The Upstream Stimulatory Factor Binds to and Activates the Promoter of the Rat Class I Alcohol Dehydrogenase Gene*

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The gene encoding rat class I alcohol dehydrogenase (ADH) is expressed primarily in the liver. Recent studies in our laboratories indicate that multiple cellular factors present in the rat liver interact with various regions of the promoter of this gene. One of the regions contains the sequence 5'-CACATG-3' that has an "E box" homology to which a number of transcription factors containing the basic helix-loop-helix motif bind. We now demonstrate that the human transcription factor, upstream stimulatory factor (USF), a basic helix-loop-helix-containing protein, binds to and activates the promoter of the rat class I ADH gene. Electrophoretic mobility shift assays of labeled oligonucleotide containing the 5'-CACATG-3' sequence within the ADH promoter revealed the formation of multiple DNA-protein complexes when nuclear extracts obtained from adult rat liver were used. The binding of proteins to the DNA could be competed away with an oligonucleotide specifying a sequence within the adenovirus major late promoter (MLP) that had previously been shown to bind USF. Similar complexes were observed when electrophoretic mobility shift assays of labeled MLP oligonucleotide were performed with rat liver nuclear extracts. Conversely, nuclear extracts isolated from La cells, cells known to have abundant USF, contain factors that interact with the sequence present in the ADH promoter. This interaction could be competed efficiently by the MLP oligonucleotide. USF synthesized in an in vitro transcription and translation system also binds to the ADH promoter as well as to the MLP. In addition, antiserum directed against USF recognizes factors present in the rat liver nuclear extracts that interact with the ADH promoter. Furthermore, transcription directed from both the ADH and the adenovirus major late promoters was inhibited by an oligonucleotide representing the USF-binding site within the ADH promoter in a cell-free in vitro transcription system. Lastly, an ADH promoter-reporter gene construct was transactivated by an eukaryotic expression vector containing USF in HepG2 cells co-transfected with the two constructs. These experiments demonstrate that USF is present in the rat liver and that it binds to and activates the promoter of the rat class I ADH gene in a sequence-specific manner.

Alcohol dehydrogenase (ADH; alcohol:NAD+ oxidoreductase, EC 1.1.1.1) is the principal enzyme responsible for ethanol oxidation. The gene encoding class I ADH is primarily expressed in the liver of a number of species including rat, mouse, and human (1-3). The 5'-flanking region of the rat class I ADH gene has been cloned and sequenced (1). The putative promoter region of this gene was found to be highly homologous to similar regions of the class I ADH gene in other species (1, 4, 5), suggesting that the control of transcription of this class of gene is similar between various vertebrates. We recently showed that the liver-specific CCAAT/enhancer binding protein (C/EBP) binds to and activates the promoter of the rat class I ADH gene through a region of DNA immediately upstream from the start site of transcription (6). A similar conclusion was obtained from studying the promoter of the human class I ADH gene (7, 8).

During the course of our study, we noted that nuclear extracts isolated from rat liver contain multiple factors in addition to C/EBP that interact with defined regions of the promoter of the rat class I ADH gene (6). One of these regions, designated region 3, is located between position −52 and −60 relative to the start site of transcription and has a perfect palindromic sequence, 5'-CACATGTG-3'. This region of DNA was shown to interact with a heat-stable protein present in rat liver nuclear extracts (6). We also noted that sequences within this region contain an "E box" homology, 5'-CANNTG-3', that was first identified as a site of DNA-protein interaction in the enhancer and promoter regions of the immunoglobulin genes (9, 10). Since its discovery, the E box homology has been found in the promoters of a growing number of other cellular genes (11-17). Further characterization of cellular factors that interact with this E box homology present in various gene promoters revealed that they belong to a class of DNA-binding proteins that contain a specific domain referred to as the basic helix-loop-helix (bHLH) motif (18). In some instances, these DNA-binding proteins and

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1 The abbreviations used are: ADH, alcohol dehydrogenase; USF, upstream stimulatory factor; MLP, major late promoter; C/EBP, C/EBP/C/EBP enhancer binding protein; bHLH, basic helix-loop-helix; bp, base pair(s); EMSA, electrophoretic mobility shift assays; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; HEPES, 2-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, [ethylene bis (oxethylenedinitrol)] tetraacetate acid; MLTF, major late transcription factor.
putative transcription factors are involved in the control of cellular proliferation or differentiation and are therefore tissue-specifically distributed (19-24). Other bHLH-containing proteins appear to be more ubiquitous in their distribution (18, 25-28). One of these ubiquitous E box-binding proteins is the upstream stimulatory factor (USF) or the major late transcription factor (MLTF) that was initially identified by its ability to bind to the sequence 5'-CAGCTG-3' within the adenovirus major late promoter (29-32). This protein was subsequently shown to bind to the promoters of a number of cellular genes that are either tissue-specifically or developmentally regulated. They include genes encoding mouse metallothionein I (33, 34), rat γ-fibrinogen (15, 35), human growth hormone (16, 36), liver-specific rat pyruvate kinase (37, 38), and human heme oxygenase (39). In this study, we show that USF or an USF-like protein present in rat liver nuclear extracts binds to and activates transcription from the promoter of the rat class I ADH gene.

MATERIALS AND METHODS

Plasmids—The full length cDNA clone encoding the 43-kDa human USF, d12 (40), was a kind gift from Dr. Michele Sawadogo of the M.D. Anderson Cancer Center, Houston, TX. The adenovirus major late promoter construct pHTXB used in the cell-free transcription reaction was obtained from Dr. Jeffrey Cordon of our Institution. This construct contains 250 bp of the adenovirus major late promoter and 540 bp of the transcribed region (41) cloned in the plasmid pH222. The 230OADH-CAT construct containing 230 bp of the rat ADH promoter sequence fused to the coding sequence of the reporter gene chloramphenicol acetyltransferase (CAT) has been described (6).

The eukaryotic expression vector RSV-USF was generated by fusing d12 (40) that had been digested with BamHI followed by filling in the 5' overhang and the blunt end of the plasmid DNA polymerase (Boehringer Mannheim) to an 800-bp DNA fragment containing the long terminal repeat of the Rous sarcoma virus (RSV), the latter derived by digesting a RSV-human myc construct PM21 (42) with Sall and HindIII followed by Klenow fill-in. The orientation of the RSV enhancer sequence in relationship to the USF coding sequence was confirmed by direct DNA sequencing (43). The ML,E,b-CAT construct was generated by fusing a tandem repeat of an oligonucleotide bearing the binding site for USF within the adenovirus major late promoter (29-32) to the minimal promoter E,b-CAT construct (44) between the Xhol and Sall sites.

Oligonucleotides—Single-stranded oligonucleotides were synthesized by the Protein/Pep tide/DNA Facility in our Institution. The sequences of individual oligonucleotides described in the text were as follows. The ADH-U oligo specifies sequence 5'-GATCTGTAGGCCACGTGACCGG-3' (coding strand, position -46 to -73, Ref. 1) and 5'-GATCCAACTGGGCCAAAGTTGCACACA-3' (noncoding strand). The MLP oligo specifies the USF-binding site (noncoding strand) with +12 to +31 on coding strand, Ref. 45), 5'-CTTATTTTTC-3' (adenovirus major late upstream, position +1 to +21 on coding strand, Ref. 41), and 5'-ACGCCCTTCTAGGTTGTTTTG-3' (adenovirus major late downstream, position +234 to +241 on noncoding strand).

The isolated nuclear extracts from the human epithelial cells HeLa were obtained similarly from cells that were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Transfection and CAT Assays—Transfection of the human hepatoblastoma cell line Hep2g was performed as described previously (6). 20 μg of reporter DNA (pUC-CAT), a promoterless CAT construct; E,b-CAT; the adenovirus E,b minimal promoter-CAT construct; 230ADH-CAT; or ML,E,b-CAT) were transfected into subconfluent Hep2g cells on 10-cm plates in the presence of 20 μg of either carrier

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RESULTS

Rat Liver Nuclear Extracts Contain an USF-like Factor That Interacts with the Rat Class I ADH Promoter—EMSA were performed on end-labeled oligonucleotide ADH-U specifying sequences encompassing region 3 of the rat class I ADH promoter (6) using nuclear extracts obtained from adult rat liver. Increasing amounts of nuclear extracts were incubated with a constant amount of labeled ADH-U oligo. Fig. 1 shows that multiple DNA-protein complexes were detected as retarded bands in a dose-dependent fashion. The specificity of each of the DNA-protein complexes was determined by competition experiments when EMSA were performed in the presence of increasing amounts of unlabeled oligonucleotide ranging between 0- and 1000-fold molar excess of the labeled probe. Fig. 2 shows that at least four complexes (indicated by arrowheads) could be competed away successfully when unlabeled ADH-U oligo was used. We then tested unlabeled MLP oligo, an oligonucleotide containing sequences between the adenovirus major late promoter with which USF had been shown to interact, for its ability to compete for the formation of complexes (29–32). The unlabeled MLP oligo not only competed for binding of proteins with the labeled probe but did so at a higher efficiency (Fig. 2). In contrast, an unrelated oligonucleotide of similar length, ADH-E, failed to compete for the formation of the complexes even when added at a

![Figure 1](image1.png)

**Fig. 1.** EMSA of oligonucleotide ADH-U that contains sequences within region 3 (Ref. 6) of the rat class I ADH promoter. End-labeled ADH-U oligo was incubated with increasing amounts (0–16 μg) of nuclear extracts obtained from rat liver and DNA-protein complexes resolved on a 5% nondenaturing polyacrylamide gel. *F* indicates the position of the free probe. Diagrammed below the autoradiogram is the proximal promoter of the rat class I ADH gene based on DNase I footprint analysis using rat liver nuclear extracts (6). Region 1 binds the transcription factor CCAAT/enhancer binding protein, C/EBP. Region 2 binds an unspecified nuclear protein. Region 3 contains the E box homology which is included in the sequences of the oligonucleotide ADH-U.

![Figure 2](image2.png)

**Fig. 2.** Competition experiments using unlabeled ADH-U, MLP, and ADH-E oligos. EMSA were performed using 12 μg of rat liver nuclear extracts and labeled ADH-U oligo. Competitions were carried out with increasing amounts (0–1000-fold molar excess) of either the homologous ADH-U oligo, the heterologous MLP oligo, or an unrelated ADH-E oligo. Specific DNA complexes formed between the nuclear extracts and the labeled probe are marked with arrowheads. Comp. indicates competitor oligos, and *x* indicates -fold molar excess. *F* indicates the position of the free probe.

![Figure 3](image3.png)

**Fig. 3.** EMSA of labeled MLP oligo using rat liver nuclear extracts. 12 μg of rat liver nuclear extracts were incubated with labeled ADH-U (*lanes* 1–4) or MLP oligo (*lanes* 5–8). Competitions were carried out using 1000-fold molar excess of unlabeled oligos as indicated. *F* denotes free probe. The free MLP probe, being smaller than the ADH-U probe, has largely migrated off the gel.

1000-fold molar excess of the labeled probe (Fig. 2). These findings suggest that factors present in the rat liver nuclear extracts that interact with the ADH-U oligo can recognize and bind to a region of the adenovirus major late promoter where USF had been shown to interact (29–32). This conclusion was supported by the reciprocal experiment when EMSA were performed with rat liver nuclear extracts and labeled MLP oligo (Fig. 3). DNA-protein complexes of similar mobilities were formed which could clearly be competed away with excessive amounts of unlabeled ADH-U oligo (compare *lanes* 5 and 6, Fig. 3). The binding affinity of proteins toward the MLP oligo was at least 20 times higher than that toward the ADH-U oligo since the unlabeled MLP oligo competed much more efficiently than the unlabeled ADH-U oligo when present in equal amounts (compare *lanes* 6 and 7, Fig. 3).
multiple DNA-protein complexes formed between rat liver nuclear extracts and labeled ADH-U or MLP oligo were probably in part due to the presence of multiple forms of USF in the cell (49). Alternatively, some of the complexes may contain heterodimer of proteins formed between USF and other bHLH proteins. The different efficiency by which the complexes were competed away by unlabeled oligos (Fig. 2) suggests that the second possibility may in fact exist.

**HeLa Cell Nuclear Extracts also Contain Factors That Interact with the Rat Class I ADH Promoter**—HeLa cells are cells in which USF was originally identified and contain abundant USF binding activity (49, 50). To test whether HeLa cells contain factors that may interact with the rat class I ADH promoter, EMSA were performed on labeled ADH-U oligo with nuclear extracts obtained from HeLa cells. Fig. 4A shows that multiple DNA-protein complexes were formed and that these complexes could be competed away effectively with either unlabeled ADH-U or MLP oligo. Conversely, a classical shift pattern for USF occurred when labeled MLP oligo was used (Fig. 4B, Refs. 29–32). Although at a lower efficiency than the MLP oligo, the ADH-U oligo was able to compete for binding to labeled MLP probe (Fig. 4B). The shift pattern for the labeled ADH-U oligo was similar to but not identical with that for the labeled MLP oligo (compare A and B of Fig. 4), suggesting that there are differences between the DNA-protein complexes formed. Be that as it may, these findings suggest that USF present in HeLa cells recognizes sequences within the rat class I ADH promoter in a sequence-specific fashion.

**USF Synthesized in Vitro Interacts with the Rat Class I ADH Promoter**—To directly demonstrate that USF can interact with the rat class I ADH promoter, the protein was synthesized in a coupled in vitro transcription and translation system and tested for its ability to bind to labeled ADH-U oligo in EMSA. A full length cDNA clone encoding the human USF, d12 (40), was used as the template from which RNA was synthesized with T7 RNA polymerase. The transcript was then translated in vitro and used for binding. As shown in Fig. 5B, protein directed by d12 (USF) bound to both the ADH-U and the MLP oligos avidly. Similar to earlier observations, there was a difference in the affinity of the protein for the two oligos (compare lanes 5 and 7, Fig. 5B). Some binding to the MLP probe was observed when protein made from a control brome mosaic virus template was used (lane 3, Fig. 5A), a phenomenon presumably due to the presence of USF-like activity in the rabbit reticulocyte lysates as previously noted (40). The increase in binding was nevertheless template-dependent (compare lanes 3 and 7, Fig. 5). These results indicate that USF, a human transcription factor, binds to the rat class I ADH promoter.

**Antiserum Directed against USF Recognizes Factors in Rat Liver That Interact with the ADH Promoter**—If factors in rat liver nuclear extracts contain USF or an USF-like protein, it would be expected that antiserum directed against the human USF would recognize them. We therefore tested the effect of a polyclonal antiserum directed against the purified 43-kD human USF (49) on the formation of DNA-protein complexes between rat liver nuclear extracts and labeled ADH-U oligo. Fig. 6 shows that addition of antiserum following incubation of rat liver nuclear extracts and labeled ADH-U oligo markedly disrupted the formation of normal DNA-protein complexes (lanes 4, Fig. 6). No significant changes in the mobility of the free probe were noted if antiserum alone was incubated with the free probe in the absence of any nuclear extracts (data not shown). An additional effect of the antiserum was the appearance of new, slow-migrating DNA-protein-antibody complexes (arrowheads, lane 4, Fig. 6), resulting in the so-called “supershift.” This effect was specific for the antiserum directed against USF as neither nonimmune serum (lane 3) nor antiserum directed against C/EBP or the myc protein (data not shown) influenced the complex formation. These results indicate the presence within rat liver nuclear extracts of one or several factors antigenically related to the human USF that binds to the ADH promoter.

**Cell-free Transcription of an ADH Promoter-Reporter Gene Construct Indicates That USF Is Important in Mediating Transcription from the ADH Promoter**—To test the ability of USF or USF-like factors in rat liver to mediate transcription directed by the ADH promoter, cell-free in vitro transcription reactions were performed using rat liver nuclear extracts in the presence or absence of various competing oligos. Two independent templates, one containing the rat class I ADH promoter and the other the adenovirus major late promoter, were used in the same reaction. As shown in Fig. 7A, nuclear
directed against the human USF was included in the absence of any competing oligonucleotides. Serum extracts from rat liver supported transcription from both promoters in similar transcription reactions (lanes 4 and 5, Fig. 7A). In contrast, a mutated ADH-E oligo failed to inhibit transcription from either promoter (lanes 1–3, Fig. 7B). These results indicate that USF or USF-like factors present in rat liver participate in the activation of transcription from promoters containing an USF-binding site.

The Rat Class I ADH Promoter Can Be Transactivated by the Human USF Expressed in Cells—To provide direct evidence that USF functions to activate the promoter of the ADH gene, co-transfection experiments were performed in HepG2 cells using an ADH promoter-CAT construct and an eukaryotic expression vector containing USF, RSV-USF. Fig. 8 shows that CAT activity directed by the ADH promoter was induced 4.4-fold by the co-introduction of RSV-USF (compare lanes 1 and 2, Fig. 8). Similarly, a heterologous promoter-CAT construct, ML2E15-XCAT, that contains two copies of the MLP sequence in tandem, was also transactivated by USF (lanes 3 and 4). In contrast, neither a promoterless CAT construct, pUC-CAT1, nor a minimal promoter-CAT construct, E15-CAT, were transactivated (lanes 5–8). We therefore conclude that USF activates the promoter of the rat class I ADH gene.

DISCUSSION

The promoter of the rat class I ADH gene contains multiple sites where interaction with rat liver nuclear factors occur (6). Previously, we showed that C/EBP binds to and activates the ADH promoter in a sequence-specific fashion (6). In this study, we identified USF as yet another rat liver nuclear protein that interacts with the class I ADH promoter by binding to a different region. We also demonstrated that USF transactivates the same promoter in transient co-transfection experiments.

USF was originally identified as a cellular protein that interacts with the core sequence of 5'–CACGTG–3' between position −55 and −60 within the adenovirus major late promoter (29–32). Since then, the protein has been purified, and extracts from rat liver supported transcription from both promoters (lane 1). When increasing amounts of competing ADH-U oligo were used in the transcription reactions, transcription from both promoters was inhibited (lanes 2 and 3, Fig. 7A). The inhibitory effect of the ADH-U oligo on transcription from the major late promoter was specific, as another oligonucleotide, ADH-E, which specifies the C/EBP binding site within the ADH promoter (6), selectively inhibited transcription from the ADH promoter but not from the major late promoter in similar transcription reactions (lanes 4 and 5, Fig. 7A).
its DNA binding properties have been characterized (49–52). It appears to exist in cells in two major forms, both of which are heat-stable proteins capable of interacting with the major late promoter (49). More recently, a full length cDNA encoding the human USF has been isolated, and its entire sequence has been determined (40). Not surprisingly, based on its DNA-binding characteristics, USF belongs to the rapidly growing family of DNA binding proteins that contain the basic helix-loop-helix motif (18). Interestingly, while USF is present in diverse cell types (33), it has been found to regulate the expression of an increasing number of genes that are tissue-specific. In this regard, it is fitting that expression of the liver-specific rat class I ADH gene is also regulated by USF. It is quite possible that USF exerts its effect cooperatively with other transcription factors whose expression is more restricted to a given tissue. The potential ability of USF to form heterodimers with other members of the bHLH proteins supports this hypothesis (40).

Recent publications indicate that USF is not the only cellular protein that binds to the MLP sequences. Several cellular factors that are distinct from USF, including TFE3 (26), TFEB (27), and BAP (53), have been identified. Of these, at least two (TFE3 and BAP) are present in the liver. In fact, based on their molecular characteristics, BAP and TFE3 may be the same protein (26, 53). However, the factor we identified that interacts with the E box homology within the rat class I ADH promoter is most likely USF. We based our conclusion on a number of observations. First, the MLP oligo competed away all DNA-protein complexes formed between rat liver nuclear extracts and labeled ADH-U oligo. This differs from the findings of Kugler et al. (53) which showed that the MLP oligo could compete for only one of two DNA-protein complexes formed between rat liver nuclear extracts and a labeled oligonucleotide containing a site within the vitellogenin gene promoter that has the core sequence of 5'-CACATG-3'. Second, HeLa cells contain factors that recognize the ADH-U oligo, and interaction of the two could be efficiently competed away with the MLP oligo. Third, antisera directed against USF disrupted the DNA-protein complexes formed between rat liver nuclear extracts and labeled ADH-U oligo. Lastly, methylation interference studies of the mouse Adh-1 gene promoter using rat hepatoma nuclear extracts and labeled ADH-U oligo. This differs from the findings of Kugler et al. (53) which showed that the MLP oligo could compete for only one of two DNA-protein complexes formed between rat liver nuclear extracts and a labeled oligonucleotide containing a site within the rat promoter. This difference in nucleotide is not a consequence of sequencing error as we have repeatedly sequenced DNA in this region of the rat promoter (data not shown). Whether this single nucleotide change results in any functional differences in interaction between USF and its binding site present in the class I ADH gene promoters of the three species remains to be tested.

Expression of the class I ADH genes is regulated by a number of hormones under physiologic conditions (56–60). With the exception of the glucocorticoids (61), the mechanism by which these hormones regulate class I ADH expression remains unclear. For example, growth hormone clearly activates transcription of the rat class I ADH gene (57), but the cellular factor that mediates its effect has not been identified. In order to understand the molecular basis by which various hormones regulate expression of the ADH gene, we recently began to characterize the transacting cellular factors that interact with its promoter. Our previous study indicates that C/EBP activates the promoter of the rat class I ADH gene (6). In this study, we identified USF as another transcription factor for the same gene. Further study of the direct effect of hormones on activities of these transcription factors may facilitate the elucidation of the molecular mechanism of hormonal regulation of ADH gene expression.

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