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Location of regulatory elements responsible for drug induction in the rat cytochrome P-450c gene

(DNA transfection/cis-acting element/gene expression/xenobiotic metabolism/inducible enhancer)

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ABSTRACT **The synthesis of cytochrome P-450c is induced remarkably in cultured cells as well as animal tissues in response to added chemicals such as 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzodioxin. To study this mechanism, we joined the sequence of 5′-flanking and upstream regions of the P-450c gene to the structural gene for chloramphenicol acetyltransferase. The fusion gene was introduced into Hepa-1 cells for the assay of the expressed acetyltransferase activity. At least three cis-acting regulatory regions that are responsible for the inductive expression were determined in the sequences from nucleotide −3674 to −3067, from −1682 to −1429, and from −1139 to −1029, relative to the transcription start site, by external deletion analysis. Further detailed analysis of the region (nucleotides −1139 to −1029) most influential on the inducibility revealed that a regulatory element consisting of 10 base pairs termed a drug regulatory element (DRE) and its homologues were tandemly arranged in this region. The consensus sequence deduced from DREs is 5′-C(G/T)(T/A)NGGGCTGGG-3′. The regulatory sequence from nucleotide −1140 to −844 is capable of conferring inducibility on a heterologous promoter in a manner independent of its orientation and distance from the subordinate promoter.**

A family of hemoproteins, collectively termed cytochrome P-450, occurs in liver microsomal membranes, and its members metabolize a number of endogenous substrates such as steroids, prostaglandins, and fatty acids and an almost infinite number of exogenous substances. The wide substrate specificity of the P-450 system results from multiple molecular forms of P-450, each of which shows different but sometimes overlapping substrate specificities (1). Of these P-450s, P-450c especially catalyzes the hydroxylation reaction of polycyclic aromatic hydrocarbons such as benzo[*a*]-pyrene and 3-methylcholanthrene (MC), and it is also present in various extrahepatic tissues (2, 3). This species of P-450 is strongly induced by a number of chemicals, including MC and 2,3,7,8-tetrachlorodibenzodioxin (TCDD). The induction of P-450c is known to be controlled at the level of transcription (4, 5). In the previous study (6), we described a transient assay system that mimics the induction process of P-450c *in vivo* in animal tissues by the administration of MC. We present in this paper the location of three cis-acting regulatory sequences in the 5′ flanking region of the P-450c gene, using the transient assay system. From the analysis of the proximal region, sequence elements of 10 base pairs (bp) that were deduced to be responsible for the induction by MC were found.

MATERIALS AND METHODS

Construction of Deletion Plasmids. Fusion genes with various external deletions were constructed as follows. pMC6.3k (6) was treated with BAL 31 exonuclease after cleavage with *Xho* I. The digested plasmids were treated with T4 DNA ligase in the presence of synthetic *Cla* I linker and then introduced into *Escherichia coli* HB101 to yield intermediate plasmids. These intermediate plasmids were cleaved with *Cla* I and *Bgl* II to generate shortened 5′ flanking sequences with a *Cla* I site at the 5′ end. The fragments were introduced into pMC6.3k by replacing the original *Cla* I–*Bgl* II region. The *Cla* I site in pMC6.3k was previously generated by converting the *Bam*HI site into the *Cla* I site with the linker ligation.

DNA Transfection and Assay of Chloramphenicol Acetyltransferase (CAT) Activity. Transfection experiments were performed essentially as described (6). The CAT activity was determined by the method of Gorman *et al.* (7). All assays were performed within the range of a linear relation of the activities with time of incubation or concentration of extracted proteins. The acetylated forms of [^{14}C]chloramphenicol were separated from unreacted [^{14}C]chloramphenicol by HPLC [46 mm × 25 cm column; 5-μm TSK gel silica-60 particles; mobile phase, chloroform/methanol, 92.5:7.5, (vol/vol)] and then quantified by scintillation spectrometry or detected by autoradiography after separation of the reaction products on TLC.

DNA Sequence Analysis. Sequences were determined by the chain-termination method as described (8, 9).

RESULTS

Location of Sequences Responsible for Drug Induction. To identify functional regions responsible for the induction in the 5′ flanking sequence of the P-450c gene, the initial set of hybrid plasmids with various external deletions was constructed as shown in Fig. 1. The extent of deletions was determined by restriction mapping and by DNA sequence analysis (Fig. 2). These plasmid DNAs were transfected into Hepa-1 cells as described and then the cells were exposed to the added inducer. The maximal MC-induced CAT activity in the cells transfected with pMC6.3k was always higher (1.5-fold) than in those transfected with pSV2-cat (6). This strong activity was decreased concomitantly with progressive deletions starting from the 5′ end of the 5′ flanking region of the P-450c gene (Fig. 1). The CAT activity was markedly decreased when the sequences were removed between nucleotides −3674 and −3067, −1682 and −1429, and −1139 and −1029 relative to the transcription start site. The observations suggested that at least three regions contained essential

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Abbreviations: CAT, chloramphenicol acetyltransferase; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; bp, base pair(s); DRE, drug regulatory element.

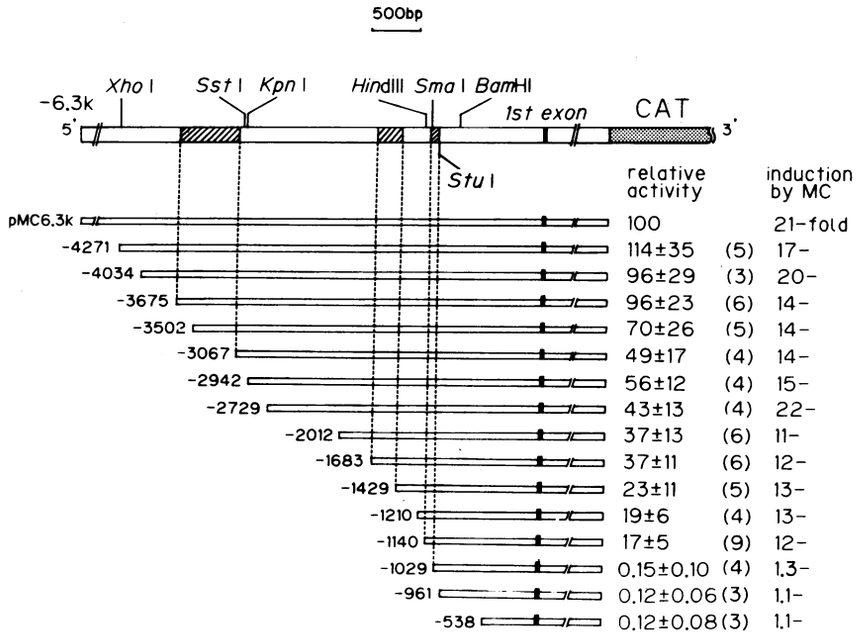

FIG. 1. Structure and activity of P-450–CAT chimeric genes. The DNA fragment from the 5′ end region of the rat P-450c gene used to construct hybrid plasmids with introduced deletions is represented at the top by a wide bar. The CAT coding sequence is denoted by the stippled bar. The DNA sequences whose deletion caused marked reduction in the induced CAT activity are indicated by hatched areas in the wide bar, and the first exon of the P-450c gene, which contains only untranslated leader sequence, is represented by a solid bar. The extent of deletions is shown by the length of narrow bars. Nucleotide positions of the 5′ flanking sequence are numbered in negative from the mRNA start site. The column immediately to the right of the bars gives the CAT activity induced by MC as mean ± SD. The data are expressed as a percentage of the activity obtained with pMC6.3k. The number of separate experiments with each deletion plasmid is designated in parentheses. The extent of induction by MC is also shown by a ratio of the induced activity to the uninduced activity. Representative restriction cleavage sites are shown above the wide bar.

DNA elements responsible for the induced expression of the P-450c gene.

In the previous study (6), we proved by the method of primer extension that the inducible transcription in the cells transfected with pMC6.3k started exclusively at the correct transcription-initiation site. In a series of deletion experiments, we confirmed by the primer extension method with the same primer as used previously (6) that the transcription of the mRNA was always initiated at the correct site (data not shown) and that the CAT activities measured, in fact, paralleled the amount of the CAT mRNA. Therefore, the CAT activity is considered to be a reasonable measure for investigating the transcriptional inducibility of the P-450c gene, although changes in mRNA stability could not be rigorously eliminated.

Regulatory Elements for Drug Induction. Further investigation to disclose the sequence essential for the regulatory function in the region extending from −1139 to −1029 whose deletion caused the most profound effect on the inducibility was carried out by using the hybrid genes with a series of fine deletions. Fig. 3 shows the construction of the hybrid genes and the quantitation of the CAT activities together with the autoradiographic results. Marked decreases in the induced activity were observed when the sequences from −1139 to −1131, −1107 to −1093, and −1092 to −1075 were deleted. Particularly, the deletion of the sequence from −1107 to −1075 resulted in remarkable reduction in the inducible activity. These observations suggested the presence of key sequences for the regulatory function in these regions. When the deletion proceeded further beyond nucleotide −1060, the resulting hybrid genes exhibited no more significant response to the inducer.

In connection with stepwise reductions in the inducibility of the CAT activity, a fine survey of the sequences in the relevant regions allows us to deduce nucleotide sequences responsible for the regulatory function. Possible regulatory DNA elements consisting of 10 nucleotides with a single C or G 12 bp upstream and two Cs or Gs 11 bp downstream from it are frequently distributed in these regions (Figs. 2 and 4). The −1139 to −1131 sequence contained one set of this DNA element as shown in Figs. 2 and 4. Interestingly, the sequence from −1107 to −1075, whose deletion caused profound effect on the inducibility of the gene, contained two sets of this DNA element in tandem; one of them was in reverse orientation. We term this possible regulatory DNA element responsive to drugs or xenobiotic inducers "DRE" (drug regulatory element). Although it has yet to be studied how the DRE sequence is involved in this induction process, a number of DNA sequences analogous to the DRE occur in either orientation in the other part of the 5′ flanking region or in the downstream region from −1029. This downstream region shows apparently no significant response to the inducer (Fig. 3). It remains also to be investigated whether the position, spatial arrangement, or cumulative number of the sequences is important for the inducibility-conferring activity, as is reported for other eukaryotic regulatory elements. As shown in Fig. 5, however, an experiment with tandemly repeated DRE-2 clearly demonstrated that the DRE sequence itself indeed has the potential of the inducibility-conferring activity. The placement of tandem repeats of synthetic decamer of DRE-2 on the 5′ end (*Stu* I site) of the −1033 truncated gene led to recovery of inducibility of the gene in response to inducer, although the induced activity was lower than that of the original hybrid gene pMC6.3k; we detected 4.5-fold induction over the control level. Possible reasons for this low inducibility will be discussed later.

Effect of the Sequence Containing DRE on a Heterologous Promoter. To investigate whether this cis-acting DNA sequence containing DREs acts on heterologous promoter(s), the *Sma* I–*Bam*HI fragment (nucleotides −1140 to −844) was placed on either the 5′ or 3′ end of the chimeric gene made from simian virus 40 early promoter and CAT gene in $pA_{10}CAT$ in both directions (Fig. 6). In all constructions, the inserted regulatory DNA sequence elevated the expression of the CAT gene in response to the addition of MC. The enhancing effect was exerted in a manner apparently independent of distance and orientation of the cis-acting DNA sequence in relation to the subordinate gene. Curiously, however, the regulatory DNA sequence inserted at the 5′ end in the inverted orientation caused a somewhat higher level of the induced activity than that in the correct orientation for unknown reasons. In general, these observations indicate that the inserted cis-acting DNA sequence *per se* is qualified as an inducible enhancer.

Expression of the CAT Activity in Mutant Cells. pMC6.3k was introduced into two derivatives from Hepa-1 cells selected for benzo[*a*]pyrene resistance (11). One mutant (C4) is supposed to be defective in translocation of the cytosolic receptor–inducer complex into nucleus, and the other (C12) is a mutant deficient in the cytosolic receptor; thus, C12

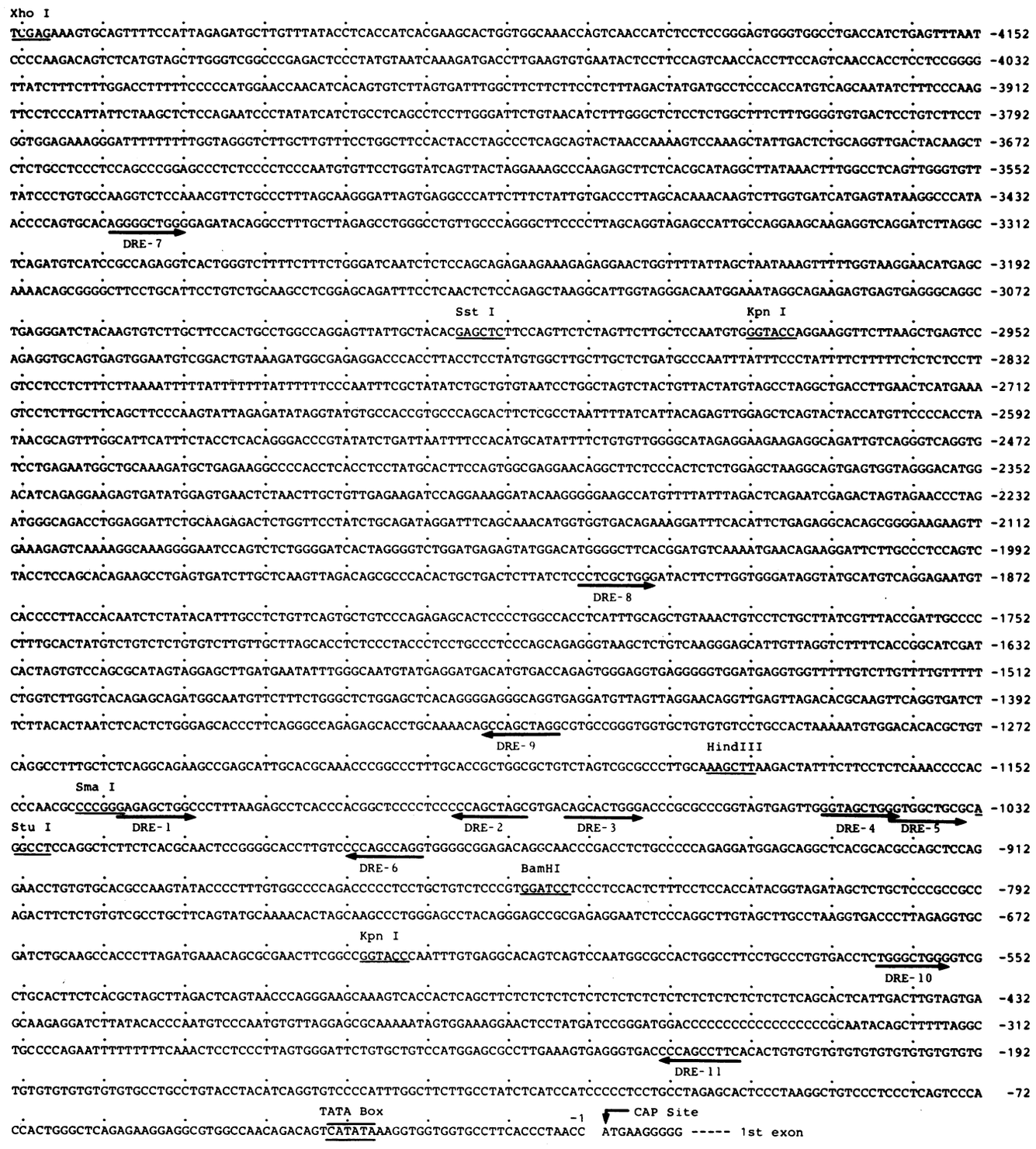

FIG. 2. Nucleotide sequence of the 5′ flanking region of the cytochrome P-450c gene. Nucleotides are numbered in negative from the mRNA start site. Arrows show the position and orientation of drug regulatory elements (DREs). Sequences were determined by the chain-termination method using the M13 vector. Almost all the sequences were determined in both orientations.

shows only about 10% of wild-type receptor levels, and therefore, 10–20% of wild-type aryl hydrocarbon hydroxylase inducibility. Accordingly, MC cannot induce at all the synthesis of endogenous P-450c in C4 and induces it very poorly in C12. The CAT activity in the C4 mutant cells transfected with pMC6.3k was not at all induced by the treatment with MC, as shown in Fig. 7. Only a low level of inducible CAT expression was observed in the C12 cells transfected with pMC6.3k, as expected from the expression of the endogenous P-450c gene; the fully induced CAT activity corresponded to about 2% of the activity in the wild-type cells. In these experiments, pMLCAT (6) and pSV2CAT were used as the negative and positive controls, respectively. These results indicate that the expression of the transfected chimeric gene was regulated in the same way as that of the endogenous P-450c gene.

DISCUSSION

In consideration of the results obtained with mutant cells described here and the previously reported ones of inducer

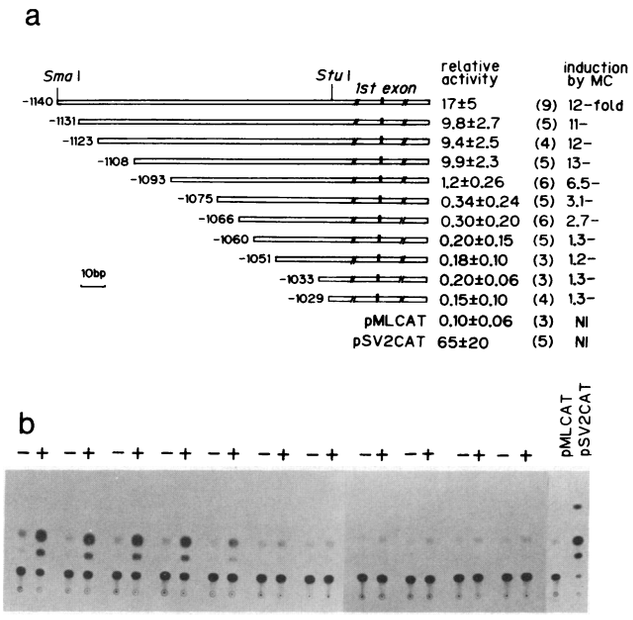

FIG. 3. Chimeric genes with fine deletions and their CAT activities. (*a*) Structure of each external deletion. The column on the right gives the CAT activity induced by MC as mean ± SD with the number of independent experiments shown in parentheses. The data are expressed as a percentage of the activity obtained with pMC6.3k. The extent of induction by MC is also shown in the same way as in Fig. 1. NI, not induced. (*b*) Autoradiogram of the CAT activity produced by the deletion plasmids in *a*. The order of the assay from left to right corresponds to the order from top to bottom in *a*. The + and − symbols indicate the presence or absence of the inducer MC in the culture medium. The products were separated with TLC (chloroform/methanol, 95:5, vol/vol) and exposed to x-ray film at −70°C for 40 hr. Spots, from bottom to top, are chloramphenicol and its 1-acetate, 3-acetate, and 1,3-diacetate derivatives. pSV2-cat (7) and pML-cat (6), which lacks the promoter sequence, were used for the positive and negative controls, respectively.

specificities, induction rate, and cell-specific expression (6), this transient assay system for the inducible expression of the CAT activity reflects the induction process of the endogenous cytochrome P-450c gene in cultured cell lines and livers of experimental animals. Experiments using transformants harboring the integrated chimeric gene (pMC6.3k) gave us essentially the same results as obtained with the transient assay system in terms of the criteria described above (data not shown). Since P-450c gene is of rat origin and Hepa-1 cells are derived from a mouse hepatoma, this expression system shows no rigid species specificity, as is often the case with the

DRE-1	-1150	C	---	11 bp	---	GAGAGCTGGC	---	10 bp	---	CC	-1117	N
DRE-2	-1076	C	---	11 bp	---	GCTAGCTGGG	---	10 bp	---	CG	-1109	R
DRE-3	-1095	C	---	11 bp	---	CAGCACTGGG	---	10 bp	---	GG	-1062	N
DRE-4	-1063	G	---	11 bp	---	GGTAGCTGGG	---	10 bp	---	GG	-1030	N
DRE-5	-1055	G	---	11 bp	---	GGTGGCTGCG	---	10 bp	---	GG	-1022	N
DRE-6	-969	G	---	11 bp	---	CCTGGCTGGG	---	10 bp	---	CC	-1002	R
CONSENSUS		G/C	---	11 bp	---	C N TA / C N GG GCTGGG	---	10 bp	---	GG/CC		
DRE-7	-3431	A	---	11 bp	---	AGGGGCTGGG	---	10 bp	---	CC	-3398	N
DRE-8	-1933	G	---	11 bp	---	CCTCGCTGGG	---	10 bp	---	GT	-1900	N
DRE-9	-1312	C	---	11 bp	---	CCTAGCTGGC	---	10 bp	---	GT	-1345	R
DRE-10	-577	G	---	11 bp	---	CTGGGCTGGG	---	10 bp	---	TT	-544	N
DRE-11	-210	C	---	11 bp	---	GAAGGCTGGG	---	10 bp	---	CT	-243	R

FIG. 4. DRE homologues in rat cytochrome P-450c gene. Sequences are written in the 5'-to-3' direction. The position of the end nucleotide of a given sequence is shown on either side of the sequence. The orientation of the sequences is indicated to the right: N, normal; R, reversed. The deduced consensus sequence is shown in the middle.

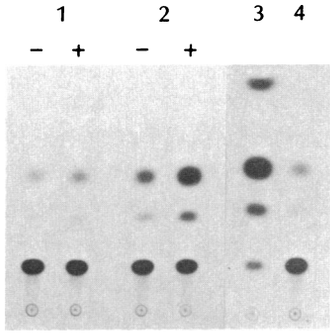

FIG. 5. Inducibility-conferring activity of repetitive sequence of DRE-2 on the homologous promoter. A synthetic DNA fragment of the DRE-2 sequence was inserted into the *Stu* I site of the −1033 truncated chimeric plasmid, which apparently lost the inducibility, by blunt end ligation with T4 ligase. A plasmid with 10 tandemly repeated DRE-2 units [determined by the procedure of Maxam and Gilbert (10)] on the 5' end of the truncated chimeric gene was used in this experiment. An autoradiogram of CAT activity products is shown. Lane 1, inducibility of CAT activity of the −1033 truncated chimeric gene; lanes 2, inducibility of CAT activity of the −1033 truncated gene with tandemly repeated DRE-2 sequence on its 5' end. The + and − symbols indicate the presence or absence of the inducer MC in the culture medium. Lanes 3 and 4, expression of pSV2-cat (7) and pML-cat (6) used for positive and negative controls, respectively.

gene expression involving RNA polymerase II. We have also found recently that the corresponding regulatory region of the human P-450c gene also confers the same inducibility on the CAT gene in the Hepa-1 system (13).

Recently, Jones *et al.* (12) and Gonzalez and Nebert (14) reported the results of similar experiments using the regulatory region of the mouse cytochrome P_1-450 gene, equivalent to the rat P-450c gene and the Hepa-1 system. The two groups have suggested the presence of the cis-acting regulatory DNA element responsive to TCDD in the upstream region of the gene. However, they observed a rather lower level of induction, as compared with the inducibility of the endogenous P_1-450 gene in cultured cells, animal livers, or our results. This is probably due to the lack of the sequence further upstream from −1642 and/or of the first intron sequence in their construct of the hybrid gene. In our deletion experiments, mainly two regions influential in the induction were localized upstream from the −1642, that is, the sequences between −3675 and −3067 and between −1683 and −1429, a part of which was contained in their construction. In addition to these two regions, we found another region, from −1140 to −1029, apparently more important in regard to the inducibility (Fig. 1). By the experiments using hybrid genes with detailed deletions, we tracked down a key sequence for the inducibility and deduced a decanucleotide

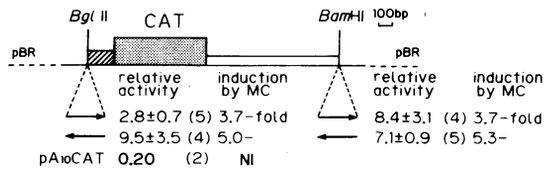

FIG. 6. Inducibility-conferring activity of the sequence of the DRE region on a heterologous promoter. The *Sma* I–*Bam*HI fragment from −1140 to −844 was excised and was placed in the *Bgl* II or *Bam*HI site of the pA_{10}CAT plasmid in both orientations as indicated by the arrows. The induced activities (mean ± SD) are expressed as a percentage of the CAT activity obtained with pMC6.3k. The number of separate experiments is designated in parentheses. The extent of induction by MC is also shown in the same way as in Fig. 1. NI, not induced.

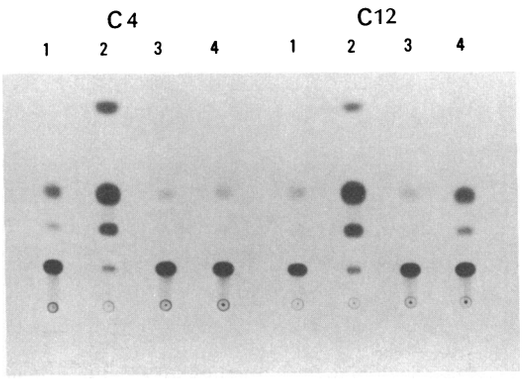

FIG. 7. Activity of pMC6.3k in mutant Hepa-1 cells. pMC6.3k was transfected into two derivatives of Hepa-1 cells: C4, a mutant in translocation of cytosolic receptor into nucleus, and C12, a mutant of decreased receptor activity (11). Reaction products were separated with TLC and exposed to x-ray film at −70°C for 24 hr. pSV2-cat (7) and pML-cat (6), which lacks the promoter sequence, were used for the positive and negative controls, respectively. Lanes 1, 2, 3, and 4 show the CAT expression of pML-cat, pSV2-cat, pMC6.3k in the absence of MC, and pMC6.3k in the presence of MC, respectively.

sequence (DRE) as shown in Fig. 4. This decanucleotide motif or its homologues are frequently distributed in the region from −1140 to −1029 and its vicinity. More interestingly, two versions of this motif occur in close proximity in the region from −1107 to −1075, the deletion of which caused the most profound effect on the inducibility. With one of them in the inverted orientation, there is a potential of forming a stem-and-loop structure in this region. In confirmation of the inducibility-conferring activity of this motif, the placement of tandem repeats of this synthetic decanucleotide on the 5′ end of the −1033 truncated gene restored the inducibility of the gene, although the fully induced activity was considerably lower than that of the original hybrid gene, pMC6.3k. This low level of induced activity is probably due to the lack of optimal arrangement of the DRE sequences, including as yet unrecognized surrounding sequence(s), and also to the absence of the sequence upstream from the site at −3 kilobases that contains other enhancer sequence(s) with presumably different effects than the DRE, as suggested in the previous paper (6). In support of the previous suggestion, a survey of the sequence reveals infrequent distribution of the DRE or its homologues in the region upstream from the −3-kilobase site (only once). Furthermore, deletion of two homologues in the region upstream from −1429 did not always concur with the reduced inducibility of the truncated gene, suggesting the presence in this upstream region of other sequence(s) influential in inducibility. The optimal number and spatial arrangement of the DRE and its interaction with the other upstream enhancer element(s) for the efficient inducible expression of the gene require further investigation. The importance of sequences other than the DRE has been suggested by placing various fragments containing DRE on 5′ end of simian virus 40 early promoter in $pA_{10}CAT$ (A.F.-S., K.S., and Y.F.-K., unpublished observations).

Several cis-acting regulatory elements responsible for the inducible expression of the genes transcribed by RNA polymerase II were identified for the most part in the 5′ flanking region of the genes, including genes for metallothioneins (15, 16), interferons (17–19), lysozyme (20), mouse mammary tumor virus (21, 22), and so on. These regulatory elements are usually 10 to 20 bp long, are repetitively arranged in the 5′ flanking region of the genes, and act on a heterologous promoter. These general properties of the regulatory elements are applied to the case of the DRE. However, the consensus sequence derived from individual DREs (Fig. 4) did not show sequence homology with any of the regulatory elements so far reported except for a slight similarity to the sequence of the immunoglobulin enhancer (23). This suggests the interaction of this DRE with unique trans-acting factors in response to xenobiotic inducers. In this context, the presence of a receptor or a protein factor that specifically binds to the inducers such as MC and TCDD has been reported (24, 25). It is not yet known whether or not, or how, this receptor–inducer complex interacts directly with the DRE sequence in the induction process. Solution of this problem is important for better understanding of the induction process of the drug-metabolizing cytochrome P-450 by xenobiotic inducers.

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