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**Regulatory DNA elements localized remotely upstream from the drug-metabolizing cytochrome P-450c gene**

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**ABSTRACT**

we have investigated regulatory DNA elements in the expression of the drug metabolizing P-450c gene of rats. After combining the 5' flanking and upstream untranslated regions of the isolated P-450c gene with structural gene for chloramphenicol acetyltransferase (CAT), the fusion genes were transfected into cultured cells (Hepa-1 and L cells) for the assay of transient expression of CAT activity. CAT activity was expressed inducibly in response to 3-methylcholanthrene only in Hepa-1 cells. At least three regions containing regulatory DNA elements were identified; one, which is present in the sequence from -44b to -0.2kb immediately upstream of the TATA box, functions in the basal level of transcription, and the other two which were located in the sequence from -0.8kb to -1.0kb and from -1.0kb to -6.3kb, enhance in combination, transcription in response to inducers in a manner independent of their orientation.

**INTRODUCTION**

Cytochrome P-450, a component of microsomal monooxygenase system, metabolizes innumerable sorts of both endogenous and exogenous substrates, including steroids, fatty acids, drugs, chemical carcinogens and other environmental pollutants. This metabolic versatility of the system can be explained in part by the presence of multiple forms of cytochrome P-450, each form of which has its own rather broad substrate specificity. Some of the P-450's involved in drug metabolism are known to be inducible enzymes. Their syntheses are specifically induced in response to exogenous inducers (1,2).

In rat livers and other tissues, P-450c and d are induced predominantly by the treatment of 3-methylcholanthrene or TCDD (2, 3, 7, 8 -tetrachlorodibenzo-p-dioxine). This inductive synthesis of the enzymes has been suggested to be mediated by an inducer-receptor complex(es) (3,4).

Recently we have isolated rat P-450c and d genes and determined their nucleotide sequences(5, 6). In order to investigate cis-acting DNA elements necessary for the inducibility of P-450c gene, we constructed fusion genes by combining the 5' flanking and untranslated upstream regions of P-450c gene with the structural gene of prokaryotic CAT (chloramphenicol acetyltransferase) and transfected them into Hepa-1 cells (a mouse hepatoma cell line) and L929 cells (a mouse fibroblast cell line).

In this paper, we report that regulatory DNA elements responsive to the inducers are localized remotely upstream from the P-450 gene. External and internal deletion experiments showed that the region up to -1.0kb was necessary for the induction, and that the sequence between -1.0kb and -3.0kb and the further upstream region from -3.0kb augmented the inducibility.

### MATERIALS AND METHODS

#### Construction

Chloramphenicol acetyltransferase (CAT) gene is derived from pA10CAT(provided by Dr. G. Khoury, NIH, reference (7)) which contains splicing signals and polyadenylation site of SV40 early gene as described previously. pMLCAT is a plasmid which contains CAT gene between HindIII and EcoR I sites of pML which was originally constructed by Lusky and Botchan (8). The original HindIII site of pMLCAT was converted to a BglII site by the linker ligation for the following constructions. In pMC6.3k, the fragment spanning from the BamHI site, 6.3kb upstream of the cap site to Sau3A1 site(+2566) adjacent to the initiation codon was inserted into pMLCAT between its BamHI and BglII sites in the correct orientation. Various derivatives, designated as pMCx(x indicates the length of the 5' flanking sequence of P-450c gene from the transcription start site), were constructed from pMC6.3k by using the restriction sites indicated in Fig.1. pMC 6.3k was partially digested with the corresponding restriction enzyme in Fig. 1d, filled up by Klenow enzyme and then was subjected to blunt-end ligation with the upstream BamHI site which had been filled up with Klenow enzyme in the case of the external deletions. For the internal deletions, pMC6.3k which

had been partially digested and filled up as above, was ligated with the upstream KpnI site which had been trimmed with S1 nuclease. In pMC6.3kop, 5.5kb BamHI fragment from -6.3kb to -0.8kb was inverted (shown by an open box with an arrow in it in Fig. 1).

#### Transfection and Induction

Hepa-1 cells (provided by Dr. C. Kasper, University of Wisconsin, Madison) were maintained by passage every 3-4 days in Dulbecco's modified Eagles medium with 10% fetal calf serum. L cells (obtained from ATCC) were grown in MEM with 10% fetal calf serum. The day before transfection, Hepa-1 cells or L cells were seeded at a density of  $3 \times 10^6$  and  $2 \times 10^6$  cells per 10 cm petri dish, respectively.

Transfection was carried out as described by Wigler et al. (9), using 10  $\mu$ g of each of the plasmid DNA's per dish. Two hrs after a treatment with glycerol, the cells were exposed to the medium containing 3-methylcholanthrene (MC) or other chemicals. Cells were harvested for the CAT assay, 40hrs after the inducer treatment.

MC (1mM in dimethylsulfoxide (DMSO)) was added to make a final concentration of 1  $\mu$ M, with the same amount of solvent DMSO being supplemented into separate dishes without inducer as control. Phenobarbital was dissolved in phosphate-buffered saline at a concentration of 100mM.  $\beta$ -Naphthoflavone was dissolved in DMSO at 10mM and isosafrole was dissolved in ethanol at 10 mM.

#### CAT Assay

The CAT activity was measured as described (10). Cell-free extracts were made from inducer-treated or non-treated cells by freeze-thawing (three times) in 200 $\mu$ l of 0.25 M Tris-HCl (pH 7.8) and centrifuging for 10 min in a microfuge at 4°C. The assay mixture contained 20  $\mu$ g of Hepa-1 cell extracts on a protein basis, 0.2  $\mu$ Ci  $^{14}$ C chloramphenicol and 0.45 mM acetyl-CoA in 180  $\mu$ l and was incubated at 37°C for 15min. In the case of L cells, usually 100  $\mu$ g of L cell extracts was incubated in 180  $\mu$ l of the substrate solution for 60 min. There was no artifactual inhibitory effects on this assay system at least in the range of 20 to 100  $\mu$ g of cell extracts.

Primer Extension Analysis

$5 \times 10^6$  Hepa-I cells transfected with pMC6.3k (50  $\mu$ g per 14 mm petri dish) were cultured in the medium with or without 1  $\mu$ M MC. Sixteen hrs later, RNA was extracted from the cells with 6 M guanidine thiocyanate as described previously (11). The isolated RNA (10  $\mu$ g) was then hybridized with 1 pmole of the 5'-end  $^{32}$ P-labeled primer in 180 mM NaCl and 6.6 mM Hepes (pH 7.5) at 45 °C for 40 min. The primer is a 35 mer synthetic oligonucleotide in the sequence of structural region of the CAT gene. The location of the primer is shown in the map by a solid bar and its sequence is underlined in Fig.3. The hybridized primer was extended by avian myeloblastosis virus reverse transcriptase (30 units) at 37 °C in 40 mM Tris-HCl (pH7.5) containing 1 mM DTT, 10 mM  $MgCl_2$ , 0.5 mM each of dATP, dTTP, dGTP and dCTP. The extended products were analyzed by electrophoresis in 6% polyacrylamide gel containing 8 M urea. The gel was dried and autoradiographed at -80 °C.

RESULTS AND DISCUSSION

Transfection of Hepa-1 and L cells with pMC6.3k

The construction of fusion genes are summarized in Fig. 1a-c. A hybrid gene pMC6.3k was constructed by inserting -6.3kb to +2566bp P-450c gene DNA sequence into the Hind III site immediately upstream of the CAT gene in pMLCAT and amplified in HB 101 (Fig. 1c). The plasmid pMC6.3k DNA was introduced into various cell lines for testing the inducibility of the CAT activity by using the calcium phosphate method(9). These cell lines included rat 5123D cells, H4IIE cells (supplied by Dr. Yoshikura, Tokyo University, Tokyo and by Dr. Kasper, respectively), mouse Hepa-1 cells and L cells. Hepa-1 cells are known to express endogenous MC-inducible P-450 gene, while L cells do not(12). As a positive and a negative control, pSV2CAT(10) and pMLCAT DNA, respectively, were treated similarly in most of the experiments. pMLCAT contained pML sequence ligated directly to the 5' end of the CAT structural gene (Fig. 1a), therefore, lacking any promoter elements for the CAT gene expression. As shown in Fig. 2a, Hepa-1 cells transfected with pMC6.3k expressed CAT activity which was inducible by the

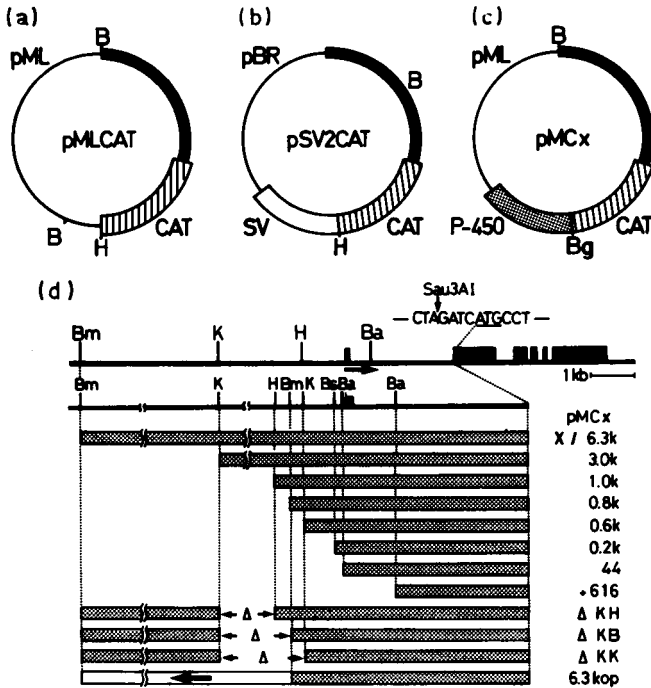


Fig. 1. Constructions of the recombinant plasmids containing fused genes. Constructions of pMLCAT (a) and pMCx (c) were described in MATERIALS AND METHODS. pSV2CAT (b) was constructed as described previously (10). (d) shows the structure of P-450c gene and regions of 5' external and internal deletions used. Solid boxes indicate the exons. The initiation codon (underlined) is located at the 15th base of the second exon, and is adjacent to the Sau 3A1 site which was used for the ligation with the CAT gene. Delta signs between the arrows indicate the regions of internal deletions. In pMC6.3kop, 5.5kb BamHI fragment from -6.3kb to -0.8kb was inverted (shown by an open box with an arrow in it). Illustrations are as follows; stippled bars, various fragments of P-450c gene; hatched bars, CAT gene; bold lines, splicing signals and polyadenylation site of SV40; thin lines, the region of pML or pBR; an open box, 5' upstream regulatory sequence of SV40. Restrictions sites are as follows; Bm, BamHI; H, HindIII; K, KpnI; Bg, Bg1II; Ba, Ba1I; Bs, BstEII.

treatment with MC. The level of maximally induced CAT activity was even higher than that of pSV2CAT-transfected Hepa-1 cells with or without MC treatment. On the other hand, L cells transfected with pMC6.3k showed a very low level of CAT activity, as compared with those transfected with pSV2CAT, irrespective of the presence or absence of the inducer (Fig.2b). Both Hepa-1 and

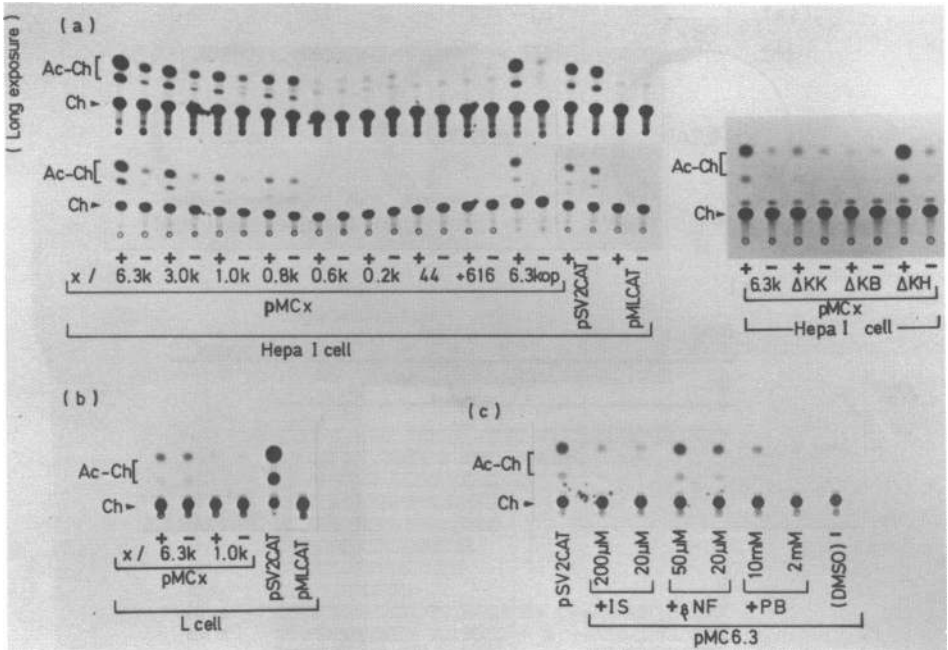


Fig. 2. Expression of CAT gene in Hepa-1 and L cells transfected with various recombinant plasmid DNAs. (a), Hepa-1 cells transfected with various recombinant DNAs. (b), L cells transfected with pMC6.3k and pMC1.0k. (c), induction of Hepa-1 cells transfected with pMC6.3k by several chemicals. Plus and minus symbols denote the presence or absence of the chemicals. In Experiment (d), chemicals are as follows; phenobarbital, PB;  $\beta$ -naphthoflavone, NF; isosafrole, IS.

L cells transfected with pMLCAT expressed practically no CAT activity. These results indicate that regulatory DNA elements responsive in a cell-specific manner to the inducer MC are present in the inserted 5' flanking and untranslated upstream regions of the P-450c gene. Hepa-1 cells were found to express the CAT activity by the treatment of MC to the same level as observed with the two rat cell lines (data not shown), suggesting that there was no species specificity in the expression of the fusion genes between rat and mouse cell lines. In view of a rapid growth rate and easy handling in transfection experiment, Hepa-1 cells were taken up for further study with L cells as a negative control. The addition of MC into culture media induced the CAT activity in Hepa-1 cells dose-dependently with a

median effective dose (ED50) of about 0.2  $\mu$ M. The fully induced activity was as high as or more than 25 fold that observed in the cells without the inducer (data not shown). This transient expression system using the fused gene showed a similar level of induction as observed with the expression of endogenous MC-inducible P-450 genes in rat livers(13) and cultured cells(14). Recently Jones et al. have reported results of a similar experiment using the CAT structural gene fused with the 5' flanking sequence of mouse P<sub>1</sub>-450 gene derived from a variant Hepa-1 cell line. However, a relatively low level(4 fold) of CAT activity induction was observed with their fused gene in Hepa-1 cells (15). From very close homology in the restriction maps of their P<sub>1</sub>-450 gene and the wild type counterpart recently reported by Gonzalez et al.(16,17), it appears that in the fusion gene constructed by Jones et al., the junctional point between the 5' upstream sequence of the P<sub>1</sub>-450 gene and the CAT gene is localized in the middle of the first intron of the former and that the primer DNA used for the primer extension experiment was localized within the first intron. Thus the possibility could be raised that the transcription may have been incorrectly initiated in the experiment reported by Jones et al. or that their P<sub>1</sub>-450 gene in the high activity variant of Hepa-1 cells could be grossly altered in the 5' flanking sequence.

Primer Extension Analysis of Fusion Gene Transcript

We used primer extension analysis to locate the transcription initiation site of pMC6.3k. A synthetic oligonucleotide of 35 mer within the CAT structural gene was used for the primer. As shown in Fig.3, the extended primer DNA observed only in the cytoplasmic RNA prepared from MC-treated Hepa-1 cells formed a single band, the length of which was estimated to be 188+3 bases long on the basis of the mobility of the size markers. This length agrees very well with the size(188 bases) expected on the assumption that the transcription started at the right site of P-450c gene and the first intron was spliced out correctly(5). The thick band which can be seen in both induced and non-induced lanes with a length of about 270 bases could be due to either a background of the primer extension experiment or an aberrant

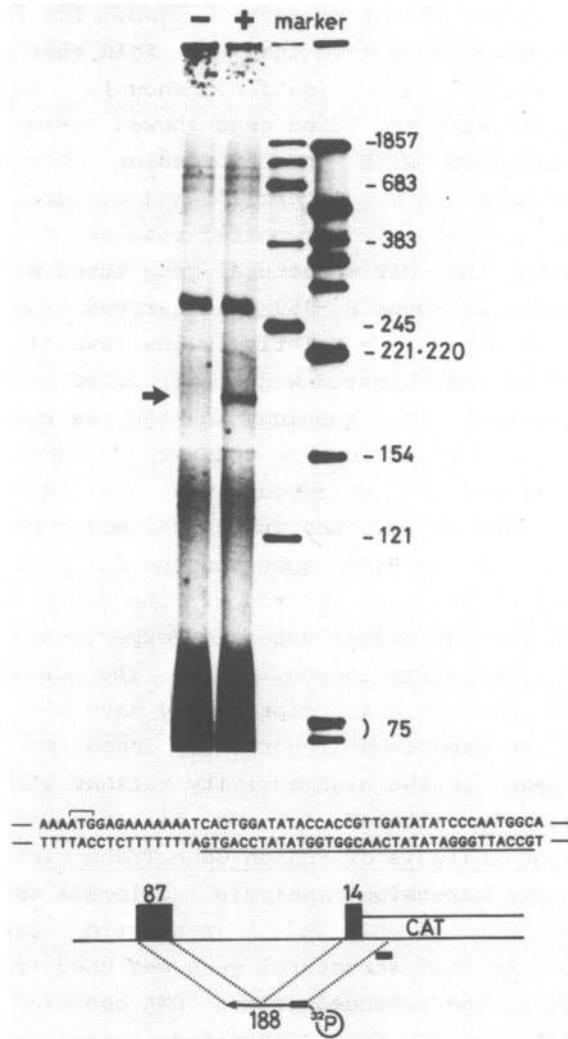


Fig. 3. Primer extension analysis of the transcripts synthesized in Hepa-1 cells transfected with pMC6.3k. Solid boxes indicate the first and the second exon, and the bar below the CAT gene shows the position of a synthetic oligonucleotide. The extended primer of the expected length (188 bases) is shown by an arrow at the bottom. The sequence of the oligonucleotide used as a primer is underlined. The autoradiogram shows the products of the primer extension experiment using either induced(+) or uninduced (-) RNA as a template. The arrow on the left of the electrophoretogram indicates the band which can be uniquely observed with the RNA prepared from MC-treated cells. Size markers are fragments of pBR322 digested either with *Hinf*I (right) or with *Bst*NI plus *Bam*HI (left).



transcription product unrelated to the induction process. The nature of this band is not known.

#### Inducer Specificity of Fusion Gene Expression

With regard to the time course of induction of the CAT gene expression, the increased activity could be already detected within 2hrs after MC treatment and then continued to rise for the ensuing 40 hrs (data not shown).  $\beta$ -Naphthoflavone, a potent inducer of P-450c in rat livers, also induced CAT activity in pMC6.3k-transfected Hepa-1 cells, whereas treatment with isosafrole and phenobarbital, known to be poor and non-inducers of P-450c, respectively, in rat livers, resulted in only a low or basal level of the CAT activity, with the exception that phenobarbital at a dose as high as 10mM enhanced the CAT activity significantly in the Hepa-1 cells (Fig. 2c). It is not clear, however, whether or not the induction of CAT activity at such a high concentration of phenobarbital has any physiological significance, because 90% of the cells were killed at this concentration. Thus, in general, induction of CAT expression shares the same inducer specificities as found for the MC-inducible P-450 gene in rat livers(13).

#### Transfection of External Deletions of pMC6.3k and pMC6.3kop

In order to localize cis-acting DNA elements responsible for the transcriptional induction of P-450c gene by MC, we constructed several recombinant plasmids containing various lengths of external deletions in the 5' flanking region of the fused gene as illustrated in Fig. 1d. The recombinant plasmids were transfected into either Hepa-1 or L cells for determination of inducibility of CAT expression. pSV2CAT DNA was also used as a positive control. Transfection experiments of each plasmid were performed at least three times. Fig. 2a shows a representative autoradiogram from these experiments and Table 1 represents the results of two independent experiments. The CAT activities were quantitated by counting the radioactivities of the reaction products separated on thin layer chromatography. A decrease in MC-induced CAT activity was observed in the cells transfected with pMC1.0k as compared with pMC6.3k and pMC3.0k, but pMC1.0k still retained some inducibility of the gene expression. With further external deletions proceeding downstream

Table 1 CAT Activities <sup>a</sup> of External Deletion Plasmids

Plasmids	Experiment I			Experiment II		
	uninduced	induced	ratio <sup>b</sup>	uninduced	induced	ratio
	%	%		%	%	
pMC6.3k	2.07	48.0	23.2	2.06	55.8	27.1
pMC3.0k	1.17	12.8	10.9	2.42	28.0	11.6
pMC1.0k	0.586	3.95	6.74	0.278	3.19	11.5
pMC0.8k	1.61	2.10	1.30	1.70	2.40	1.41
pMC0.6k	0.076	0.068	0.89	0.100	0.110	1.10
pMC0.2k	0.123	0.090	0.73	0.110	0.140	1.27
pMC44	0.033	0.034	1.03	0.115	0.086	0.75
pMC+616	0.053	0.057	1.08	0.217	0.208	0.96
pMC6.3kop	0.562	15.8	28.0	1.94	32.1	16.5
pMCΔKH	1.47	48.9	33.3	2.80	44.8	16.0
pMCΔKB	0.158	0.362	2.29	0.90	1.07	1.19
pMCΔKK	1.62	2.71	1.67	1.75	2.36	1.35
pSV2CAT	11.4	10.54	0.92	18.1	18.6	1.03
pMLCAT	0.045	0.035	0.78	N.D. <sup>c</sup>	N.D.	

- a. CAT activities were assayed by counting the radioactivities of the reaction products separated on thin layer chromatography and are expressed as the percentage of the acetylated forms of chloramphenicol.
- b. "ratio" indicates the level of the induction (induced/non-induced).
- c. not determined.

to yield pMC0.8k 0.6k and 0.2k, practically no more inducibility was retained in these plasmids. The slightly higher expression observed either in the presence or absence of the inducer with cells transfected with pMC0.8k was reproducible, but significance of this effect remains to be investigated. Thus, regulatory DNA elements responsive to the inducer could be localized in the sequence -6.3kb to -0.8kb upstream from the P-450c gene. When this DNA sequence (-6.3kb to -0.8kb) was fused to the rest of the hybrid gene in the opposite direction as shown in Fig.1d and Table 1, pMC6.3kop still retained as high a responsiveness to MC as pMC6.3k. Considered together with the fact that this DNA fragment, when inserted into pA10 CAT(7) in front of the SV40 promoter-CAT gene, allowed the CAT gene to be inducibly expressed in response to MC (unpublished data), these results suggest the presence of MC-dependent enhancer elements in this 5.5kb DNA fragment. The property of the fragment that renders heterologous promoters inducible at various lengths

upstream of the TATA box is analogous to those reported for regulatory elements in the upstream region of human metallothionein IIA gene (18,19) and in LTR of MMTV(20-22). A basal level of the CAT gene expression without MC-treatment was always observed in this series of experiments except for the pMC44-transfected cells. Their expressions of CAT activity were well above that of pMLCAT-transfected cells used as the negative control, but the activity in the pMC44-transfected cells fell down to the level of the negative control. This indicates that the sequence between -0.2kb and -44b may be involved in the basal level of expression, as is the case with most of the eukaryotic genes(23-25).

#### Transfection of Internal Deletions of pMC6.3k

In order to further characterize the regulatory sequence, internal deletions were introduced into the 5' flanking region of the P-450c gene. As shown in Fig. 2a, when the sequences from -0.6kb to -3kb or from -0.8kb to -3kb were deleted, resultant forms of the fusion gene (pMC $\Delta$ KK and pMC $\Delta$ KB) were found to lose largely responsiveness to MC treatment, whereas deletion of the -1kb to -3kb sequence (pMC $\Delta$ KH) did not affect or even enhanced the responsiveness of the gene. These results suggested that the sequence from -0.8kb to -1.0kb together with the region upstream from -3kb, was indispensable for an apparent full inducibility of the gene. The high level of the CAT expression of pMC $\Delta$ KH (the deletion of the -1kb to -3kb sequence), does not necessarily mean that the region between -1kb and -3kb is non-functional for the induced expression, because the external deletion experiments showed that the sequence from -1kb to -3kb had also an enhancing activity for the inducibility. A closer approximation of the region upstream from -3kb to the sequence of -0.8kb to -1.0kb or the promoter region may exert an enhancing effect more efficiently in the induced CAT expression to compensate the deletion of the -1.0kb to -3kb sequence. More precise location and characterization of these regulatory DNA elements, their sequence analysis and their mode of interaction with a inducer-receptor complex will shed light on the mechanism of the induction of this drug-metabolizing enzyme.

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