We determined by footprint analysis that these mutations also blocked binding of either LAP or DBP at the site mutated (data not shown), as previously observed for C/EBPα (43). The mutants were analyzed for transactivation by C/EBPα, LAP, DBP, and a 1:1 mixture of C/EBPα and LAP (Fig. 5). Surprisingly, the downstream mutant *ADH2-cat*(−272)A-A responded like the wild type to both LAP and DBP, with inductions of about 14- and 9-fold, respectively. This muta-

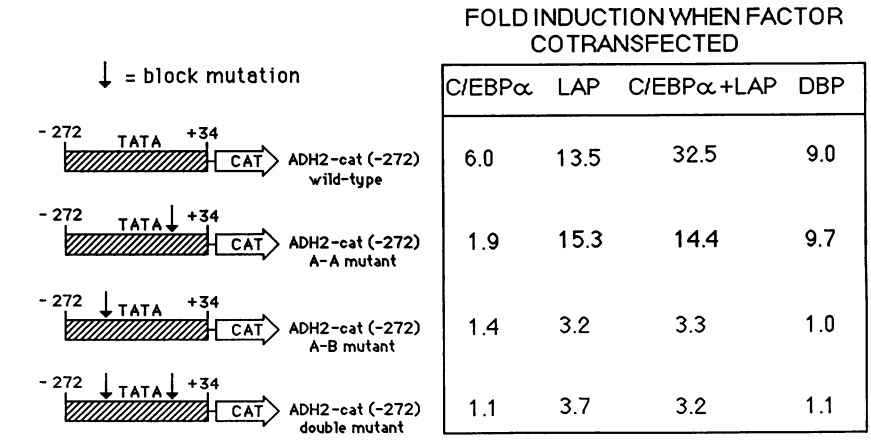
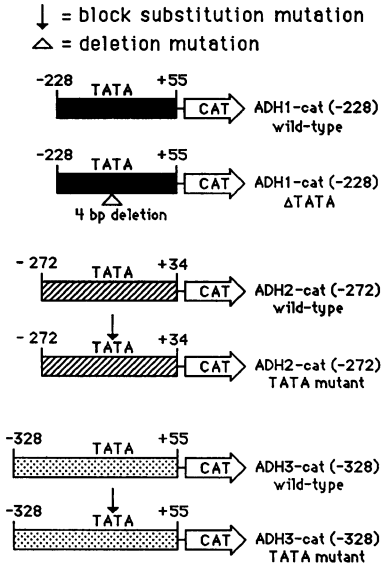


FIG. 5. Effect of *ADH2* promoter-proximal mutations on transactivation by bZIP factors. Mutant forms of *ADH2-cat*(−272) which eliminate the bZIP-binding sites flanking either side of the TATA box were subjected to cotransfection analysis as described for Fig. 3. The fold inductions for the various transcription factors are indicated.



FOLD INDUCTION WHEN FACTOR COTRANSFECTED

C/EBPα	LAP	C/EBPα+LAP	DBP	HNF-1
1.6	25.3	26.0	7.2	4.1
4.5	35.0	84.0	7.8	4.1
6.0	13.5	32.5	9.0	1.0
5.1	14.8	27.5	11.5	ND
1.5	3.5	20.0	19.5	1.0
1.6	3.5	18.7	16.0	1.4

FIG. 6. Effects of ADH TATA mutants on transactivation by liver transcription factors. Cotransfection analysis using C/EBPα, LAP, DBP, and HNF-1 expression vectors was performed as described for Fig. 3 on the wild-type and mutant *ADH-cat* fusions indicated. Mutants included a 4-bp deletion in the *ADH1* TATA box and 11-bp block substitutions in the *ADH2* and *ADH3* TATA boxes. The fold inductions for the various transcription factors are indicated. ND, not determined.

tion did reduce the response to C/EBPα as well as the synergistic effect between C/EBPα and LAP, thus suggesting that the region downstream of the TATA box is important for C/EBPα transactivation even when this factor is in the form of a presumptive C/EBPα-LAP heterodimer.

The upstream mutant *ADH2-cat*(−272)A-B responded very poorly to LAP and was unresponsive to both C/EBPα and DBP (Fig. 5). Similarly, a double mutant in which both sides of the TATA box were mutated responded poorly. Thus, the upstream binding site appears to be the single most important element controlling *ADH2* transcription by bZIP factors. The position of this element at about −40 bp places it in an ideal location to interact with the basal transcription machinery.

TATA mutations in *ADH1*, *ADH2*, and *ADH3*. Because of the presence of bZIP binding sites both upstream and downstream of the ADH TATA box, we had previously prepared TATA mutants of the *ADH1* and *ADH2* promoters. The *ADH1* promoter (normally unresponsive to C/EBPα) naturally contains 4 extra bp in the TATA region relative to *ADH2* and *ADH3* (see Fig. 7), but removal of these bases allowed C/EBPα to now transactivate, possibly as a result of bringing the two flanking C/EBPα-binding sites one-half turn of the DNA helix closer together (43). We now show that *ADH1-cat*(−228)ΔTATA, containing this 4-bp deletion, has a synergistic response to a 1:1 mixture of C/EBPα and LAP (Fig. 6). This mutant now responds much more like the *ADH2-cat*(−272) construction. This finding further reinforces our view that the precise location of bZIP-binding sites around the three ADH TATA boxes contributes to differential expression of these genes.

We previously showed that a block substitution mutation of 6 bp in the *ADH2* TATA box did not effect C/EBPα transactivation (43). To further examine this issue, we prepared 11-bp block substitution mutations in both the *ADH2* and *ADH3* TATA boxes to ensure that every possible A and T in the vicinity was disturbed. In both cases, the TATA mutations did not effect the response to C/EBPα, LAP, the C/EBPα-LAP mixture, or DBP (Fig. 6). Thus, human ADH promoters may not require this TATA element to respond to the bZIP transcription factors examined. Another possibility is that transcription of both *ADH2-cat* and *ADH3-cat* fusions is not initiated at the correct ADH cap sites in the transfection assay. However, vitro transcription assays of *ADH2-cat*(−272) indicate that C/EBP induction occurs using the correct initiation site (40a).

DISCUSSION

We have analyzed several liver transcription factors to determine their roles in the sequential turn-on of human ADH genes during liver development. The bZIP transcription factors C/EBPα, LAP, and DBP, as well as the homeodomain transcription factor HNF-1, were examined. Analysis of the human *ADH1*, *ADH2*, and *ADH3* promoters has revealed a differential activation by these factors, which can roughly explain why they are expressed in the sequence observed. A summary of the promoter elements involved indicates that each of the three promoters has a unique set of functional elements which allow sequential turn-on during early fetal liver (*ADH1*), late fetal liver (*ADH2*), and postnatal liver (*ADH3*) development (Fig. 7). Thus, *ADH1* contains an element near −200 bp (corresponding to site F identified by liver nuclear extract footprinting) which allows it to respond to HNF-1 transactivation. Evidently, the homologous HNF-1-binding sites in the *ADH2* and *ADH3* promoters are insufficient to activate transcription. The expression of *ADH1* in early fetal liver correlates with the expression of HNF-1, which has also been shown to be expressed at very early stages of fetal liver development (11, 24). *ADH2* contains several elements rendering it responsive to C/EBPα homodimers, unlike either the *ADH1* or *ADH3*

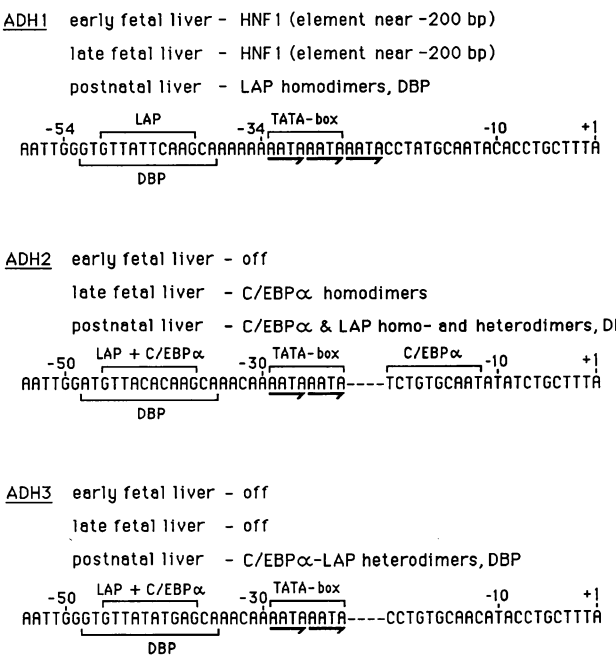


FIG. 7. Summary of promoter elements involved in the sequential expression of human class I ADH genes. In early human fetal liver development (second trimester), *ADH1* is expressed, with the additional expression of *ADH2* during late fetal liver development (third trimester) and the additional expression of *ADH3* during postnatal liver development at about 5 months after birth (39). A model of the liver transcription factors which may be involved with this sequential turn-on is presented. The functional binding sites for the factors C/EBPα, LAP, DBP, and HNF-1 in the three ADH promoters are indicated, along with the time during development when they are likely to exert their strongest effect on ADH transcription.

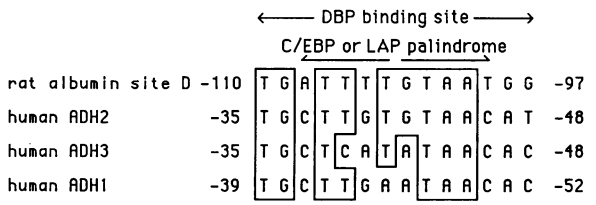


FIG. 8. Homology of the critical bZIP protein binding sites in ADH and albumin promoters. The transfection and DNA-binding studies performed herein indicate that the bZIP protein-binding site centered around −40 bp in the ADH promoters is the most critical element for transcriptional regulation. These elements in the human *ADH1*, *ADH2*, and *ADH3* promoters are shown aligned with the comparable element from the D-binding site of the rat albumin promoter. Conserved base pairs are boxed between the various promoters. The perfect C/EBP-LAP palindrome (ATTGCGCAAT) element is approximated in all four promoters indicated by the inverted repeat arrows. This palindromic element is also an integral part of the DBP-binding site, as shown previously (31).

promoter. The late fetal liver appearance of *ADH2* expression coincides with a burst of C/EBPα expression which also occurs in late fetal liver development (2). LAP begins to be expressed during late fetal liver development but accumulates to much higher levels postnatally (10). Also, DBP is not expressed until adult liver development (31). Thus, these factors may not be present in high enough amounts to effect ADH gene expression prenatally. The restriction of *ADH3* expression to early postnatal liver can thus be attributed to its lack of response to HNF-1 as well as C/EBPα and LAP homodimers. The strong synergistic transactivation of *ADH3* that we observed by using cotransfection of a mixture of C/EBPα and LAP (presumably forming heterodimers) could conceivably occur in vivo during early postnatal liver development, when there is an increase of LAP relative to C/EBPα. Thus, *ADH3* expression could be delayed until a critical ratio of LAP and C/EBPα was achieved postnatally.

After their initial turn-on in the liver, all three human ADH genes continue to increase in expression until they reach maximal expression at about 5 years of age (39). This increase correlates with the strong transactivation of all three promoters by DBP, which is expressed highly only late in liver development. Thus, it can be proposed that HNF-1, C/EBPα, and C/EBPα-LAP are instrumental in the establishment of *ADH1*, *ADH2*, and *ADH3* transcription, respectively, during early liver development and that DBP is instrumental in the maintenance of high-level transcription of all three ADH genes during late liver development. It is also conceivable that the other factors HNF-1, C/EBPα, and LAP continue to play a role in maintaining ADH transcription in adult liver in conjunction with DBP.

The results of the mutational analysis of *ADH2* indicate that the bZIP-binding site just upstream of the TATA box near −40 bp is the most critical for transactivation by LAP and DBP and is also needed for C/EBPα transactivation. This site in *ADH2*, as well as the homologous sites in *ADH1* and *ADH3*, share extensive homology with the D-binding site of the rat albumin gene, which binds all three of these bZIP proteins (Fig. 8). Interestingly, these sites in the three ADH genes lie very close to the TATA box and may replace its function, since we have shown that TATA substitution mutants of *ADH2* and *ADH3* still respond to these three bZIP proteins. This phenomenon is not restricted to transactivation by bZIP proteins because we have shown that the retinoic acid response element located at −289 bp in the *ADH3* promoter (14) still functions in the TATA mutant (44a). Thus, bZIP proteins binding to the −40 bp element may be able to communicate with the basic transcription apparatus in the absence of a TATA box for binding of TFIID and may be able to facilitate the transactivation of a distantly bound factor such as the retinoic acid receptor, which has a zinc finger DNA-binding domain. Transcription from a TATA-less promoter has recently been examined in detail and found to still require a functional TFIID complex of which there exist many subforms (36). This finding suggests that TFIID does not always need to bind to the TATA box to help assemble the transcription complex and may instead communicate with other DNA-binding proteins in the vicinity of the transcription initiation site. Despite ADH promoters appearing to have TATA boxes, they may not be used as DNA-binding sites for TFIID during the establishment of high-level transcription in the liver. Instead, the putative ADH TATA boxes may be used only in other tissues in which ADH is expressed at low levels and in which the bZIP proteins examined here are at low levels or nonexistent. In liver, the critical promoter element for ADH transcription complex formation appears to be the −40 bp site which binds the bZIP proteins C/EBPα, LAP, and DBP.

The transfection experiments performed in this study, using C/EBPα and LAP alone as well as a 1:1 mixture of each, indicated that the *ADH1*, *ADH2*, and *ADH3* promoters have evolved to differentially respond to these factors. If we assume that the factors transfected individually produce

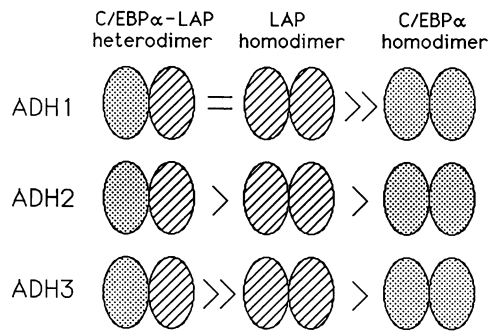


FIG. 9. Hierarchy of ADH promoter responsiveness to C/EBPα and LAP. C/EBPα and LAP are known to form both homo- and heterodimers (5, 9). On the basis of the results of *ADH-cat* cotransfection with C/EBPα alone and LAP alone (which presumably produces homodimers of each factor), and a 1:1 mixture (which presumably produces some heterodimers), we have ranked the *ADH1*, *ADH2*, and *ADH3* promoters according to their responsiveness to particular dimeric forms. The dotted circles correspond to C/EBPα monomers, and the hatched circles correspond to LAP monomers. =, approximately the same response; >, greater response, >>, much greater response.

homodimers and that the mixture produces heterodimers as previously shown by Williams et al. (46), we can establish a hierarchy of responsiveness of each of the promoters to the homo- or heterodimers (Fig. 9). In particular, *ADH1* has a very good response to both the heterodimer and the LAP homodimer relative to the C/EBP homodimer. *ADH2* is a little more responsive to the heterodimer than to the LAP homodimer and shows a relatively weak response to the C/EBPα homodimer. *ADH3* responds strongly only to the heterodimer, with the LAP and C/EBP homodimers giving weak responses. Thus, C/EBPα and LAP do appear to have a synergistic effect on *ADH2* and especially *ADH3* transcription. Also, according to our results, the human albumin promoter responds to the heterodimer with a synergistic effect similar to that of the human *ADH2* promoter (Fig. 4). Since previous studies on the mouse albumin promoter did not indicate any synergism between C/EBPα and LAP (46), this finding may reflect an evolutionary difference between the mouse and human promoters.

In summary, the human ADH multigene family has provided examples of classes of promoters which clearly respond differentially to C/EBPα and LAP homo- and heterodimers, DBP and HNF-1. We suggest that the differential responses of *ADH1*, *ADH2*, and *ADH3* to these liver transcription factors which we have observed in transfection assays may be physiologically significant for the sequential turn-on of ADH genes during liver development. Since ADH plays a role in the synthesis of retinoic acid (12), a compound implicated in development of the epithelial cells of the liver as well as many other developmental processes, the sequential turn-on of three ADH genes in human liver may be needed as part of the establishment of retinoid homeostasis for correct human development.

ACKNOWLEDGMENTS

We thank S. McKnight for the C/EBPα expression vectors, U. Schibler for the LAP and DBP expression vectors, A. Nicosia for the HNF-1 expression vector, and D. Wilson for the human albumin promoter fused to the *cat* gene.

This study was supported by Public Health Service grant AA07261 from the National Institute on Alcohol Abuse and Alcoholism. G.D. is a recipient of Research Scientist Development Award K02 AA00119 from the National Institute on Alcohol Abuse and Alcoholism.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
2. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3**:1146–1156.
3. **Blomhoff, R., and K. Wake.** 1991. Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J. **5**:271–277.
4. **Brown, C. J., L. Zhang, and H. J. Edenberg.** 1992. Human alcohol dehydrogenase gene expression: tissue specificity. J. Cell. Biochem. **16A**:68.
5. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5**:1538–1552.
6. **Carr, L. G., and H. J. Edenberg.** 1990. *cis*-acting sequences involved in protein binding and *in vitro* transcription of the human alcohol dehydrogenase gene *ADH2*. J. Biol. Chem. **265**:1658–1664.
7. **Chang, C.-J., T.-T. Chen, H.-Y. Lei, D.-S. Chen, and S.-C. Lee.** 1990. Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. Mol. Cell. Biol. **10**:6642–6653.
8. **Courtois, G., S. Baumhueter, and G. R. Crabtree.** 1988. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. Proc. Natl. Acad. Sci. USA **85**:7937–7941.
9. **Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler.** 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes Dev. **4**:1541–1551.
10. **Descombes, P., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell **67**:569–579.
11. **De Simone, V., L. De Magistris, D. Lazzaro, J. Gerstner, P. Monaci, A. Nicosia, and R. Cortese.** 1991. LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. EMBO J. **10**:1435–1443.
12. **Duester, G.** 1991. Human liver alcohol dehydrogenase gene expression: retinoic acid homeostasis and fetal alcohol syndrome, p. 375–402. *In* R. R. Watson (ed.), Drug and alcohol abuse reviews, vol. 2. Liver pathology. Humana Press, Clifton, N.J.
13. **Duester, G.** 1991. A hypothetical mechanism for fetal alcohol syndrome involving ethanol inhibition of retinoic acid synthesis at the alcohol dehydrogenase step. Alcohol. Clin. Exp. Res. **15**:568–572.
14. **Duester, G., M. L. Shean, M. S. McBride, and M. J. Stewart.** 1991. Retinoic acid response element in the human alcohol dehydrogenase gene *ADH3*: implications for regulation of retinoic acid synthesis. Mol. Cell. Biol. **11**:1638–1646.
15. **Eklund, H., P. Müller-Wille, E. Horjales, O. Futer, B. Holmquist, B. L. Vallee, J.-O. Höög, R. Kaiser, and H. Jörnvall.** 1990. Comparison of three classes of human liver alcohol dehydrogenase. Emphasis on different substrate binding pockets. Eur. J. Biochem. **193**:303–310.
16. **Felder, M. R., W. K. Church, and J. B. Shaffer.** 1982. Analysis of genetically defined liver enzymes in transplantable hepatomas and developing liver of the mouse. Oncology **39**:382–390.
17. **Friedman, A. D., W. H. Landschulz, and S. L. McKnight.** 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. **3**:1314–1322.
18. **Frolik, C. A.** 1984. Metabolism of retinoids, p. 177–208. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), The

retinoids, vol. 2. Academic Press, Orlando, Fla.
19. **Giguere, V., E. S. Ong, P. Segui, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) **330**:624–629.
20. **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.
21. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39**:653–662.
22. **Knowles, B. B., C. C. Howe, and D. P. Aden.** 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science **209**:497–499.
23. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
24. **Kuo, C. J., D. B. Mendel, L. P. Hansen, and G. R. Crabtree.** 1991. Independent regulation of HNF-1α and HNF-1β by retinoic acid in F9 teratocarcinoma cells. EMBO J. **10**:2231–2236.
25. **Laimins, L. A., G. Khoury, C. M. Gorman, B. H. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
26. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**:1681–1688.
27. **Li, T.-K.** 1977. Enzymology of human alcohol metabolism. Adv. Enzymol. **45**:427–483.
28. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
29. **Mendel, D. B., L. P. Hansen, M. K. Graves, P. B. Conley, and G. R. Crabtree.** 1991. HNF-1α and HNF-1β (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. Genes Dev. **5**:1042–1056.
30. **Mezey, E., and P. R. Holt.** 1971. The inhibitory effect of ethanol on retinol oxidation by human liver and cattle retina. Exp. Mol. Pathol. **15**:148–156.
31. **Mueller, C. R., P. Maire, and U. Schibler.** 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell **61**:279–291.
32. **Petkovich, M., N. J. Brand, A. Krust, and P. Chambon.** 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (London) **330**:444–450.
33. **Poli, V., F. P. Mancini, and R. Cortese.** 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell **63**:643–653.
34. **Potter, J. J., D. Cheneval, C. V. Dang, L. M. S. Resar, E. Mezey, and V. W. Yang.** 1991. The upstream stimulatory factor binds to and activates the promoter of the rat class I alcohol dehydrogenase gene. J. Biol. Chem. **266**:15457–15463.
35. **Potter, J. J., E. Mezey, R. J. Christy, D. W. Crabb, P. M. Stein, and V. W. Yang.** 1991. CCAAT/enhancer binding protein binds and activates the promoter of the rat class I alcohol dehydrogenase gene. Arch. Biochem. Biophys. **285**:246–251.
36. **Pugh, B. F., and R. Tjian.** 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes Dev. **5**:1935–1945.
37. **Roberts, A. B., and M. B. Sporn.** 1984. Cellular biology and biochemistry of the retinoids, p. 209–286. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), The retinoids, vol. 2. Academic Press, Orlando, Fla.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Smith, M.** 1986. Genetics of human alcohol and aldehyde dehydrogenases. Adv. Hum. Genet. **15**:249–290.
40. **Sporn, M. B., and A. B. Roberts.** 1991. Interactions of retinoids and transforming growth factor-β in regulation of cell differentiation and proliferation. Mol. Endocrinol. **5**:3–7.
40a. **Stewart, M., and G. Duester.** Unpublished data.
41. **Stewart, M. J., M. S. McBride, L. A. Winter, and G. Duester.** 1990. Promoters for the human alcohol dehydrogenase genes *ADH1*, *ADH2*, and *ADH3*: interaction of CCAAT/enhancer binding protein with elements flanking the *ADH2* TATA box. Gene **90**:271–279.
42. **Stewart, M. J., M. L. Shean, and G. Duester.** 1990. *trans*-activation of human alcohol dehydrogenase gene expression in hepatoma cells by C/EBP molecules bound in a novel arrangement just 5′ and 3′ to the TATA box. Mol. Cell. Biol. **10**:5007–5010.
43. **Stewart, M. J., M. L. Shean, B. W. Paeper, and G. Duester.** 1991. The role of CCAAT/enhancer-binding protein in the differential transcriptional regulation of a family of human liver alcohol dehydrogenase genes. J. Biol. Chem. **266**:11594–11603.
44. **Toniatti, C., A. Demartis, P. Monaci, A. Nicosia, and G. Ciliberto.** 1990. Synergistic *trans*-activation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites. EMBO J. **9**:4467–4475.
44a. **van Ooij, C., and G. Duester.** Unpublished data.
45. **Wahli, W., and E. Martinez.** 1991. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. FASEB J. **5**:2243–2249.
46. **Williams, S. C., C. A. Cantwell, and P. F. Johnson.** 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev. **5**:1553–1567.