Tissue-specific and Androgen-repressible Regulation of the Rat Dehydroepiandrosterone Sulfotransferase Gene Promoter*

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Dehydroepiandrosterone sulfotransferase (Std) catalyzes sulfonation of androgenic steroids and certain aromatic procarcinogens. In rats, this enzyme is selectively expressed in the liver, and its expression is strongly repressed by androgens. DNase I footprinting and electrophoretic mobility shift analyses revealed two hepatocyte nuclear factor-1 (HNF1), three CCAAT/enhancer-binding protein (C/EBP), and one consensus palindromic thyroid hormone response elements within the first 215 base pairs (bp) of the promoter sequence of rat Std. This promoter is normally inactive in fibroblast-derived NIH 3T3 cells. However, overexpression of HNF1 and C/EBP resulted in synergistic activation of the Std promoter in this cell type, indicating essential roles of these two trans-regulators in liver-selective expression of the rat Std gene. On the other hand, point mutations at any one of five cis elements proximal to the −215 bp region markedly reduced reporter gene expression, suggesting that all of these sites are important for overall promoter function. Androgen repression of the Std gene in rat liver can be recapitulated in androgen receptor (AR)-negative HepG2 hepatoma cells after cotransfection with an AR expression plasmid. Functional assay of a nested set of 5′-deleted promoters mapped the negative androgen response region between positions −235 and −310. Antibody supershift and oligonucleotide competition identified three OCT-1 and two C/EBP elements between bp −231 and −292. An additional OCT-1 site was found to overlap with a C/EBP element at the −262/−252 position. Mutational inactivation of any one of five cis elements within the −231/−292 region abolished negative androgen response. However, none of these cis elements showed DNase I protection by recombinant AR in footprinting assay, suggesting the absence of a direct AR-DNA interaction. Thus, these studies on rat Std promoter function indicate that (i) HNF1 and C/EBP are responsible for liver specificity of the rat Std gene; (ii) androgen repression of the gene requires the presence of all of the OCT-1 and C/EBP elements between positions −231 and −292; and (iii) AR may exert its negative regulatory effect indirectly through transcriptional interference of OCT-1 and C/EBP rather than through a direct DNA-AR interaction.

Dehydroepiandrosterone sulfotransferase (EC 2.8.2.2) is a cytosolic sulfoconjugating enzyme that catalyzes sulfonation of a number of endogenous hydroxysteroids as well as polycyclic xenobiotics such as certain aromatic carcinogens (1, 2). Preferred endogenous substrates for this enzyme include dehydroepiandrosterone (DHEA), various androgenic hormones, and bile acids. Sulfate conjugation renders these substrates biologically nonfunctional. Thus, the sulfated forms of testosterone and 5α-dihydrotestosterone (DHT) are receptor-inactive, and sulfonated drugs and xenobiotics are mostly devoid of biological activity (1, 3–5). However, in contrast to the inactivation of steroids and drugs, sulfonation enhances the carcinogenic/mutagenic potential of a group of polycyclic aromatic hydrocarbons by converting them to DNA-reactive metabolites (6, 7).

We showed earlier that the androgen sensitivity of the rat liver is reciprocally correlated with the hepatic expression of the dehydroepiandrosterone sulfotransferase gene (designated as Std), and the androgen-mediated down-regulation of Std ensures that a maximum state of androgen responsiveness of the liver could be attained during the animal’s post-pubertal young adult life (8–10). Furthermore, using a transiently transfected cell culture system, we have recently provided evidence that Std can attenuate androgen receptor function as reflected by the loss of androgen induction of the probasin gene promoter (11). This finding lends further credence to a physiological role of Std in modulating androgen sensitivity in selected target tissues.

Mammalian Std expression is under tissue-specific and hormonal control. The rodent enzyme is synthesized exclusively in the liver, whereas in humans, both the liver and adrenal cortex are the major sites of its expression, although minor amounts of Std are also expressed in the intestine and several other tissues (1, 2, 12, 13). The liver and intestinal expression of Std relates primarily to the metabolic inactivation of drugs and steroids and to the bioactivation of aromatic carcinogens, while the adrenal Std acts on locally synthesized DHEA. The significance of the high plasma levels of DHEA and DHEA-sulfate in human physiology remains unclear, although they are thought to be linked to the risk factors for neoplasia and cardiovascular diseases and to the humoral regulation of pregnancy, and they may also play roles in organizing the neocortex in developing brain (1, 14). Interestingly, a number of human breast cancer cell lines exhibit altered Std expression, suggesting that Std

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2 The abbreviations used are: DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; HNF, hepatocyte nuclear factor; C/EBP, CCAAT/enhancer-binding protein; AR, androgen receptor; nARR, negative androgen response region; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; ARE, androgen response element; CAT, chloramphenicol acetyltransferase; bp, base pair(s).
activity may contribute to the differences in sex steroid sensitivity of the normal versus neoplastic breast tissue (15). Since DHEA also acts as a prohormone in the synthesis of androgens and estrogens in peripheral tissues (16), the suggested role of Std in the local modulation of tissue androgen sensitivity is especially intriguing. In the rodent liver, the Std gene is regulated by both androgens and growth hormone. Std is expressed at severalfold higher levels in females than adult males, and either hypophysectomy or androgen supplementation following ovariecotomy causes a marked down-regulation of this gene in the liver (9, 17, 18).

In this article, we describe identification and characterization of the regulatory elements that provide the liver-specific and androgen-repressible regulation of the rat Std gene. Multiple cis elements cognate to the liver-enriched hepatocyte nuclear factor-1 (HNF1) and CCAAT/enhancer binding protein (C/EBP) are needed for appropriate expression of this gene, and both HNF1 and C/EBP are essential for the liver-selective promoter activity. Our results also show that androgenic inhibition of the Std promoter occurs in the absence of any identifiable binding site for the androgen receptor (AR) within the negative androgen response region (nARR) and that a composite interaction involving multiple OCT-1 and C/EBP elements is responsible for the mechanism of the hormonal repression.

**EXPERIMENTAL PROCEDURES**

**Nuclear Extract and DNase I Footprinting**—The liver nuclear extract was prepared as described by Hattori et al. (19) with minor modifications (20) and was dialyzed against 20% (v/v) glycerol, 20 mM HEPES, pH 7.6, 0.1 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium molybdate. The nuclear lysis buffer and all subsequent solutions contained 2 μg/ml each of aprotinin, leupeptin, and bestatin as protease inhibitors, and all manipulations were performed at 2–4 °C.

The end-radiolabeled DNA fragment (50,000 cpm, 10 fmol) was incubated with 50 μg of nuclear extract in a 50-μl reaction mixture at 10 mM HEPES, pH 7.6, 60 mM KCl; 5% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 2 μg of poly(dI-dC)-double-stranded DNA. The reaction was preincubated for 10 min at room temperature without the DNase I and then treated with the labeled DNA for 20 min, and the reaction was continued on ice for 30 min. The reaction mixture was then brought to 1 mM CaCl2 and 5 mM MgCl2 and incubated at room temperature for 1 min, after which the protein-bound DNA was digested with 0.02–0.1 μg of DNase I (30 to 2 min, room temperature) under standard buffer conditions (20). For incubations with BSA, 10-fold less DNase I was used. Digested DNA fragments were extracted with phenol/chloroform and analyzed on a sequencing gel.

**Electrophoretic Gel Mobility Shift Assay (EMSA) and Antibody Supershift**—The 32P-labeled double-stranded DNA probe (50,000 cpm, 10 fmol) was incubated with nuclear extract (5 μg) under previously described conditions (21). For oligonucleotide competition, the unlabeled homologous or heterologous DNA was added during preincubation. In supershift assays, the nuclear extract was preincubated with the antibody (1–3 μg) for 10 min before addition of the DNA probe. Antibodies to HNF1β and HNF1-β were gifts from Dr. Gerald Crabtree, and the antibodies to other transcription factors were purchased commercially (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Bacterial Expression of Recombinant AR and OCT-1 as Thioredoxin Fusion Proteins**—The expression vectors pThioHis and pTrxFus (Invitrogen, CA) were used for Escherichia coli expression of rat AR and human OCT-1, respectively. The 1.1-kilobase pair rat AR cDNA containing the DNA-binding and ligand-binding domains of AR was generated by PCR using the full-length AR cDNA (a gift from Dr. S. Liao) as a template. The 1.1-kilobase pair rat AR cDNA was cloned at the BglII site of pThioHis to create pThioHis-AR, which contains an in-frame fusion of the rat AR cDNA with the His-Tag thioredoxin start sequence. The 2.3-kilobase pair full-length OCT-1 cDNA (gift from Dr. W. Herr) was cloned into the BamHI site of pTrxFus to create pTrxFus-OCT-1, which has OCT-1 and thioredoxin cDNAs in the same reading frame. Thioredoxin-AR was expressed in the E. coli strain TOP10 by induction of the log phase bacterial culture with 1 mM isopropyl-1-thio-β-ν-galactopyranoside (room temperature, 7 h). Harvested cells were lysed by sonication and freeze-thaw. The supernatant of the cell lysate was purified by affinity chromatography on ProBond™ (Invitrogen). The expressed fusion protein was analyzed by gel shift and antibody supershift assays, using a 41-base pair androgen response element (ARE) from the tyrosine aminotransferase gene (22) and an anti-AR antibody (JAR C-19, Santa Cruz Biotechnology), which was raised against the C-terminus epitope of AR. For OCT-1 expression, the E. coli strain G1724 was transformed with pTrxFus-OCT-1, and the transformed bacteria were grown at 30 °C to mid-log phase and induced with tryptophan (100 μg/ml) at 37°C for 4 h. Harvested cells were osmotically shocked, and the supernatant from the cell lysate was used as the source for OCT-1. The expressed fusion protein was characterized by Western blot using anti-OCT-1 antibody (Santa Cruz Biotechnology).

**Plasmid Constructs and Site-directed Mutagenesis**—A nested set of 5′-deleted promoters were prepared from the −1970/+38 rat Std gene fragment by PCR, using the plasmid pSMPA-CAT as the template. The PCR products were subcloned in the reporter vector pSVOCAT-reverse (8) at the SsiI site. The sitesensitive marker protein SME-2, initially identified as an age-dependent liver protein, was later established to be DHEA-sulfotransferase, and thus the SME promoter is equivalent to the Std promoter (8, 23, 24). The PCR products and the promoter/vector junctions were authenticated by DNA sequencing.

Point mutations were introduced at specific regulatory elements by PCR-mediated splicing after overlap extension (25). In initial steps, the left arm of the PCR product was generated from the wild type template, using a vector-derived sense primer and an antisense primer containing desired base changes corresponding to a specific protein-binding site of the Std promoter; similarly, the right arm of the PCR product was generated using the sense primer containing the mutant oligo sequence and the vector-derived antisense primer. Amplified DNAs were gel-purified, and vector-based sense and antisense primers were used to splice the left arm and right arm DNA products by overlap PCR. Mutant oligonucleotides for the individual sites (mutant bases in boldface type and underlined) are as follows: A, GAGGATCTCAGATTCTTTTAACT; B, AGTACTGGTTCAGTGCGACT; C, GTGTGGCTCTCGATTATTTATTC; D, GTGAAAGGCATATTATTATTTCT; E, GTGTGGCTCTCGATTATTTATTC; F, AATTATTTATTTATTC; G, CCTTTGATGCAGAGAGCTATGC; H, AAGGATCTCAGATTCTTTTAACT; I, AATTATTTATTTATTC; J, CCTTTGATGCAGAGAGCTATGC. The vector-derived sense primer, TCTGTCTCAGTGCCTGATCT, and the vector-derived antisense primer (located within the chloramphenicol acetyltransferase (CAT) coding sequence), 5′-CGGTTACCTATAGTATGTCGACCA-3′, were used. The amplified DNAs were sequenced prior to their subcloning into pSVOCAT-reverse.

**Transfection and Enzyme Assay**—The cell lines were from ATCC and were grown either in Dulbecco’s modified Eagle’s medium/F-12 (1:1) with 5% fetal bovine serum (HepG2 cells) or in minimum essential medium with 10% fetal bovine serum (NIH 3T3 cells). The cells were seeded at 5 × 10^5/well in six-well plates (Falcon), cultured overnight, and transfected with plasmid DNAs by calcium phosphate-DNA coprecipitation (26). The CAT-plasmid was at 2 μg/well, and expression plasmids for HNF1-α (pBSV-HNF1-α; a gift from Dr. Gerald Crabtree), OCT-1, and C/EBP-α (pMSV-C/EBP-α; a gift from Dr. Steven McKnight) were each at 1 μg/well. The DNA amount per well was equalized to a total of 4 μg by adding the vector plasmid. Cells were harvested 36 h after transfection, and CAT activity in the cell extract was assayed using C-chloramphenicol and n-butylryl coenzyme A substrates. Radiolabeled products were quantified by liquid scintillation spectrometry of xylene-extracted n-butylryl chloramphenicol. Alternatively, reaction products were separated by thin layer chromatography on a silica gel plate (Sigma) and visualized by autoradiography. The protein content was measured by the Bradford assay (27).

**RESULTS**

**Liver-specific and Androgen-repressible Activity of the Rat Std Promoter in Transfected Cells**—In our earlier studies, we observed that the rat Std promoter from −170 to +35 is functional in hepatoma but not fibroblast-type cell lines (8). In this article, we show that the liver-specific activity of this gene is retained even with a much shorter promoter fragment as evident from Fig. 1A, both −1023/+38 rat Std-CAT and −215/+38 rat Std-CAT plasmids are active in expressing CAT in transfected HepG2 (human hepatoma) but not NIH 3T3 (mouse fibroblast) cells (lanes 1 and 2 versus lanes 5 and 6), although both cell types could support the ubiquitous expression of the SV40 viral promoter (lanes 4 and 8). The rat Std promoter is
also inactive in several other nonhepatocytic cell lines such as COS-1, T47D, LNCaP, and HeLa, which are derived from diverse tissues. Thus, the DNA sequence between −215 and +38 contains sufficient regulatory information for the liver-selective transcriptional activation of the Std promoter.

Androgen-dependent down-regulation of Std gene expression observed in vivo in the rat liver (9,10) can be recapitulated in transfected liver cell lines. As shown in Fig. 1B, upon cotransfection of HepG2 cells with an AR expression plasmid and the Std-CAT reporter construct, CAT gene expression directed by either the −1970/+38 or the −1023/+38 promoter fragment was inhibited by 80% in the presence of 10⁻⁸ m DHT. Androgenic inhibition was also observed at 10⁻⁹ m DHT, albeit to a lesser extent. Because of the higher level of steroid-metabolizing enzymes in the liver and liver-derived cells, generally a higher level of the hormonal ligand is necessary for a maximal hormonal response in these cell lines. Selectivity of AR-mediated trans-repression of the Std promoter was established by the observed lack of any inhibitory effect by dexamethasone, progesterone, or estrogen in HepG2 cells cotransfected with the corresponding receptor expression plasmids. Interestingly, a DNA-binding mutant of AR, which carries a single amino acid substitution (Ala → Thr) at the 579-position, also failed to mediate the DHT-dependent down-regulation of the Std promoter (Fig. 1B, inset), indicating the important role of this domain of the receptor in the negative hormonal regulation.

Liver-specific Regulatory Elements within the Std Promoter and Authentication of HNF1 and C/EBP Binding Sites—The regulatory elements conferring the liver-specific activity to the Std gene were initially investigated by DNase I footprinting of the −215/+38 promoter in the presence of rat liver nuclear extracts (Fig. 2). Results show five strong footprinted regions at −325/−55 (site A), −58/−78 (site B), −92/−113 (site C1), −120/−138 (site C2), and −173/−193 (site D). Same DNA sequences were also protected for the antisense strand. However, no gender- and age-dependent differential nucleosome protection of the −215/+38 DNA was detected (lane 2 versus lanes 3 and 4), indicating that this promoter fragment lacks the necessary cis elements that confer the age- and sex-dependent regulation of Std gene expression.

The site A (−325/−55) shows close similarity to the HNF1-binding elements of a number of hepatocyte-specific genes (28), and sites B (−58/−78), C1 (−92/−113), and C2 (−120/−138) show 100% homology to the C/EBP consensus element ((G/A)TTGCG(C/T)AA(C/T) or T(G/T)NNG(C/T)AA(T/G)) (29,30). In addition, the sequence GTTACAATATTTAT from positions −102 to −105 shows an overall similarity to the prototypic HNF1-binding sequence, despite having the half-sites separated by two bases instead of one; interestingly, examination of the promoters of a series of HNF1 target genes also revealed occasional examples of variable spacer bases within an HNF1 element (28). Finally, the D site at the −173/−193 footprinted sequence is identical to the TREalt, an inverted repeat of the core hepatocyte consensus sequence (AGGTCA) that is activated by the thyroid hormone receptor, retinoic acid receptor, and the retinoid X receptor (31).

The putative cis-acting sites summarized in Table I were authenticated by oligonucleotide cross-competition and antibody supershift analyses (Figs. 3-5). Fig. 3 shows that the binding site A yielded multiple gel-retarded complexes (lanes 1), which were competed out with an excess of the unlabeled homologous sequence as well as the HNF1 consensus sequence (lanes 2 and 4) and not by the heterologous D element (lane 3). Moreover, as evident from the antibody supershift data, the major component of the A complex (marked with an asterisk) specifically immunoreacted with the antisera to either HNF1-α or HNF1-β (lanes 6 and 7). Three additional complexes with faster electrophoretic mobilities are most likely the proteolytic fragments of HNF1 that are not recognized by the epitope-specific HNF1 antibodies. Intensities of these additional bands varied in different batches of nuclear extracts, possibly due to differences in the extent of proteolysis.

Binding of C/EBP-α and C/EBP-β to site B was established in a similar manner (Fig. 3, lanes 9–15). The B complex was competed out by homologous and consensus C/EBP sequences (lanes 10 and 11) but not by the D site oligonucleotide (lane 12). The closely migrating multiple DNA-protein complexes at site B may result from different isoforms of C/EBP-α and C/EBP-β, which arise due to translation initiation at the multiple iner-
nul AUG sites of the C/EBP-α and C/EBP-β mRNAs (32). The anti-C/EBP-α yielded one supershifted band (arrowhead, lane 13); the anti-C/EBP-β yielded two supershifted bands (arrowheads in lane 14), one of which migrated similarly to the anti-C/EBP-α-mediated upshifted band of lane 13. The immune recognition is specific, since the nonimmune serum did not recognize any of these bands (lane 15). C/EBP-α and C/EBP-β also bind to the C1 and C2 sites (Fig. 4). The upper band within the C1 complex was selectively supershifted by the C/EBP-α antibody (the band with an asterisk, lane 8). The C/EBP-β antibody specifically removed the two lower bands (lane 10). When the assay included both α and β antibodies, most of the C1 complex was supershifted (lane 9, two arrowheads). Oligonucleotide competition of the C1 complex also shows the presence of a C/EBP element within the C1 site (lanes 2, 3, and 5). The faster migrating band in lanes 1–12 may result from nonspecific DNA–protein interaction. The unlabeled C2 oligonucleotide also competed for protein binding at the C1 complex (lane 4). The presence of a C/EBP site within the C2 element was confirmed from competition and antibody supershift assays of the C2 complex (Fig. 4, lanes 13–19). Since not all bands within the C2 complex were removed by the unlabeled C/EBP consensus oligo (lane 15), it is likely that the C2 complex contains additional proteins that are unrelated to C/EBP. Alternatively, C/EBP may have a higher affinity for the C2 element compared with its consensus sequence.

In agreement with a predicted HNF1 site within C1 (Table I), a minor part of the C1 complex was supershifted by anti-HNF1-α (lane 12) and anti-HNF1-β (data not shown). However, the unlabeled HNF1 consensus failed to compete out the major complex at the C1 site even at a 400-fold molar excess (lane 6). This lack of competition may indicate that either the HNF1-associated complex constitutes a minor part of C1 or the C/EBP-HNF1 complex is poorly resolved from the dimeric C/EBP at the C1 element. As will be described later, an HNF1 binding site within C1 was also evident from the loss of nuclelease protection at site A (HNF1 element) when a competitor C oligonucleotide (with both C1 and C2 sequences) was added in the footprinting assay shown in Fig. 6 (lane 4).

The nuclear protein(s) that specifically interact with the D site to yield a gel-retarded doublet can be competed out by both an unlabeled D element and a TREpal consensus sequence (lanes 2 and 7) but not by several other sequences that were tested as shown in Fig. 5 (lanes 3–6, 8, and 9). The protein cognate to the D site is expressed ubiquitously, since gel shift assay with nuclear extracts from several nonhepatocytic cell lines also produced similarly migrating retarded doublet bands. Preliminary data suggest that the protein bound to the D site is not recognized by the commercially available antibodies to thyroid hormone receptor, retinoic acid receptor, and retinoid X receptor, and delineation of the role of this protein in the functionality of the D element awaits further characterization of this regulatory site.

The HNF1 and C/EBP sites were also authenticated by oligonucleotide competition for the DNase I footprinted sequences

### Table I

#### Putative cis-acting sequences at the DNase I-footprinted sites of the −215/+38 rat Std promoter

| Site | Sequence | Description |
|------|----------|-------------|
| A    | AGAAAGTAATGATTACCTTATTAACT | (HNF1 consensus) |
| B    | AGTTAGTTTCACACAGAAC | (C/EBP consensus) |
| C1   | GACTGTTTTCCAAATTTAACT | (C/EBP consensus) |
| C2   | GAATTGCTCAATAATGACAT | (C/EBP consensus) |
| D    | GGTTGCGGTTGATCAGATCT | (TREpal consensus) |
The unlabeled C/EBP consensus oligonucleotide abolished the protection entirely at the site B, and it slightly reduced nuclease resistance at C1, although the C2 footprint remained fully protected (Fig. 6, lane 5); this may be due either to binding of additional protein(s) at C2 or to differential affinity of C/EBP for sites B, C1, and C2. Failure of the C/EBP consensus element to completely compete out the gel-retarded C2 complex, as shown earlier (Fig. 4, lane 15), is consistent with the competition data presented in Fig. 6. When a competing oligonucleotide containing the −87−140 DNA sequence (designated as C) was used, protection was completely lost at B, C1, and C2 (lane 4). The observation that the C oligonucleotide competed for protein binding at the footprinted A site provides additional support for the presence of an HNF1-binding site within the C1 element. In the presence of the D competitor oligonucleotide, resistance to nuclease digestion was maintained at sites A, B, C1, and C2 (Fig. 6, lane 5) but not at the homologous D site (data not shown).
Activation of the Std Promoter in Fibroblasts by Co-expression of C/EBP and HNF1—Since the proximal \(-215/-38\) Std promoter contains multiple binding sites for HNF1 and C/EBP, the two liver-enriched transcription factors, it was of interest to determine their roles in the liver-specific activation of Std. As seen in Fig. 7A, although expression of C/EBP-\(\alpha\) in 3T3 fibroblasts caused limited activation of the Std promoter, and HNF1-\(\alpha\) expression by itself was almost noneffective, coexpression of HNF1-\(\alpha\) and C/EBP-\(\alpha\) in these cells synergistically enhanced the promoter activity by 70–100-fold over that re-

![EMSA with the D element (–173/–193). The DNA-protein complex at D, appearing as a doublet, was challenged with cold oligonucleotide duplexes at 100-fold excess. Lane 1, no competition; lane 2, D; lane 3, A; lane 4, B; lane 5, C; lane 6, C\(_2\); lane 7, TRP\(_{me}l\) (5'-AAGATTCAGGTCATGACCTGAGGAGA); lane 8, GRE (5'-AGAG-GATCTGTACAGGATGTTCTAGAT); lane 9, AP1(5'-CGCTTGATGA-CTCAGCCGGA).](image)

![Oligonucleotide competition of the DNase I footprints at the –215/+38 Std promoter. The probe was incubated with either 50 \(\mu\)g of RLNE (lanes 2–5) or BSA alone (lanes 1 and 6). The G + A ladder (lane 7) serves as DNA markers. Competitor oligonucleotides were at 200-fold molar excess. Lane 2, no competition; lane 3, C/EBP consensus oligo; lane 4, C oligo (–37/–140) spanning both C\(_1\) and C\(_2\) elements; lane 5, D oligo (–173/–193). The D footprint does not appear as part of this autoradiographic picture.](image)

![Promoter activation from the p(–215/+38) Std-CAT plasmid by transfected HNF1-\(\alpha\) and C/EBP-\(\alpha\) expression constructs. A, 3T3 cells; B, HepG2 cells. Cells were cotransfected with 2 \(\mu\)g of the Std-CAT construct and 1 \(\mu\)g each of the HNF1-\(\alpha\) and C/EBP-\(\alpha\) expression plasmid, either singly or in combination. CAT activity was expressed as counts/min of radioactivity of the xylene-extracted acylated chloramphenicol, normalized to an equal protein amount. Data show -fold activation over either the activity in HNF1 transfected 3T3 cells (A) or basal activity in HepG2 cells (B). Each bar graph represents the average of four (3T3 cells) or three (HepG2 cells) independent transfections, performed in duplicate. The points in the histograms are values from individual experiments. H, HNF1-\(\alpha\); C, C/EBP-\(\alpha\).](image)

![Reduced CAT expression from mutant Std-CAT constructs. Point mutations are depicted by the mt subscript. Data show percentage of CAT activity from mutant constructs relative to the wild type (WT) construct. 3T3 cells were cotransfected with 2 \(\mu\)g of reporter plasmid and 1 \(\mu\)g each of HNF1-\(\alpha\) and C/EBP-\(\alpha\) expression plasmids; CAT activities were normalized to equal protein amounts. Each bar graph shows the average of two independent transfections that are carried out in duplicate.](image)
resulting from HNF1-α expression alone. Functional synergy between these regulatory proteins in the activation of the Std promoter was also observed in other nonhepatocytic cells such as kidney-derived COS-1 and mammary gland-derived T47D. Even in HepG2 cells, expression of either HNF1-α or C/EBP-α increased CAT expression by 2.4- and 10.5-fold, respectively (Fig. 7B). Nevertheless, simultaneous overexpression of HNF1 and C/EBP did not cause any additional increase in the promoter activity, most likely due to a saturating endogenous level of HNF1 in HepG2 cells. Thus, the HNF1 and C/EBP elements are the primary determinants for the liver-specific expression of Std. However, overall activity of the Std promoter is regulated by all five protein-binding elements, since point mutations at either the D site or any one of the several HNF1 and C/EBP sites reduced reporter gene expression by about 80% (Fig. 8).

Regulatory Elements Conferring Androgenic Repression of the Std Promoter—Despite its liver specificity, the 21970/138 Std promoter was not inhibited by androgen in HepG2 cells cotransfected with an AR encoding plasmid. This finding contrasts with the results presented in Fig. 2 showing that the reporter CAT expression from the 21023/138 and 2310/138 promoters is suppressed by as much as 80% in DHT-treated HepG2 cells. To identify the negative androgen response region (nARR) responsible for the hormonal repression, a nested set of 5'-deleted Std promoter fragments were tested for activity in HepG2 cells. Androgenic repression of 70% or higher was consistently observed for the deletion constructs containing up to position 310 of the upstream sequence (Fig. 9). Shortening the promoter from -310 to -235 greatly reduced the inhibitory response. Thus, a nARR appears to be located within the -235/-310 sequence.

DNase I footprinting of a promoter fragment containing the nARR showed an extended nuclease-protected area from -231 to -292 (Fig. 10). Such a long protected region is suggestive of closely spaced multiple protein binding sites, and due to its upstream location from the D element, the -231/-292 footprinted sequence is designated as site E. This upstream site contains several putative binding elements for the liver-enriched bZip transcription factor C/EBP and the ubiquitous POU domain trans-regulator OCT-1 (Table II). The two C/EBP sites (E3 and E4b) are flanked by one downstream (E5) and two
Regulation of DHEA-sulfotransferase Gene Expression

In order to obtain additional insights into the mechanism of the AR-mediated trans-repression of the Std gene at the nARR, we have used bacterially expressed recombinant AR for its potential interaction with this region. As shown in Fig. 11A, the bacterially expressed AR but not the vector-expressed bacterial protein extract produces two retarded bands in the presence of an oligonucleotide duplex containing the ARE of the tyrosine aminotransferase gene promoter (lanes 1 and 2). Both of these retarded bands were specifically competed by an excess of the homologous oligonucleotide but not an oligonucleotide containing the estrogen response element (ERE) of the vitellogenin promoter (lanes 3 and 4). However, only the slower migrating band was supershifted by the polyclonal antibody directed toward a C-terminal epitope of AR. Thus, the faster migrating band may either be due to an AR fragment produced by endopeptidase action or by an alternate translational initiation/termination product that is not recognized by this particular anti-AR antibody. Furthermore, when tested with another well characterized ARE-containing promoter, i.e. androgen-inducible rat probasin (33), the recombinant AR was able to confer DNase I resistance to the androgen response region, which includes ARE-2 (−117 to −140) of this gene. The DNA sequence of ARE-2, shown in Fig. 11B includes a 15-base pair sequence (GGTTCTGGAGTACT; the three bases separating the six-base half sites are shown in lower case) that has a substantial similarity to the consensus ARE (5'-GG(A/T)A-CANNNTGTTC)., as established by Roche et al. (34), using a DNA-binding site selection assay. However, despite its specific interaction with two AREs from two different gene promoters (i.e. tyrosine aminotransferase and probasin), the recombinant AR did not confer any DNase I resistance to the Std promoter within the nARR (Fig. 11C, lane 3). On the other hand, the recombinant OCT-1 rendered protections to three different sites, i.e. −271 to −299, −231 to −254, and −254 to −271 (lane 4). The liver nuclear extract also produced a nuclease-resistant DNA ladder pattern similar to that generated by OCT-1 (lanes 4 and 5), although the extent of protection by liver nuclear proteins was less complete. These results lead us to conclude that there may be no direct interaction of AR with the DNA sequence located within nARR.

Interacting Role of OCT-1 and C/EBP in the Negative Androgen Response—The putative cis elements within the E site, as summarized in Table II, are also shown to bind the cognate transcription factors in vitro (Fig. 12). The E1 and E2 complexes were specifically supershifted by the OCT-1 antibody; similar analysis also identified E3 as an OCT-1 binding site. Binding of C/EBP-α and C/EBP-β (but not C/EBP-γ) at E3 and E4 elements is indicated by the immunoreactivity of the corresponding antibodies to the gel-retarded C/EBP complex that resulted from specific binding of liver nuclear proteins to either E3 or E4 elements (Fig. 12). Competition with homologous and heterologous DNAs also supported the antibody supershift results. Fig. 13 shows that an OCT-1-specific antibody inhibited the formation of the E4 complex, whereas neither the anti- OCT-2 (lane 3) nor nonimmune serum (lane 4) influenced the intensity of the E4 complex. The E4 site also binds to recombinant OCT-1 (lanes 1 and 6), recombinant thioredoxin (lane 2), thioredoxin-fused recombinant AR (lane 3), thioredoxin-fused recombinant OCT-1 (lane 4), and RLNE (lane 5). Recombinant proteins were at 25 µg/lane; RLNE and BSA were each at 50 µg/lane. Numbers at the extreme right mark various base positions within the footprint.
with high specificity (data not shown), further confirming the presence of an OCT-1 element at E4.

Since multiple OCT-1 and C/EBP sites are identifiable within the nARR, it was important to examine which of these elements are potentially involved in the negative androgen regulation of the Std promoter. Androgen repressibility of the wild type −310/+38 Std promoter was compared with that of a series of mutant promoters containing point mutations at the individual protein-binding elements within the −231/−292 E footprint (Fig. 14). For each mutant construct, two or more bases within the core transcription factor recognition sequence of a particular cis element were changed, as described under “Experimental Procedures.” The mutation at E4 was not investigated in this study. As shown in Fig. 14, contrary to the wild type (WT) promoter, which is inhibited by 70% or more in the presence of DHT, mutations within E1, E2, E4, and E5 sequences abolished the negative androgen response. Thus, all of the OCT-1 and C/EBP elements are integrally related to the mechanism underlying androgenic repression of the Std promoter.

DISCUSSION

Androgen sensitivity of target tissues is largely determined by spatio-temporal expression of the androgen receptor, receptor-associated factors, and androgen-activating/inactivating enzymes. Enzymatic activation of testosterone to DHT, its more potent receptor-active form, by 5α-reductase has been extensively investigated (35). However, less is known about selective enzymatic inactivation of androgens in target cells. A number of enzyme-catalyzed modifications such as oxido-reduction by hydroxysteroid dehydrogenase, glucuronidation by UDP-glucuronosyl transferase, and sulfonation by dehydroepiandrosterone sulphotransferase can convert both testosterone and DHT into receptor-inactive forms (5). In earlier reports, we had shown an inverse age-dependent correlation between the expression of Std in the liver and demonstrated further that Std mRNA expression in the liver is down-regulated in androgen-treated rats (8–10, 23). Our recent results on the Std-mediated attenuation of androgen receptor transactivation function in transfected cells also support a role of Std in modulating androgen action (11). In order to delineate the physiologic underpinning of the tissue-selective expression of Std in relation to the role of this enzyme in the regulation of androgen action in the liver, we have characterized the regulatory elements involved in the liver-selective and androgen-suppressible expression of the rat Std gene promoter.

Hepatocyte specificity of gene expression is generally dictated by the interplay of a small number of liver-enriched transcription factors, which include the isoforms of HNF1; HNF3, HNF4, and HNF6; C/EBP-α and -δ forms; and the proline and acidic amino acid-rich bZip proteins such as DBP (36–39). In the case of Std, our results indicate that synergistic interaction between HNF1-α and C/EBP-α is sufficient to activate this promoter in fibroblast-type NIH 3T3 cells. Functional synergy between HNF1-α and C/EBP-α or C/EBP-β has previously been established for the phosphoenolpyruvate carboxykinase gene promoter in mouse hepatoma cells (40). Furthermore, it is known that HNF1 interacts cooperatively with glucocorticoid receptor to influence the liver-specific promoter function of insulin-like growth factor-1 in hepatoma cells (41). In the case of the rat Std gene, it appears that a yet to be characterized nuclear receptor may interact with HNF1 and/or C/EBP to regulate the basal activity of the promoter. This possibility stems from the consideration that the palindromic organization of the AGGTCA motif at the D element is a likely binding site for a member of the nuclear receptor superfamily (31). In addition, in the chromatin context, cross-talk of other liver-enriched transcription factors may play important roles in the liver-restricted Std expression. Functional cooperativity among transcription factors appears to be a general feature for tissue-specific regulation of eukaryotic genes (42).

Results of this study also show that it is possible to mimic the in vivo androgenic repression of Std in cultured HepG2 cells, and progressive deletions of the promoter at the 5’-end enabled us to map a nARR between the −310 and −235 positions. Multiple OCT-1 sites flanking two C/EBP elements have been identified within the nARR. Mutational inactivation of any one of the OCT-1 and C/EBP elements localized within the nARR abolished DHT inhibition, indicating a mechanism that integrates cross-talks among all of the OCT-1 and C/EBP elements. However, based on DNase I footprinting with recombinant AR (Fig. 11), no ARE was identifiable within the entire nARR.

The absence of an ARE at the nARR of the Std promoter
suggests that the ligand-activated AR interferes with the transactivating roles of OCT-1 and C/EBP at the −231/−292 sequence to bring about androgenic repression. Intriguingly, a mutant AR containing alanine to threonine substitution at the 579 amino acid position, which inactivates its DNA binding function, was unable to cause down-regulation of the Std promoter (Fig. 1B, inset). It is possible and likely that such a mutation also causes changes in the overall conformation of the receptor protein. Thus, a specific conformation of the receptor may be necessary for the AR-mediated interference with positive trans-regulations at the nARR. Furthermore, both a weak DNA-protein interaction and a strong protein-protein association, as established for the ADF-AF(HNF1) interaction at the DNA-protein interaction and a strong protein-protein association of the AR-Ets receptor protein. Thus, a specific conformation of the receptor may be necessary for the AR-mediated interference with positive trans-regulations at the nARR. Furthermore, both a weak DNA-protein interaction and a strong protein-protein association, as established for the ADF-AF(HNF1) interaction at the DNA-protein interaction and a strong protein-protein association of the AR-Ets reporter construct is active in DHEA-sulfotransferase Gene Expression

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Regulation of DHEA-sulfotransferase Gene Expression

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