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Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: a similarity to glucocorticoid regulatory elements

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ABSTRACT

The DNA element governing the inducible expression of drug-metabolizing P-450c gene by xenobiotic treatments was investigated by gene transfer methods. A variety of dissected fragments from -844 to -1140bp region which was essential for the inducibility of P-450c gene were placed on the heterologous SV40 promoter for testing the inducibility. Mapping studies in combination with gel retardation assay defined the presence of the two xenobiotic responsive elements(XRE, XRE1, -1007 - -1021bp; XRE2, -1088 - -1092bp) composed of about 15 nucleotides which expressed the enhancer activity in response to xenobiotic inducers. The two XREs share 10 nucleotides in common out of 15 as expressed in the sequence CG/CTG/CC/TTG/CTCACGCT/AA and are arranged in the inverse orientation. They are different from DREs(drug responsive element) proposed previously(Sogawa, K. et al. Proc. Natl. Acad. Sci. 83, 8044-8048(1986)) and expressed a strong enhancer activity in response to 3-methylcholanthrene. The XRE shows a significant homology with glucocorticoid regulatory elements and apparently needs normal functions of a putative xenobiotic receptor for the inducible enhancer activity.

INTRODUCTION

Rat cytochrome P-450c is a monooxygenase with a broad substrate specificity. Nowadays, this hemoprotein has evoked a general interest, because of its activity which is directed toward detoxication of xenobiotic substances on one hand and metabolic activation of carcinogenic polycyclic aromatic hydrocarbon including benzo(a)pyrene and 3-methylcholanthrene(3-MC) on the other(1,2). The transcription of the P-450c gene is induced remarkably in rat livers(3) by the treatment of 3-MC or TCDD(2,3,7,8-tetrachlorodibenzo-dioxin). This induced expression of the gene is suggested to be somehow associated with the susceptibility of host animals to those carcinogenic substances(1,4).

The modulation of the gene expression by the inducers is believed to be mediated by intracellular receptor protein(s) which associates with a certain regulatory region of the gene as a complex with an inducer molecule (2,5,6). To define cis-acting elements responsive to 3-MC in P-450c gene,

we have introduced a chimeric gene composed of the upstream region of the P-450c gene DNA and chloramphenicol acetyltransferase (CAT) gene into mouse Hepa-1 cells. Transfection of a series of deletion mutants of the upstream region have delineated at least three regions of the DNA sequence essential for the inducible transcription(7,8), and deduced DRE sequence of 10bp apparently responsible for the inducibility. However, because of the following reasons, 1) DRE sequence itself shows only a weak enhancer activity even in the tandem arrangement, 2) its sequence is not conserved well in the upstream regulatory region of the human gene, we have analysed in detail sequence from -844 to -1140bp by using the heterologous promoter system of SV40 early gene. This sequence was apparently the most effective for the induction and was shown to confer the inducibility on the heterologous promoter of SV40 early gene with faithful reflection of the original gene(8). It was expected, therefore, that the regulatory activity of various subfragments derived from the sequence was precisely determined on the heterologous gene which did not respond to 3-MC.

We report here that the two regions of no more than 33 nucleotides from -997 to -1029 and from -1069 to -1092 act as tissue-specific inducible enhancers and that their effect largely depends upon the normal function of the putative receptor for the inducers.

The two regions share a conserved pentadecanucleotide in reverse orientation with each other. This conserved sequence was found at the corresponding position of the equivalent genes of human(9) and mouse(10,11) and shows some similarity to the glucocorticoid regulatory elements(GRE).

MATERIALS AND METHODS

Construction of Plasmids

pUH and pDE vectors(Fig. 1) used to measure the enhancer activity are the derivatives of pA10CAT(12, kindly provided by Dr. G. Khoury, NIH) in which pBR portion of pA10CAT was replaced by pUC18. The BglII-BamHI fragment of pA10CAT which contains the fusion gene of SV40 promoter without its enhancer sequence and the CAT structural gene was filled up by Klenow enzyme and inserted into the flush-ended unique EcoRI site(pUH) or HindIII site(pDE) of pUC18 to place the polylinker cloning site either on the 5'-end of the SV40 promoter in pUH or on the 3'-end of the polyadenylation site in pDE as shown in Figure 1.

All of the fragments in Figure 2 were inserted into the flush-ended unique BamHI site(filled by Klenow enzyme) of pUH in the inverse orientation

to the original, relative to the transcription except for the fragments, SmBm and N15. In the case of SmBm, the 300bp SmaI-BamHI fragment was inserted in between SmaI and BamHI sites of pUH. The names of the fragments are derived from the restriction sites at 5' and 3' ends of the fragments used for the plasmid construction. The resultant plasmids are designated as the names of fragments prefixed with p . For example, SmBm indicates the SmaI-BamHI fragment and pSmBm, a resultant plasmid. Sm, SmaI; Bm, BamHI; St, StuI; Bn, BanI; Nh, NheI; Fn, FnuDII. To make N-15, the plasmid which contains a fragment NhFn was cleaved processively by Bal 31 from the FnuDII site to the 15 nucleotides of the other end, ligated with BglII linker, and then was fused again to the repaired BamHI site in polylinker sequence of pUH.

To construct the plasmids which contain XREs downstream from the CAT gene, the fragments were inserted into the BamHI site of pDE.

As a positive control, Xho I-linked SV40 enhancer(13, kindly provided by W. Schaffner, University of Zürich) was inserted into the unique Sal I site of pUH.

Transfection and Induction

Mouse Hepa-1 cells, their derivatives C4 and C12, and rat H4IIE cells were propagated in Dulbecco's modified Eagles medium with 10% fetal calf serum. Mouse L929(obtained from ATCC) and mouse C2C12 were grown in MEM with 10% fetal calf serum.

DNA transfections were performed as described by Wigler et al.(14), using a mixture of 1 µg of a test plasmid and 4 µg of sonicated bacterial DNA as carrier. After glycerol shock, The cells were exposed to the medium with or without 1 µM 3-MC and then were harvested for the CAT assay 40 hours after treatment with the inducer.

CAT Assay

CAT activity was assayed as previously described(7,15). Samples of 10-100 µg of proteins were used in 180 µl assay mixture to obtain conversion rates within a linear correlation with the enzyme activity.

Gel Retardation Assay

Nuclear extracts were prepared from Hepa-1 cells as described by J.D. Dignam et al.(16). Probes for gel retardation assay were prepared as follows. The SmFn fragment was subcloned into the flush-ended BamHI site in either orientation and then the plasmid was subjected to the digestion with HindIII, followed by the treatment of alkaline phosphatase. The treated plasmid was kinased at the 5' end with (γ-^{32}P)ATP and subsequently digested

with SacI, whose site located near the other side of the SmFn fragment. The labeled HindIII-SacI fragment containing the SmFn sequence was isolated by electrophoresis in low melting point agarose gel.

The ^{32}P-labelled fragment(specific radioactivity, $3x10^6$ cpm/pmole) was incubated with nuclear extracts of Hepa-1 cells treated with 3-MC in the absence or presence of competitor DNAs. Binding reaction(10µl) was started by preincubating at 0 ^{0}C for 15min the mixture containing 10 mM HEPES(pH7.9),10%(V/V) glycerol, 50 mM KCl, 0.1mM EDTA, 0.5 mM DTT, 3 mM $MgCl_2$, 4 mM spermidine, 4 µg poly(dI-dC) (17, purchased from Boeringer Manheim), 1 µg yeast tRNA, 50 ng salmon sperm DNA, and 20 or 100 fold excess (molar ratio) of competitor DNA, and then was followed by adding the labeled probe DNA fragment($2x10^4$ cpm). After 20 min incubation at 24 ^{0}C, the resulting complexes were resolved in a 5% polyacrylamide gel containing 20 mM Tris-HCl(pH7.5), 10 mM Na-acetate and 1 mM Na-EDTA. After electrophoresis, the gel was dried and autoradiographed at -70 ^{0}C with an intensifying screen.

RESULTS AND DISCUSSION

Core Sequence of Inducible Enhancer in Heterologous Promoter

We divided the regulatory sequence of -844 to -1140bp in the upstream region of P-450c gene into various fragments and placed them separately on the 5' end of the SV40 promoter without its enhancer in pUH(Fig. 1) for the assay of the inducibility of the CAT gene. All of the fragments were inserted in the inverse orientation to the original, relative to the transcription and the result is shown in Figure 2. The two halves of the

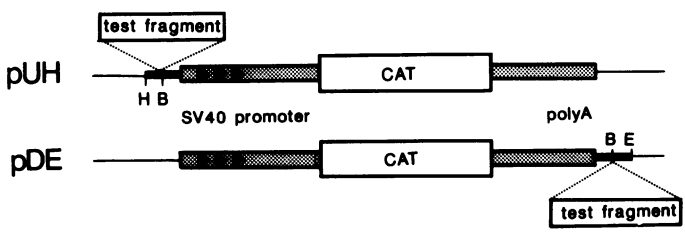

Fig. 1. Diagram of the vectors to assay the enhancer activity. pUH and pDE are the derivatives of pA10CAT(see Materials and methods). Oper. boxes denote the structural gene of CAT. Stippled boxes represent the SV40 sequence which includes its promoter, splicing site, and polyadenylation site. The triangles represent 21bp repeats of SV40. The filled boxes and thin lines denote the polylinker site and the rest portion of pUC18, respectively. Test fragments were inserted into the BamHI site in the polylinker either in pUH or in pDE. Restriction sites : H, HindIII; B, BamHI; E, EcoRI.

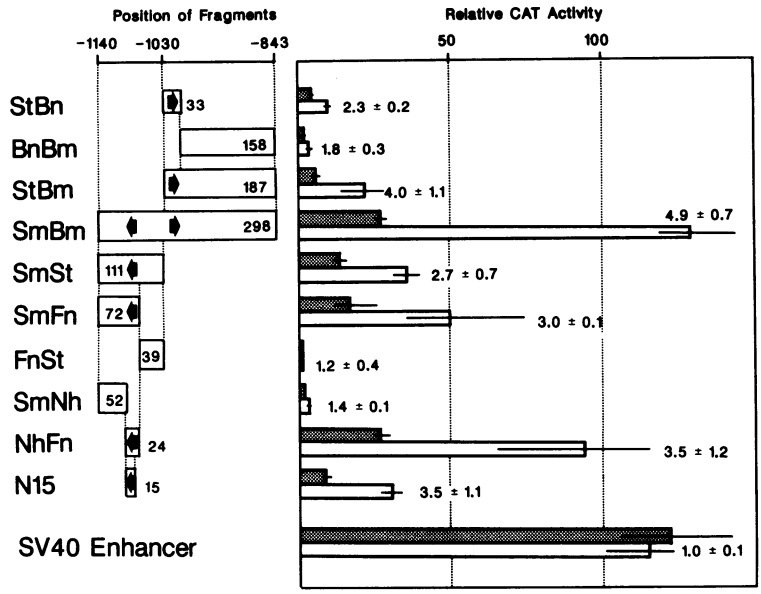

Fig. 2. The inducibility and the enhancer activity of a series of fragments in Hepa-1 cells.

All of the test fragments were inserted into pUH in the inverse orientation to the original in relation to the transcription. Open boxes on the right side to the names of fragments(see Materials and methods for the nomenclature of the fragments) indicate their lengths and positions in the original gene. Arrows in the boxes indicate the position of XREs described in text. The length of bars in the diagram indicates the corrected activity, expressed as the ratio of each test plasmid to that of pUH in which SV40 promoter without its enhancer is fused to the CAT gene. Each value is the average of 3 experiments. Open and stippled bars represent the corrected CAT activities in the presence and absence of 3-MC, respectively, and the figures show the inducibility of each plasmid expressed as the ratio of the induced to the non-induced CAT activity with standard deviation. The activity of SV40 enhancer inserted into the pUH was used as a positive control.

sequences (SmSt) and (StBm) retained the enhancer activity in response to 3-MC, although not only the ratios of the induction (induced versus uninduced activity) but also the uniduced levels of their activities were lowered by the division. Sum of the two activities in either induced or constitutive expression is below the original activity of the regulatory sequence(SmBm), suggesting the presence of synergistic effect or cooperative interaction between the two fragments. Further subdivision of each fragment was performed to define the location of core sequence(s) essential for the inducibility or the enhancer activity. The two fragments(StBn sequence from -997 to -1029 and NhFn from -1069 to -1092) as small as 33 and 24bp,

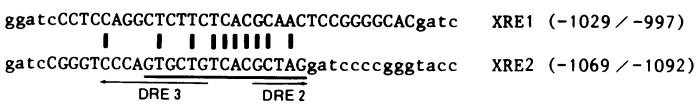

Fig. 3. Comparison of DNA sequences of XRE1 and XRE2.
XRE1 and XRE2 correspond to the restriction fragments, StBn and NhFn, respectively. Sequences of the plus and minus strands of XRE1 and XRE2, respectively, were compared. Small letters indicate flanking polylinker sequences. Vertical lines represent matched bases between the two. The underline indicates the sequence of the fragment N15(XRE2'). Arrows indicate DRE3 and a part of DRE2.

respectively, were found to express the enhancer activity in response to the inducer. The BnBm fragment(-844 to -1001) also expressed a slight inducibility.

Eventually, the 24bp fragment(NhFn) was found to generate an active subfragment(N15) no longer than 15bp by further subdivision(Fig. 2). The enhancing effect was also augmented in response to the inducer in such a short fragment,although the fully induced activity was reduced considerably. A preliminary experiment indicated that the deletion of 4 base pairs from the 5' end of the N15 fragment abolished the enhancer activity and the inducibility.

Comparison of the DNA sequence between the two active subfragments(StBn) and NhFn) in either orientation revealed the presence of a homologous sequence with a short alternate purine/pyrimidine stretch of CACGC(A)(Fig. 3). The two homologous sequences are inversely oriented with each other in the original regulatory sequence(SmBm). These observations lead us to designate the homologous sequences as xenobiotic responsive element(XRE), that is, sequences derived from StBn and NhFn fragments are designated XRE1 and XRE2, respectively, as shown in Figure 3. The fragment N15(XRE2') which is located in the region whose deletion caused a most profound effect on the inducibility of the gene shared 5 and 6 nucleotides with DRE2 and DRE3(Fig. 3, ref. 8), respectively, on the both sides. These DRE sequences were deduced to be drug responsive elements which were found frequently around the -1kb region from the previous experiment and they showed some weak enhancer activity in their tamdem arrangement(8). However, the N15 fragment which destroyed two DREs on both ends still possessed a significant inducibility to 3-MC and XRE1 does not contain DRE sequence. Taken together, we consider that the DRE motif may not a main principle for the inducibility of the gene but DRE2 and 3 could constitute a part of XRE2 for potentiating the enhancer activity. The

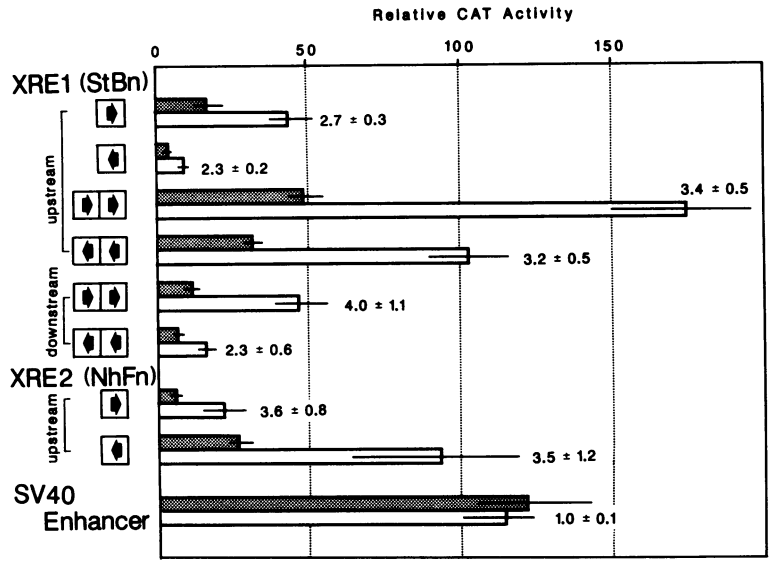

Fig. 4. Effects of the position and orientation of XREs on the enhancer activity.

A box represents a unit of inserted XRE. Rightward arrows in boxes denote the natural orientation of the inserted XREs relative to the transcription, and leftward arrows, the inverse orientation to the original. The length of bars indicates the corrected CAT activity of each plasmid, the ratio of the CAT activity of each plasmid to that of pUH in the case of the XREs inserted upstream of the SV40 promoter or to that of pDE in the case of the XRE placed downstream of the fusion gene. The result are the average of 3 experiments. The figure was depicted as Fiure 2.

sequence of XRE was found to be conserved in the corresponding positions in the human(9) and the mouse equivalent genes(10, 11).

Enhancing Activity of Xenobiotic Regulatory Element

The enhancing effect of the XRE sequences was investigated in regard to the orientation, the position in relation to the transcriptional promoter and the copy number. Either StBn(XRE1) or NhFn(XRE2) fragment was inserted into the BamHI site of pUH or pDE. As shown in Figure 4, both of the two XREs acted on the heterologous promoter in both orientations but the extent of their effects somewhat varied. XRE1 worked more efficiently, when placed in the original orientation relative to the transcription while the reverse held true for XRE2.

The effect of copy number of the regulatory elements on the activity was tested by placing the StBn fragment containing XRE1 in various

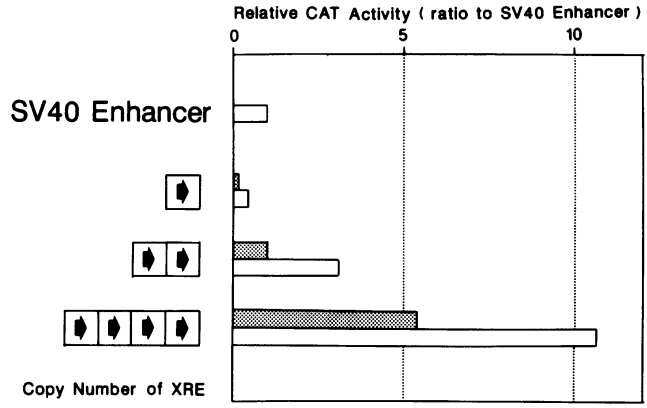

Fig. 5. The effect of copy number of XREs on the enhancer activity. The enhancing activities of one, two, and four repeats in tandem arrangement of XRE1 inserted in the pUH are expressed as ratios of the CAT avtivities in the presence(open bars) or absence(stippled bars) of 3-MC to that of the plasmid with SV40 enhancer inserted upstream of the fusion gene in pUH. One tenth the amount(0.1μg) of each plasmid DNA routinely used for the DNA transfection was mixed with 5μg of bacterial DNA as carrier and was used in this experiment.

multiplication on the 5' end of the SV40 promoter in pUH. A tandem arrangement of the two XRE1 sequences exhibited the induced CAT activity about 4 fold higher than the single, with a concomitant increase in the constitutive CAT expression(Fig. 4). Surprisingly, even higher induced CAT activity was observed with 4 fold tandem repeat of XRE1. A fully induced CAT activity of this repeated XRE1s was about 10 times as high as that of SV40 enhancer used as a positive control(Fig. 5).

For reasons as yet to be studied, the XRE1 repeat also enhanced the constitutive expression of the CAT activity without 3-MC-treatment. These phenomena were also observed with XRE2(data not shown).

The similar results have been recently reported with GRE(glucocorticoid regulatory element) placed immediately upstream of the HSV TK promoter. The tandem duplication of the GRE-containing fragment increased the strength of its constitutive enhancing activity without augmenting its inducibility in response to glucocorticoid hormone(18).

Figure 4 also shows that XREs were able to manifest their enhancing effect in the position downstream of the CAT gene. Since XREs exerted their effect in a fashion relatively independent of orientation and distance in relation to the transcription start site(Fig. 2, ref. 8) they may well be designated inducible enhancer.

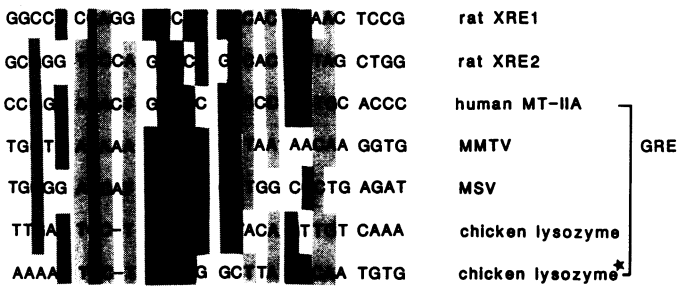

Fig. 6. Comparison of DNA sequences of XREs and several GREs. DNA sequences of human metallothionein IIA(MT-IIA) gene from -264 to -236(20), mouse mammary tumor virus(MMTV) DNA from -186 to -158(26,27), Molony murine sarcoma virus(MSV) DNA from -175 to -203(18, 28), and the sequence of chicken lysozyme gene from -50 to -77 and from -191- to -164(29) were compared with XRE1 and XRE2. The distal GRE in chicken lysozyme(marked with an asterisk) is also recognized by progesterone receptor(30). Thick stippled lanes indicate the positions in which a single nucleotide occupies 5 or more positions out of 7 and light stippled lanes, the positions in which either of the two alternative nucleotides occupies 6 or more positions out of 7.

Primer extension analysis by using an end-labeled synthetic oligonucleotide in the CAT sequence(7) revealed that the transcription of the fusion gene with a dimer of XRE1 was faithfully initiated at the same position as described previously(19). The level of 3-MC induction quantitated by the primer extension was correlated well with that measured by the CAT activity(data not shown).

Sequence Similarity between XRE and GRE

From the comparison of XRE sequence with the regulatory sequences of the various genes so far reported, the motif of XREs was found to show some similarity with the sequence of DNA binding site(GRE) for glucocorticoid receptor. Figure 6 shows the comparison of XREs with several DNA binding sites for glucocorticoid receptors. Although XRE1 and 2 have 1 and 2bp mismatches with the concensus sequence for the GRE(CN_{3-4}TGTT/CCT(20)), the sequence similarity appears to extend to the flanking sequences on both sides of the concensus. It has been recently reported that a trans-acting factor or 3-MC(or TCDD) receptor which is believed to interact with XRE sequences(see also the following section) has many physicochemical and biochemical properties(molecular weight, molecular structure, intracellular location of liganded and unliganded molecule and others) in common with the glucocorticoid receptor(5,21,22,31,32). From the investigations of the poly-

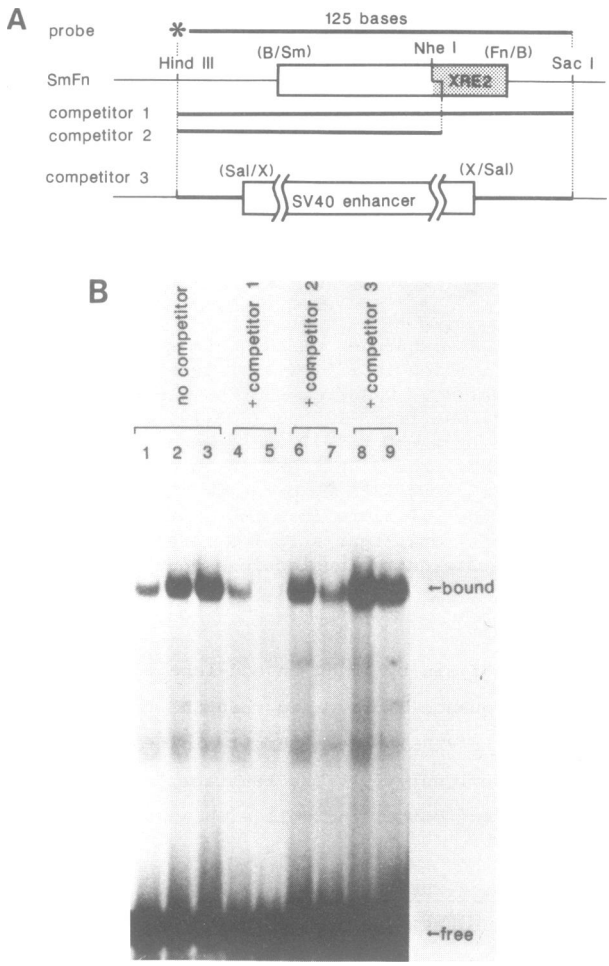

Fig. 7. Gel retardation assay.
(A) Diagram of the probe and competitor DNAs for gel retardation assay.
The probe is composed of SmFn fragment which contains XRE2(a stippled portion) and flanking polylinker site of pUH. Competitor 1 is the same as the probe. Competitor 2 is a restriction fragment cleaved by HindIII and NheI so that XRE2 was eliminated. Competitor 3 is SV40 enhancer sequence and the flanking polylinker. Restriction sites: B, BamHI; Sm, SmaI; Fn, FnuDII; Sal, SalI; X, XhoI.
(B) Autoradiogram of gel retardation assay.
Arrows indicate the positions of free and retarded probes discussed in the text. Lane 1, 2, and 3 show the gel electrophoresis of the DNA probe incubated with 1, 2 and 3μg of nuclear extracts, respectively, without competitors. In lanes 4-9, the reaction mixture contained the DNA probe and nuclear extracts(2μg) with various competitor DNAs. Lane 4,6 and 8 show electrophoresis of the DNA probe incubated with 20 fold molar excess competitors, and lane 5, 7, and 9, electrophoresis of DNA probe with 100 fold molar excess competitors.

anionic properties, effects of limited proteolysis and other treatments, and hydrodynamic properties of the two receptors, Gustafsson et al. drew a conclusion that the TCDD(or 3-MC) receptor and the glucocorticoid receptor appear to be structurally and functionally closely related proteins(22,31). Considered together, sequence homology between XREs and GREs leads us to a notion that the inducible system of XRE and 3-MC(or TCDD) receptor shares a common ancestor with that of GRE and glucocorticoid receptor. This notion will have to be finally testified by cloning the xenobiotic(3-MC or TCDD) receptor gene.

Cell Type Specific Expression of XRE and Interaction with Transacting Factor

As previously reported, the upstream regulatory sequence of P-450c gene exhibited the strict cell type specificity for the expression of the subordinate gene(CAT gene)(7). Likewise, neither XRE1 nor XRE2 fused to the chimeric gene in pUH could drive the subordinate gene in L929 mouse fibroblast or C2C12 myoblasts(data not shown), in contrast to the case of Hepa-I and H4IIE cells (a rat hepatome cell line)(data not shown).

When XRE1 or XRE2 fusion gene was transfected into the mutant cells (C4 and C12) with a defective receptor function as described previously(23), virtually no expression of the CAT activity was observed even in the presence of 3-MC. These observations suggest that the XRE is a principle with which a receptor-inducer complex or trans-acting factor interacts for the tissue-specific and inducer-specific expression.

To search for factor(s) interacting with the XRE motif, we used a protein-DNA mobility shift assay(17,24,25), with nuclear extracts from Hepa-1 cells. The probe and competitor DNAs used in this study were depicted in Figure 7. Without any competitor DNAs, a major band(s) was found to be decreased in the mobility in the gel electrophoresis by incubating with the nuclear extracts, indicating the interaction of the probe DNA with some protein factor(s). The competition assay was performed to assess the specificity of the interaction. An efficient competition was oberved with about 100 fold molar excess of the cold DNA used as the probe(competitor 1), whereas a slight and no competition were observed with competitor 2 containing the DNA sequence adjacent to the XRE and competitor 3 containing SV40 enhancer sequence, respectively. These observations suggest that the DNA probe interacted with a factor(s) largely in the region of XRE. A slight competition with competitor 2 may imply that the binding site for the factor spans beyond XRE2 so that competitor 2 interfered partially with the interaction of the factor and the XRE. The band shifted in the same or

similar position can be found also with the extract from uninduced cells, but not with the extract from L929 fibroblasts. Preliminary experiment using DNase I footprint analysis and exonuclease III protection analysis suggests that there is some quantitative difference of the factor between the extracts from uninduced and induced cells. In addition, it is possible that free receptors without ligands can bind to XREs in vitro but not in vivo similar to the case of steroid hormone receptors (33,34) or that there is other factors than the receptor which interact with the XRE sequence in a sequence specific manner. Further characterization and purification of the XRE-binding factor will be necessary to clarify whether or not it is xenobiotic receptor itself.

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