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Functional Analysis of the Transcriptional Promoter for the *CYP1A1* Gene

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In mouse hepatoma cells, the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases the transcription rate of the *CYP1A1* gene, which encodes a cytochrome P-450 enzyme. In this study, we analyzed the DNA region immediately upstream of the *CYP1A1* gene. A domain that extends upstream to nucleotide −166 was found to function as a transcriptional promoter. The promoter was silent when uncoupled from the dioxin-responsive enhancer located farther upstream. DNase footprinting experiments indicated that nuclear proteins interact with distinct domains of the promoter in a TCDD-independent fashion. Mutational analyses indicated that the *CYP1A1* promoter contains at least three functional domains, including a TATAAA sequence, a CCAAT box transcription factor/nuclear factor I-like recognition motif, and a guanine-rich G box.

The widespread environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) elicits a variety of responses, including enzyme induction, immunosuppression, birth defects, and cancer. An intracellular protein, the Ah receptor, binds TCDD saturably and with high affinity and mediates many, if not all, of the biological effects of the dioxin. We envision that many of these effects result from receptor-dependent changes in gene expression (35, 46, 48, 54).

The induction of aryl hydrocarbon hydroxylase activity in mouse hepatoma cells constitutes a useful response for analyzing the mechanism of TCDD action. In these cells, TCDD induces hydroxylase activity by increasing the rate of *CYP1A1* gene transcription in a receptor-dependent fashion (27, 28). The *CYP1A1* gene encodes a form of cytochrome P-450 that catalyzes the oxygenation of polycyclic aromatic hydrocarbons such as the environmental carcinogen benzo(a)pyrene (17). Studies in Ah receptor-defective cells reveal that the transcriptional response requires the formation of a TCDD-receptor complex, followed by the binding of the liganded receptor to a component of the cell nucleus (24, 27, 28).

Gel retardation analyses indicate that the liganded receptor binds to a specific DNA recognition sequence, which is present in multiple copies upstream of the *CYP1A1* gene (5–7, 15, 25, 52). A 500-base pair (bp) domain, located between nucleotides −800 and −1300, contains four receptor-binding sites and functions as a TCDD-inducible, Ah receptor-dependent transcriptional enhancer (13, 13a, 14, 32, 33, 43, 44). Thus, the induction of *CYP1A1* gene transcription by TCDD involves a dioxin-responsive receptor-enhancer system.

Transfection experiments reveal that the receptor-enhancer component of the dioxin-responsive system is inactive unless it is linked to a second component, which acts as a transcriptional promoter (32). Therefore, understanding the mechanism of dioxin action requires knowledge of the organization and function of the *CYP1A1* promoter. Here, we show that the DNA region immediately upstream of the *CYP1A1* transcription start site has the properties of a transcriptional promoter. The promoter interacts in vitro with nuclear proteins in TCDD-independent fashion, consists of (at least) three distinct functional domains, and is inactive when uncoupled from the dioxin-responsive receptor-enhancer system.

MATERIALS AND METHODS

Plasmid construction. The properties of the recombinant plasmids used are summarized below. A detailed account of their construction appears elsewhere (K. W. Jones, Ph.D. thesis, Stanford University, Stanford, Calif., 1989).

Because we use the bacterial chloramphenicol acetyltransferase (CAT) gene as a reporter, all of the plasmids are derived from pSV0cat, which lacks the simian virus 40 (SV40) enhancer-promoter region (23). To eliminate readthrough transcription originating within the vector, we inserted a termination signal at a suitable site upstream of the CAT gene. The terminator cassette was isolated from plasmid pSV0A/LS2 (8) by digestion with *Hin*dIII. The fragment was made blunt ended, and *Nde*I linkers were added. After digestion with *Nde*I, the resulting fragment, which contains a doublet of the polyadenylation signal for the SV40 small-t antigen gene, was inserted into the unique *Nde*I site of various CAT plasmids described below. This positions the terminator cassette upstream of the promoter DNA being analyzed for function, thereby minimizing potential artifacts due to readthrough transcription (see Results).

The plasmids constructed to analyze the function of the *CYP1A1* promoter are derived from the plasmid designated −55cat. This plasmid was constructed by isolating a 2.7-kilobase-pair (kb) *Hin*dIII-*Bam*HI fragment from the deletion mutant pCPMD 15 (33), making it blunt ended, and adding *Sma*I linkers. Digestion of the resulting DNA with *Sma*I and *Pvu*II generated a 1.22-kb fragment that was inserted between the *Pvu*II and *Hin*dIII sites of pSV0cat after conversion of its *Hin*dIII site to a *Sma*I site. The resulting plasmid, designated −55cat, contains the *CYP1A1* sequence from nucleotides −55 to +1022 inserted immediately upstream of the CAT gene. Insertion of the terminator cassette into −55cat generates the plasmid designated −55cat/t. In both plasmids, the upstream end of the *CYP1A1* DNA contains a unique *Sma*I site into which additional DNA sequences can be inserted. For example, to analyze the effect of a dioxin-responsive enhancer (DRE) on promoter activity, we inserted into the *Sma*I site a 202-bp

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fragment containing two binding sites for the liganded Ah receptor. The resulting plasmid, designated −55/DRE/t, contains the 202-bp enhancer element located immediately upstream of the *CYP1A1* promoter.

To analyze *CYP1A1* promoter function, we constructed derivatives of −55/DRE/t. The general approach was to digest the plasmid with *Sma*I and *Ppu*MI and to replace the domain spanning these restriction sites with a different domain, generated by digesting the deletion mutant pCPMD 8 (33) with an appropriate combination of restriction enzymes. These procedures have the effect of regenerating in the recombinant plasmids the native *CYP1A1* promoter sequence, the upstream end of which varies, according to the restriction enzyme used to generate the 5′ end. The recombinants are designated −X/DRE/t, where X indicates the 5′ end of the promoter sequence in nucleotides upstream of the transcription start site.

We used an analogous procedure to construct a series of enhancerless plasmids except that we used plasmid −55cat/t instead of −55/DRE/t to generate the recombinants.

The plasmid used as a spacing control for plasmid −166/DRE/t was constructed by ligating the 130-bp *Pvu*II-*Sma*I insert from pUC18 (56) into the *Sma*I site of plasmid −35/DRE/t. The recombinant, designated −35/DRE/pUC/t, contains the DRE spaced 165 bp upstream of the transcription start site.

Plasmid pHAVcat has been described previously (33). It contains about 2.6 kb of *CYP1A1* DNA (including about 1.6 kb of upstream DNA containing the promoter and enhancer) inserted into the *Hin*dIII site of pSV0cat.

Transfections and CAT assays. Mouse hepatoma (Hepa 1c1c7) cells were maintained in monolayer culture as previously described (40). Plasmid preparation, DNA transfection, and isolation of stable transformants by cotransfection with pSV2neo and selection in G418 were done as previously described (32, 34, 53). CAT assays were done as previously described except that the cell extract was heated (65°C, 10 min) before the assay, and the concentration of acetyl coenzyme A was reduced to 5 mM (13a, 23, 32). Protein concentrations were measured by the method of Bradford (3).

RNase protection analyses. The RNase protection technique was used as previously described (26, 39). To generate a *CYP1A1* antisense RNA probe, the *Sma*I-*Ppu*MI fragment was isolated from −55cat, made blunt ended, and inserted into the *Sma*I site of pGEM3. The resulting plasmid was linearized by digestion with *Bam*HI, and a 140-nucleotide transcript was synthesized by using T7 RNA polymerase and [α-^{32}P]UTP. The transcript contains 10 nucleotides of polylinker, 99 nucleotides of *CYP1A1* sequence, and 31 nucleotides of vector sequence. To generate a γ-actin antisense RNA probe, the plasmid pSP6-γ-actin, which contains human γ-actin cDNA (12), was linearized by digestion with *Hin*fI, and a 145-nucleotide transcript was synthesized by using SP6 RNA polymerase and [α-^{32}P]UTP. The transcript contains 80 nucleotides of γ-actin sequence homologous to the mouse transcript and 65 nucleotides of nonhomologous sequence. The γ-actin probe was used to control for possible differences in loading among the RNA samples analyzed.

DNase protection analyses. A 2.5-kb *Pst*I-*Ppu*MI fragment was isolated from plasmid −166/DRE/t; after treatment with alkaline phosphatase, the DNA was labeled by using polynucleotide kinase and [γ-^{32}P]ATP. The DNA was then digested with *Nde*I, and the 463-bp *Nde*I-*Ppu*MI fragment, containing the enhancer plus the promoter sequence from nucleotides −166 to +44, was used in the footprinting studies. Nuclear extracts from uninduced and TCDD-induced cells were prepared as previously described (5). The DNase footprinting technique was used as previously described (16, 45), with the following modifications: the reaction was in 50 μl of 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–100 mM KCl–1 mM dithiothreitol–1 mM EDTA–6% glycerol–1.4 μg of poly(dI-dC)–2.5 mM $MgCl_2$–25 μg of nuclear extract protein. Digestion with 0.5 U of DNase I (Worthington Diagnostics) was for 20 s at 25°C. The reaction was stopped as previously described (45).

TABLE 1. Mutant primers

Mutated domain	Mutant primer[a]	Restriction site in primer
TATA box	5′-CACAGAGTCCCTGCAGGGTG-GTGGTGCC-3′	*Pst*I
Proximal CTF/NFI	5′-TGGGCTCAGATAACGACGCG-TGTACGTATGACACAGAGTCC-3′	*Sna*BI
Distal CTF/NFI	5′-GGTATCCGGTATTCCTTCTA-CTAGTTCTCCCCCCT-3′	*Spe*I
G box	5′-GGCTTCTTGCCTATCTATCG-ATTACTCATTGCCTAGAGC-3′	*Cla*I
Distal CTF/NFI/G box	5′-GGTATCCGGTATTCCTTCTA-CTAGTTCTATCGATTACTCA-TTGCCTAGAGC-3′	*Spe*I-*Cla*I

[a] Mutated bases are underlined.

In vitro mutagenesis. The general approach has been described previously (57). To prepare the single-stranded DNA template, a 459-bp *Hin*dIII-*Ppu*MI fragment was isolated from the deletion mutant pCPMD 9 (33), the *Ppu*MI site was converted to a *Sma*I site, and the DNA was inserted between the *Hin*dIII and *Sma*I sites of plasmid M13mp18. Single-stranded DNA was isolated from the recombinant plasmid by standard techniques (4).

The single-stranded template was annealed to a universal M13 primer (M13[−40] primer; New England BioLabs, Inc.) that binds upstream of the *CYP1A1* insert in the recombinant plasmid and to a synthetic oligonucleotide that was mutated at a specific sequence of the *CYP1A1* promoter. The mutant primers used are shown in Table 1. To facilitate subsequent screening for plasmids containing mutated *CYP1A1* DNA, the primers were designed such that the mutated sequence contained a unique restriction site, as indicated in Table 1.

After second-strand synthesis, the DNA was purified by extraction with phenol-chloroform and precipitation with ethanol. The DNA was digested with *Hin*dIII and *Sma*I, and the 459-bp fragment was inserted between the *Hin*dIII and *Sma*I sites of plasmid pUC19 (56). After growth in *Escherichia coli* DH5, the recombinants were screened for the presence of mutant *CYP1A1* DNA by using the restriction enzymes indicated above. Plasmids containing the desired mutation were isolated by standard techniques (36). They were digested with *Rsa*I and *Ppu*MI to release the mutated DNA, and the resulting 211-bp fragment was inserted between the *Sma*I and *Ppu*MI sites of plasmid −55/DRE/t (see above) for functional analysis. The plasmids were sequenced by standard techniques (50).

RESULTS

Measurement of promoter function. Previously, we used an RNase protection technique to identify the start site of *CYP1A1* gene transcription in the mouse hepatoma cells used here (26). Because we have indirectly analyzed the

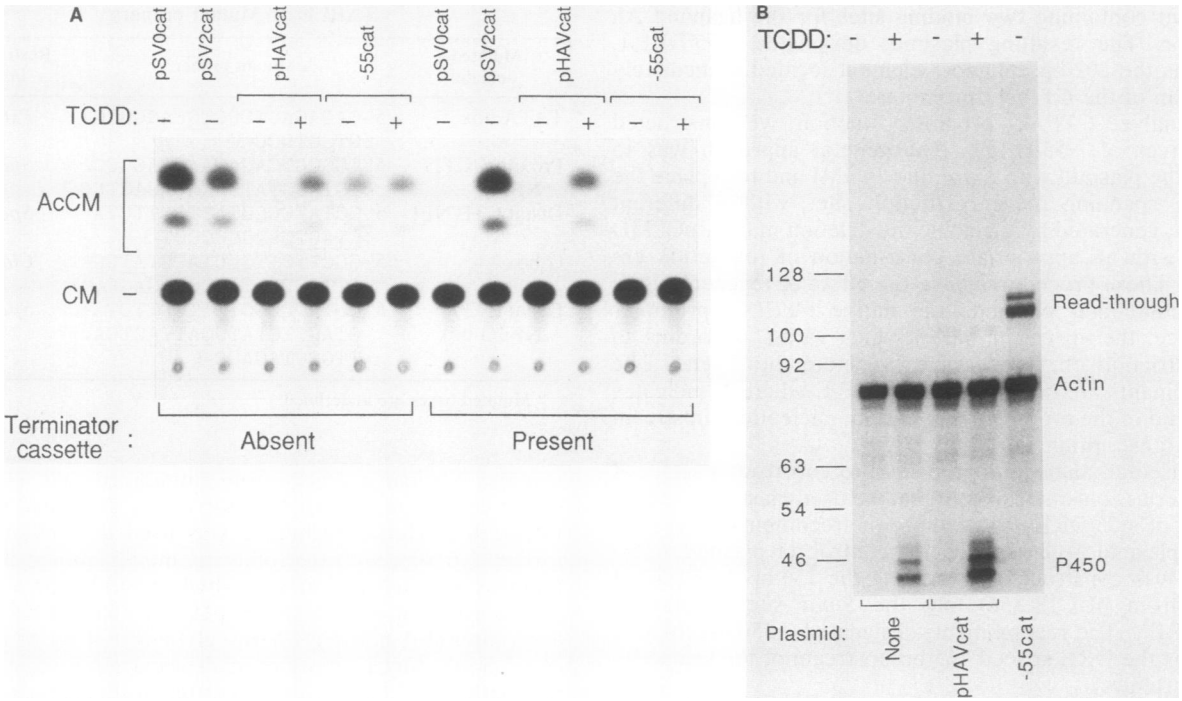

FIG. 1. Reporter gene expression in mouse hepatoma cells. (A) CAT activity. Cells were transfected transiently with the indicated plasmids, and CAT activity was measured in extracts from uninduced (−) and TCDD-induced (+; 1 nM, 16 h) cells. CM, Chloramphenicol; AcCM, acetylated chloramphenicol. (B) RNase protection. Cells were transfected stably with the indicated plasmid, and the transcriptional start site was mapped by using RNA purified from uninduced (−) and TCDD-induced (+; 1 nM, 16 h) cells.

function of the *CYP1A1* promoter by measuring the expression of a reporter (CAT) gene, we performed preliminary studies designed to minimize potential artifacts due to incorrectly initiated transcripts.

Our preliminary studies unexpectedly revealed that the plasmids pSV0cat and pSV2cat could activate the CAT gene to similar extents, even though the SV40 enhancer-promoter region had been deleted from pSV0cat (Fig. 1A). This finding implied that the vector contained other sequences capable of initiating transcription and activating CAT gene expression. RNase protection studies confirmed that spurious initiation does occur and complicates the interpretation of the transfection data. For example, analyses of wild-type mouse hepatoma cells (Fig. 1B) revealed the protection of two TCDD-inducible bands, 44 and 46 nucleotides in length, indicative of correct initiation at the endogenous *CYP1A1* promoter (26). In cells stably transfected with plasmid pHAVcat (see Materials and Methods), these same two bands were increased in intensity, reflecting correct initiation at both the endogenous P-450 gene and the stably integrated P-450–CAT fusion gene (Fig. 1B). Thus, there was no evidence of incorrectly initiated transcripts in cells transfected with pHAVcat. In contrast, analysis of cells stably transfected with plasmid −55cat, which contains less *CYP1A1* 5′-flanking DNA, revealed a major band at 109 nucleotides, indicating the presence of transcripts that originate from within the vector and elongate through the *CYP1A1* DNA into the CAT gene (Fig. 1B). This readthrough transcription was associated with a substantial level of constitutive CAT activity in the cells transfected with −55cat (Fig. 1A).

Therefore, to minimize potential artifacts arising from such readthrough transcription, we inserted a polyadenylation (terminator) signal into the unique *Nde*I site of pSV0cat, pSV2cat, pHAVcat, and −55cat. Transient expression experiments revealed that the terminator sequence abolished the constitutive CAT activity associated with pSV0cat (Fig. 1A). In addition, the terminator sequence increased the CAT activity associated with pSV2cat; this finding may reflect more efficient use of the SV40 enhancer-promoter in the absence of readthrough transcription (Fig. 1A). The terminator sequence has little effect when inserted into pHAVcat, implying that readthrough transcription does not occur with this plasmid (Fig. 1A). These findings are consistent with the RNase protection results (Fig. 1B). In contrast, the terminator sequence abolished CAT activity when inserted into −55cat (Fig. 1A). These findings demonstrate that readthrough transcription can complicate the functional analysis of *CYP1A1* promoter DNA. For example, incorrectly initiated transcripts may contribute to "constitutive" activity observed in previous analyses of the *CYP1A1* promoter (14, 22, 33, 44). For this reason, all plasmids used in the experiments described below contain the terminator sequence, inserted in the analogous *Nde*I site immediately upstream of the *CYP1A1* DNA being analyzed for function. RNase protection experiments confirmed that readthrough transcription does not occur in such plasmids (data not shown; see Fig. 5).

Promoter function is enhancer dependent. The results shown in Fig. 1A indicate that the DNA domain extending to nucleotide −55 upstream of the *CYP1A1* transcription start site has neither constitutive nor TCDD-inducible activity. To determine whether neighboring sequences confer activity upon this domain, we performed analogous experiments using plasmids in which the *CYP1A1* DNA had been extended upstream to nucleotides −166 and −419. In neither case did the recombinant plasmids exhibit constitutive or TCDD-inducible CAT activity in transfection experiments

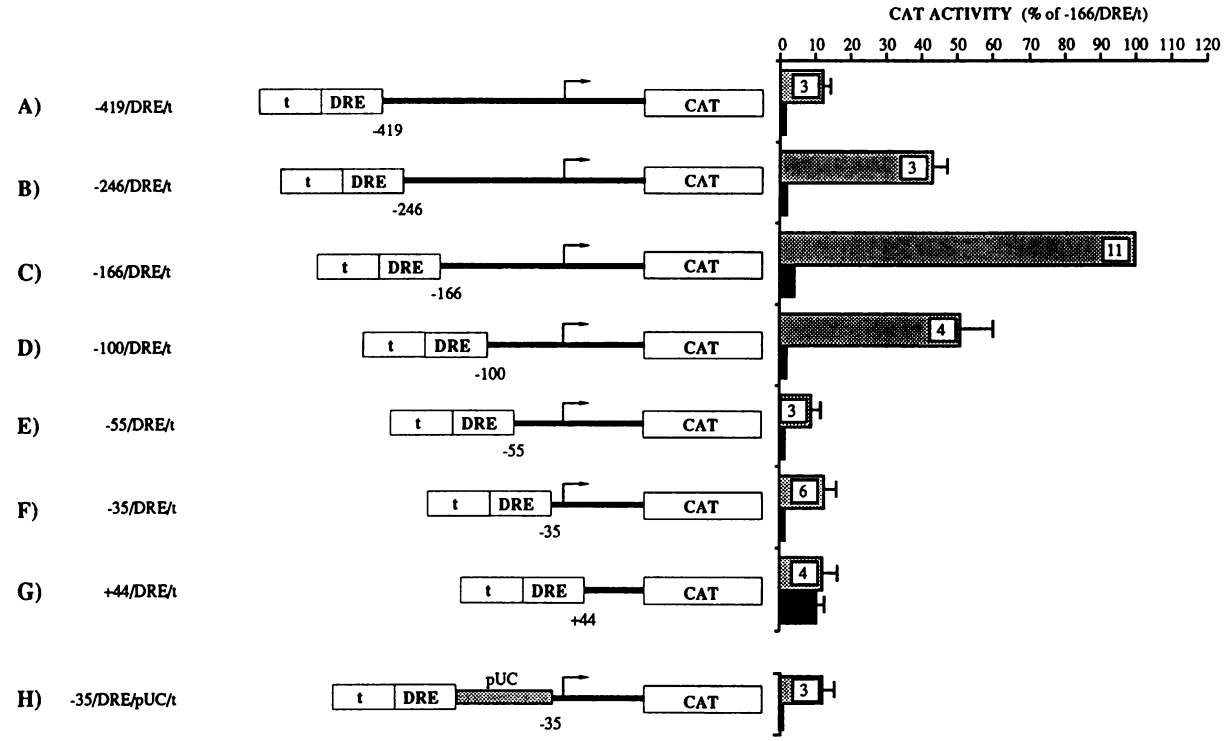

FIG. 2. *CYP1A1* promoter function. Mouse hepatoma cells were transfected transiently with the indicated plasmids, and CAT activity was measured in extracts from uninduced (■) and TCDD-induced (1 nM, 16 h) (□) cells. Numbers on the bar graph indicate the number of experiments performed. Brackets indicate the standard error of the mean. t, Terminator sequence; pUC, pUC 18 sequence.

(data not shown). These findings imply that the DNA located immediately upstream of the *CYP1A1* gene has no intrinsic promoter activity and suggest that additional sequences are important for promoter function.

To test this possibility, we analyzed a series of recombinant plasmids, each of which contains a TCDD-inducible, Ah receptor-dependent enhancer linked to varying amounts of *CYP1A1* promoter DNA. In interpreting these experiments, it is important to realize that the CAT activity in uninduced cells is both enhancer dependent (i.e., it is not observed in the absence of the enhancer) and Ah receptor dependent (i.e., it is not observed in receptor-defective cells). It reflects the presence of an inducer(s) in the cell culture medium (A. G. Miller, Ph.D. thesis, Stanford University, Stanford, Calif., 1982). Thus, "basal" activity in this system does not reflect constitutive activity of the *CYP1A1* promoter. The results shown in Fig. 2 can be summarized as follows. (i) Plasmid +44/DRE/t, which contains the enhancer but no promoter DNA, failed to respond to TCDD (Fig. 2G). Thus, in the absence of the promoter, the enhancer cannot confer TCDD responsiveness upon the target gene. Therefore, the activation of *CYP1A1* gene transcription by TCDD requires two control elements, the enhancer and the promoter. (ii) Plasmids −35/DRE/t and −55/DRE/t, which contain the *CYP1A1* TATAAA sequence but no other obvious promoter regulatory sequence(s) functioned in a TCDD-responsive fashion when the promoter DNA was linked to the enhancer element (Fig. 2F and E). Thus, the TATAAA sequence can function in an enhancer-dependent fashion and in the apparent absence of other promoter components. (iii) Plasmids −100/DRE/t and −166/DRE/t, in which the *CYP1A1* promoter DNA extends upstream to nucleotides −100 and −166, functioned in a TCDD-responsive fashion and activated the CAT gene to greater extents than did the plasmid in which the promoter DNA extends only to nucleotide −55 (Fig. 2D and C). This finding implies the existence of additional DNA domains, located between nucleotides −55 and −166, which increase promoter activity. (iv) Plasmids −246/DRE/t and −419/DRE/t, in which the *CYP1A1* DNA extends to nucleotides −246 and −419, exhibited lower levels of CAT activity than did plasmid −166/DRE/t (Fig. 2B and A). This observation suggests that there are no additional DNA domains upstream of nucleotide −166 that increase promoter activity. The mechanism governing the decrease in CAT gene expression is unknown. The decrease could reflect the existence of a DNA element(s) that inhibits promoter function. Alternatively, the decreased CAT activity could simply reflect the increased distance between the enhancer element and the transcription start site. Our data do not permit us to distinguish between these possibilities.

Effect of spacing between the enhancer and the transcription start site. In principle, the increase in CAT activity observed when the *CYP1A1* promoter DNA is extended upstream to nucleotide −166 (Fig. 2) could reflect a more optimal spatial relationship between the enhancer and the TATAAA sequence rather than the presence of additional promoter components. To address this possibility, we analyzed plasmid −35/DRE/pUC/t, which is analogous to −166/DRE/t in its spacing between the enhancer and the TATAAA sequence but contains 130 bp of pUC18 DNA instead of the 131-bp *CYP1A1* domain spanning nucleotides −35 to −166. The results of transfection experiments (Fig. 2H) indicate that this change in spacing between the enhancer and the transcription start site is not associated with an increase in the level of CAT gene expression. Therefore, we infer that the increase in CAT activity observed when the *CYP1A1* DNA is extended from nucleotide −35 to −166 reflects the

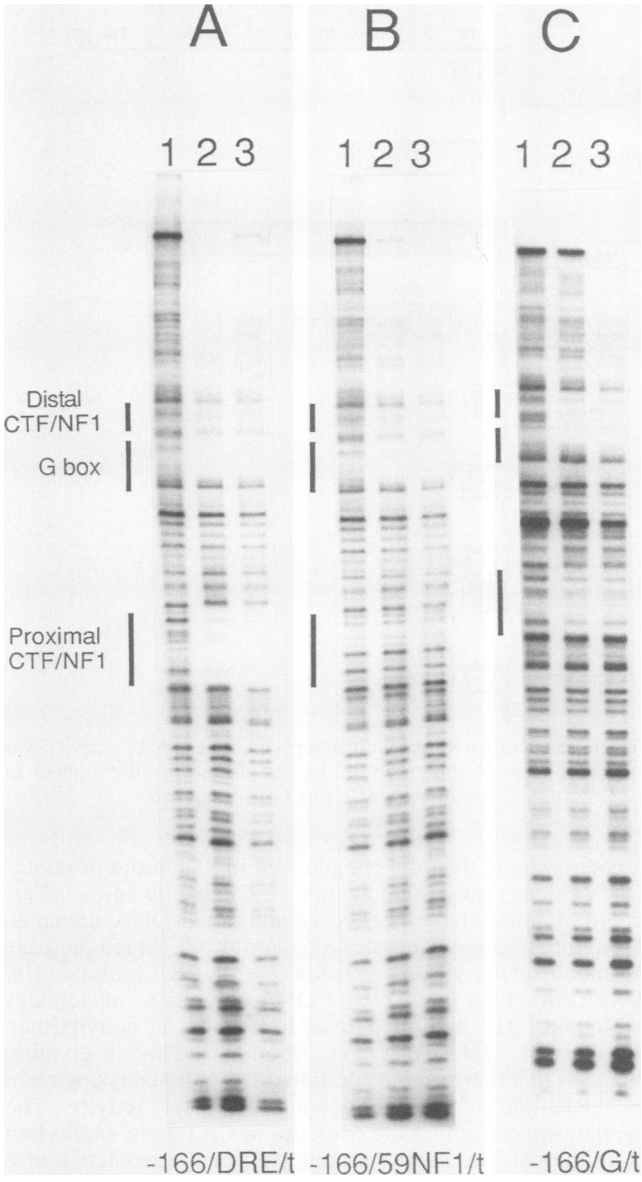

FIG. 3. Protein-DNA interactions at the *CYP1A1* promoter in vitro. The 463-bp *Nde*I-*Ppu*MI fragments from the indicated plasmids were end labeled with ^{32}P, incubated with nuclear extracts from uninduced or TCDD-induced (1 nM, 16 h) mouse hepatoma cells, and analyzed for protein-DNA interactions, using DNase I protection. Lanes: 1, no nuclear extract; 2, nuclear extract from uninduced cells; 3, nuclear extract from TCDD-induced cells.

presence of additional functional elements within this DNA region.

Functional components of the *CYP1A1* promoter. We used a DNase footprinting technique to identify regions of promoter DNA that interact with nuclear proteins and therefore are likely to be functionally important. These studies revealed two regions of DNA that were protected by nuclear proteins from DNase digestion; one spanned nucleotides −45 to −59, and the other spanned nucleotides −110 to −130 (Fig. 3A). It is noteworthy that these protein-DNA interactions did not depend on prior exposure of the cells to TCDD. This observation implies that the promoter-binding proteins are expressed constitutively. Examination of their nucleotide sequences reveals that the proximal footprint contains a sequence that exhibits substantial homology with the recognition sequence for CCAAT box transcription factor/nuclear factor I (CTF/NFI) (31, 47, 51), whereas the distal footprint overlaps a stretch of guanine residues, or G box (1, 19, 29). In addition, sequence analyses revealed two additional potential regulatory elements: the TATAAA sequence, spanning nucleotides −25 to −30, and a second CTF/NFI-like recognition site, spanning nucleotides −131 to −136. To examine their potential contribution to promoter function, we analyzed mutants of these four elements.

Mutation of the proximal CTF/NFI-like recognition sequence led both to a loss of the DNase footprint (Fig. 3B) and to an 80% decrease in the activity of the *CYP1A1* promoter, as measured by the response of the CAT gene to TCDD (Fig. 4B). These findings imply that the binding of a CTF/NFI-like transcription factor to the recognition sequence centered at nucleotide −46 makes a substantial contribution to *CYP1A1* promoter activity.

In contrast, mutation of the distal CTF/NFI-like recognition sequence had no effect on the DNase footprint pattern (data not shown) and had little effect on promoter activity (Fig. 4C). Mutation of the G box, which abuts the distal CTF/NFI-like site, altered the footprint pattern in that the protected region shifted away from the G box and toward the distal CTF/NFI-like site (Fig. 3C). This observation suggests that the loss of protein binding at the G box allows a protein-DNA interaction to occur at the neighboring CTF/NFI-like site. However, this change in the protein-DNA interaction pattern was not associated with a substantial alteration in promoter activity (Fig. 4D). These observations suggested that the G box and the distal CTF/NFI-like site might be functionally equivalent. To test this possibility, we analyzed a double mutant that contains alterations at both sites. The double mutation produced about a 50% decrease in promoter activity (Fig. 4E). These findings imply that both the G box and the distal CTF/NFI-like recognition sequence (together with their cognate binding proteins) have the potential to increase promoter activity. They appeared to be functionally interchangeable, because mutation of both components was necessary to produce a decrease in promoter function.

Mutation of the TATAAA sequence produced about an 80% decrease in promoter activity (Fig. 4F). Because the TATAAA sequence and its cognate binding protein(s) influenced the position at which transcription initiates, we performed RNase protection experiments to analyze the transcription start sites in the wild-type and mutant promoters. The two expected protected bands, 44 and 46 nucleotides in length, were detected in cells stably transfected with a plasmid (−166/DRE/t) containing the wild-type promoter (Fig. 5). In contrast, in cells transfected with the TATAAA mutant (−166/TATA/t), the bands representing correctly initiated transcripts were decreased in intensity and were accompanied by the appearance of two new bands, which represent transcripts initiated 9 and 27 nucleotides upstream of the correct start site (Fig. 5). Densitometric scanning of the autoradiogram revealed that the TATAAA mutation produced about a 75% decrease in the amount of correctly initiated transcripts (data not shown), which is in good agreement with the CAT data (Fig. 4F). These findings indicate that mutation of the TATAAA sequence influences both the strength of the *CYP1A1* promoter and the position of the transcription start site.

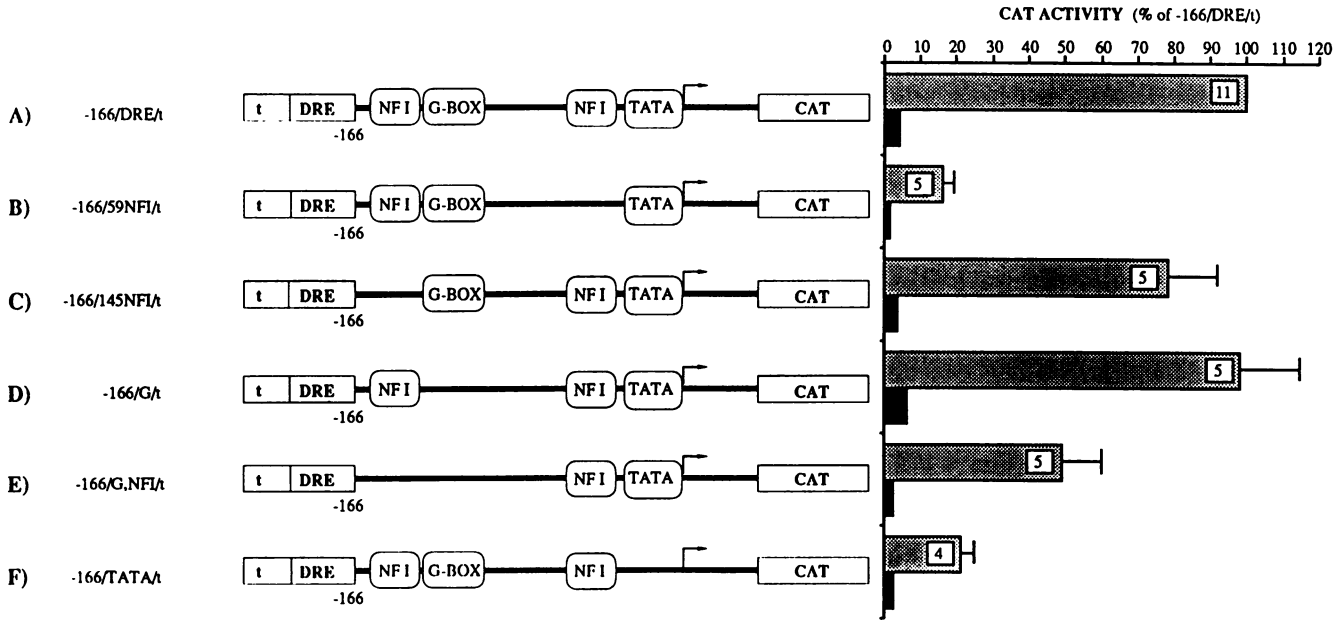

FIG. 4. Function of a mutated *CYP1A1* promoter. Mouse hepatoma cells were transfected transiently with the indicated plasmids, and CAT activity was measured in extracts from uninduced (■) and TCDD-induced (1 nM, 16 h) (□) cells. Numbers on the bar graph indicate the number of measurements made. Brackets indicate the standard error of the mean. t, Terminator sequence; NFI, CTF/NFI-like recognition motif; G box, guanine-rich sequence; TATA, TATAAA sequence.

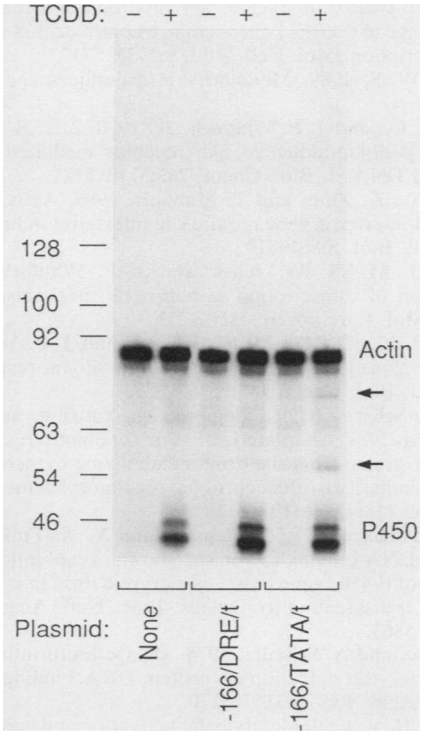

FIG. 5. Initiation of transcription in a TATAAA mutant. Cells were transfected stably with the indicated plasmids, and the transcriptional start site was mapped by using RNA purified from uninduced (−) and TCDD-induced (+; 1 nM, 16 h) cells. Arrows indicate the positions of two new protected RNA species in the mutants. Sizes (in nucleotides) are shown on the left.

DISCUSSION

We have shown previously that the *CYP1A1* transcription start site in mouse hepatoma cells is virtually identical to that reported by others for mouse liver (21, 26). Furthermore, the DNA sequence of the region upstream of the *CYP1A1* transcription start site is very similar in both rodents and humans (20). We infer, therefore, that the observations reported here for mouse hepatoma cells are not peculiar to this experimental system but are applicable to the *CYP1A1* gene in general.

Using cells stably transfected with an analogous plasmid, we demonstrated previously that an increase in the rate of CAT gene transcription accounts for the TCDD-induced increase in CAT enzyme activity (13). Therefore, we assume that in the transient expression studies reported here, the observed changes in CAT activity reflect corresponding alterations in CAT gene transcription. Our findings also indicate that readthrough transcription can introduce artifacts into studies that use transfection and the expression of a reporter gene. In particular, deletion analyses of *CYP1A1* regulatory DNA may not be amenable to unambiguous interpretation if the transcription start site has not been verified.

The control regions for many genes that are transcribed by RNA polymerase II have a modular organization and characteristically contain a linear array of protein-binding sites (10, 30, 37, 41, 49). Thus, in this general sense, the organization of the *CYP1A1* promoter appears typical in that it contains multiple, discrete functional modules. The individual components of the *CYP1A1* promoter (e.g., TATAAA sequence, G box, and CTF/NFI-like sites) also appear in other mammalian promoters; however, it is possible that the particular combination observed here is unique to the *CYP1A1* gene.

The sequence 5′-TATAAA-3′ is common to numerous eucaryotic promoters and represents a binding site for the

transcription factor TFIID, which influences both promoter strength and the position of the transcription start site (2, 18, 38, 42, 49). Our findings are consistent with these previous observations and imply that TFIID (or a related factor) contributes to *CYP1A1* gene transcription. Mutation of the TATAAA sequence is associated with the appearance of two new transcripts, which are initiated 31 and 36 bp downstream of the sequences 5′-CACAGA-3′ and 5′-GATAA-3′, respectively. This finding suggests that these sequences may have the potential to function (albeit weakly) in place of the TATAAA sequence.

The protein designated CTF/NFI is multifunctional and participates in the regulation of adenovirus replication and in the initiation of mammalian transcription. CTF/NFI represents a family of DNA-binding proteins, generated by alternative RNA splicing, that bind to the consensus recognition motif 5′-GCCAAT-3′ (51). Our analyses of the *CYP1A1* transcriptional promoter demonstrate the existence of functionally important protein-DNA interactions at a proximal site, containing the sequence 5′-GCCAAC-3′, and at a distal site, containing the sequence 5′-GCCTAT-3′. The similarities among the recognition sequences suggests that the cognate binding proteins are related to CTF/NFI. However, we have not tested this hypothesis directly. Furthermore, Yanagida et al. have interpreted gel retardation analyses of the rat *CYP1A1* promoter to mean that the proximal site binds a transcription factor distinct from CTF/NFI (55). Thus, this question warrants additional study.

Other investigators have reported that DNA domains containing stretches of guanine residues (e.g., G_6) interact with nuclear proteins both in vitro and in vivo (1, 19, 29). The findings reported here are consistent with these observations. In addition, we have extended the earlier findings by illustrating the functional role of the G box in the context of the *CYP1A1* promoter. Our DNase footprinting studies suggest that the binding of a protein(s) to the G box blocks the binding of a CTF/NFI-like factor to an adjacent site. Furthermore, our transfection studies imply that the protein-DNA interaction that occurs at the G box is functionally equivalent to that which occurs at the neighboring CTF/NFI-like site when the G box is mutated. The functional equivalence of the two interactions might reflect a structural similarity between the respective DNA-binding proteins; this may be an interesting area for future research.

Our DNase footprinting studies reveal that TCDD produces no detectable change in the pattern of protein-DNA interactions at the promoter in vitro. This observation implies that the proteins which bind to the *CYP1A1* promoter are present and active in uninduced cells. If so, these findings impose constraints upon the possible mechanisms of TCDD action. For example, our results argue against the idea that TCDD activates gene expression by altering the profile of promoter-binding proteins present within the cell. In addition, our functional studies indicate that the *CYP1A1* promoter is silent when uncoupled from the DRE. These findings suggest that in the absence of the enhancer, the promoter-binding proteins do not have access to their DNA targets. Therefore, we envision that the enhancer acts to increase the accessibility of promoter DNA to its cognate binding proteins. In fact, exposure of cells to TCDD produces a rapid, receptor-dependent alteration in *CYP1A1* chromatin structure such that the promoter DNA becomes more accessible to restriction endonucleases (9). Furthermore, binding of the liganded Ah receptor to its recognition motif bends the DNA in vitro (11). Therefore, we envision that in vivo, the receptor-enhancer interaction deforms the DNA; the deformation generates a structural alteration in chromatin that extends to the promoter, allows constitutively expressed transcription factors to bind the DNA, and produces enhanced expression of the *CYP1A1* gene.

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