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A DNA-binding factor specific for xenobiotic responsive elements of P-450c gene exists as a cryptic form in cytoplasm: Its possible translocation to nucleus

(gel shift assay/enhancer-binding protein/dioxin receptor/gene regulation)

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ABSTRACT **Transcription of the drug-metabolizing cytochrome P-450c gene is induced by 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Previously, we defined two xenobiotic responsive elements (XREs) of ≈15 base pairs, both of which activate transcription in cis in response to these xenobiotics. Using a gel mobility shift assay, we have identified a factor that specifically binds to the XREs. This factor appears in nuclei of mouse hepatoma cell line Hepa-1 only when the cells are treated with the xenobiotics, while the factor is undetectable in the nuclei of a 3-methylcholanthrene-treated mutant of Hepa-1 with defective function of a xenobiotic receptor. In addition, the nuclear factor bound to the XRE in the gel was found to be associated with [^{3}H]TCDD when the cells were treated with it, suggesting that the xenobiotic receptor is at least a component of the DNA-binding factor. The cytoplasmic fraction from nontreated Hepa-1 cells also contains the factor as a cryptic form and prominently reveals its DNA-binding activity by incubation with 3-methylcholanthrene *in vitro*. These results not only suggest the involvement of the XRE-binding factor in transcriptional activation via XREs but also provide evidence that the binding of ligands to the preexisting factor in a cryptic form induces its XRE-binding activity, which is probably followed by its translocation from cytoplasm to nucleus.**

To elucidate the mechanism of transcriptional activation of the rat cytochrome P-450c gene with xenobiotics including 3-methylcholanthrene (3-MC) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (for review, see ref. 1), we have previously defined two xenobiotic responsive elements (XREs) of ≈15 base pairs (bp), both of which are localized 1 kilobase (kb) upstream of the P-450c gene and activate transcription in cis in response to the inducers (2).

Considerable evidence has been accumulated to support the existence of a receptor for the xenobiotics such as 3-MC and TCDD with apparently similar properties to those of steroid hormone receptors and to suggest the involvement of the xenobiotic receptor in the transcriptional regulation of P-450c and other 3-MC-responsive genes (3–6). The observation that XREs fail to function as inducible enhancers in recessive mutant cells of Hepa-1 (C4 and C12; ref. 6) defective in xenobiotic receptor function (2) indicates that the receptor regulates the transcription positively by interacting with the XRE in either a direct or an indirect manner. However, successful purification of the receptor has not been reported.

Under these circumstances, to identify trans-acting factors governing inducible expression of the P-450c gene, we have taken an alternative approach, a gel mobility shift assay (7–9) with XRE used as a probe. In the present paper, we demonstrate the presence of a XRE-binding factor(s) that appears in nuclei of Hepa-1 cells only when the cells are treated with 3-MC or TCDD. The cytoplasmic fraction from the nontreated cells also contains this DNA-binding factor as a cryptic form and its XRE-binding activity is dramatically revealed by incubation with 3-MC *in vitro*. These results imply that the activation of a cryptic form of the XRE-binding factor by association with ligands is accompanied by its intracellular translocation, finally leading to the inducible expression of the P-450c gene.

MATERIALS AND METHODS

Preparation of Extracts. Hepa-1, C4, HTC, and L929 cells were maintained as described (2, 10). Nuclear extracts and cytoplasmic fractions (S100) were prepared from cells grown in the presence or absence of 3-MC (1 μM) or [^{3}H]TCDD (1 nM; 20.5 Ci/mmol; 1 Ci = 37 GBq; Cambridge Isotope Laboratories, Woburn, MA) for 10 hr as described by Dignam *et al.* (11) except that the final dialysis buffer contained 50 mM KCl.

Treatment with Inducers *in Vitro*. Nuclear extract or cytoplasmic fraction (1–2 μg/μl) was incubated with 3 μM 3-MC for 2–2.5 hr (except as noted in the figure legends) at 25°C and then applied to the gel mobility shift assay.

Gel Mobility Shift Assay. Probe 1 was prepared as follows. The plasmid, pUHStBan, which contains a single copy of XRE-1 (2), was digested with *Bam*HI, end-labeled with [γ-^{32}P]ATP, and then digested with *Hpa* II. The radiolabeled fragment (3–5 × 10^6 cpm/pmol) was isolated by polyacrylamide gel electrophoresis. For probe 2, both strands of XRE-1 (Fig. 1) were synthesized by T. Takeda and T. Yamamoto (Tokyo University) with the DNA synthesizer (Applied Biosystems, Foster City, CA), annealed, and then end-labeled with [γ-^{32}P]ATP. The assay was performed as described (2) with the following modifications. The reaction mixture (10 μl) contained 12 μg (Fig. 2*A*) or 8 μg (Figs. 2*B*, 3, and 4) of nuclear or cytoplasmic extracts, 6 μg of poly(dI-dC)·poly(dI-dC) and probe 1 (6 × 10^5 cpm in Fig. 2 and 2 × 10^5 cpm in Figs. 3 and 4). When competitor DNA was included in the mixture, salmon sperm DNA was added to adjust the total amount of DNA to 1 μg. To examine the association of [^{3}H]TCDD and the XRE-binding factor, the nuclear extract or cytoplasmic fraction prepared from Hepa-1 cells treated with [^{3}H]TCDD was incubated with or without unlabeled probe 2 (0.1 μM) and gel electrophoresis of the samples was carried out as described above except that 120 μl of binding mixture was loaded on a slot (2 × 30 mm). After

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Abbreviations: 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

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5'-CCTCCAGGCTCTTCTCACGCAACTCCGGGGCAC-3'        XRE-1 ( -1029/-997 )
     |   |  | ||||||  |
5'-CGGGTCCCAGTGCTGTCACGCTAG-3'                 XRE-2 ( -1069/-1092 )

5'-gatcCCTCCAGGCTCTTCTCACGCAACTC
      GGAGGTCCGAGAAGAGTGCGTTGAGGC-5'           probe-1

5'-gatcCCTCCAGGCTCTTCTCACGCAACTC
      GGAGGTCCGAGAAGAGTGCGTTGAGgatc-5'         probe-2
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FIG. 1. Sequences of XREs and probes used for gel mobility shift assay. Sequences of the plus and minus strands of XRE-1 and XRE-2, respectively, were compared. The locations of XREs are indicated in parentheses. Vertical lines denote matched bases between the two. Probe 1 is a restriction fragment that contains most of the XRE-1 sequence (uppercase letters) and 4 bases derived from polylinker (lowercase letters). Probe 2 is a synthetic oligonucleotide, the sequence of which is the same as that of probe 1 except for 4 bases flanking the 5′ end of the minus strand.

the gel was dried, the radioactivity of every 4-mm slice was measured.

RESULTS

Detection of a 3-MC-Inducible XRE-Binding Factor. To detect factors that interact with the XRE sequence, we performed gel mobility shift assay (7–9) by using nuclear extracts from Hepa-1 cells treated with or without 3-MC for 10 hr.

When the DNA fragment containing XRE-1 was used as a probe (Fig. 1, probe 1), a specific retarded band was reproducibly observed only with the nuclear extract from the 3-MC-treated cells (Fig. 2*A*). It was found that not only 3-MC but also TCDD could induce the band with the same mobility (data not shown). On the other hand, this band was undetectable with the nuclear extract from 3-MC-treated C4, a mutant of Hepa-1 defective in the xenobiotic receptor function (6), or L929 fibroblasts, in either of which XREs were inactive for the inducible expression of the subordinate gene, and the retarded band was extremely faint with the extract from the treated HTC, a rat hepatoma, in which the enhancer activity of the XREs is expressed only marginally. Thus, the presence of the nuclear factor specifically interacting with the XRE is intimately correlated with the expression of the inducible enhancer activity of XREs in the cells.

Relationship Between Xenobiotic Receptor and XRE-Binding Factor. Considering the role of xenobiotic receptor as a positive transcriptional regulator via XREs, the XRE-binding factor should be expected to carry TCDD or 3-MC if it contains a xenobiotic receptor domain as a part of a molecule or constitutes a complex with the receptor molecule. The nuclear extract prepared from Hepa-1 cells treated with [^{3}H]TCDD was incubated with unlabeled XREs and then applied to gel electrophoresis together with the nuclear extract incubated with ^{32}P-labeled XRE probe in parallel. [^{3}H]TCDD instead of [^{3}H]3-MC was used because the higher specific activity of the former was obtained and the two compounds are known to share the same receptor molecule (4). As a result, the prominent peak of [^{3}H]TCDD coincided exactly with the position of the XRE-binding factor bound to ^{32}P-labeled XRE probe in duplicate experiments (Fig. 2*B*). In addition, such a peak could not be detected when the extract was incubated in the absence of the XRE, indicating that a receptor–ligand complex, which is localized in the treated nuclei, could not enter the gel in the absence of XREs, probably because of the properties of the receptor itself. These experiments strongly suggested that the XRE-binding factor presented here is a xenobiotic receptor itself or includes the receptor as a component of the XRE-binding complex. On the other hand, no prominent peak of [^{3}H]TCDD was observed when the cytoplasmic fraction of the treated cells was incubated with the XRE in the same way, showing the nuclear localization of the ligand-bound receptors with the XRE-binding activity in the treated cells. From the specific activity of [^{3}H]TCDD and ^{32}P-labeled XRE probe, it was roughly estimated that one or a few ligands

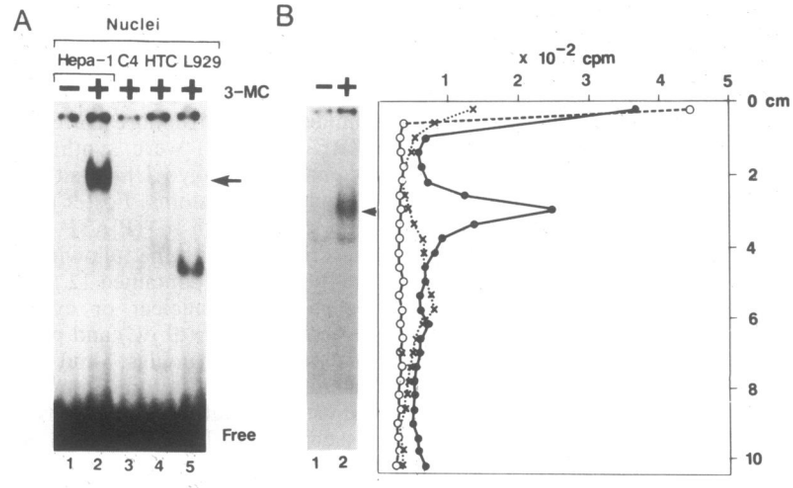

FIG. 2. (*A*) Binding activity of various nuclear extracts to probe 1. Various cell lines were grown in the absence (−) or presence (+) of 3-MC for 10 hr and then nuclear extracts were prepared. Arrow indicates the band that is specifically increased in intensity by treatment of Hepa-1 cells with 3-MC. Lanes: 1 and 2, nontreated and 3-MC-treated Hepa-1, respectively; 3–5, 3-MC-treated C4, HTC, and L929 cells. (*B*) Comigration of [^{3}H]TCDD and the XRE–factor complex. (*Right*) Distribution of [^{3}H]TCDD after gel electrophoresis of the nuclear extract or cytoplasm prepared from Hepa-1 cells grown in the presence of [^{3}H]TCDD. ●, Nuclear extract incubated with the XRE (unlabeled probe 2); ○, nuclear extract incubated without the XRE; ×, cytoplasmic fraction incubated with the XRE. (*Left*) Autoradiogram of the gel mobility shift assay in which ^{32}P-labeled probe 2 was incubated with nuclear extracts prepared from Hepa-1 cells grown in the absence (lane 1) or presence (lane 2) of TCDD. Arrowhead indicates band induced by treatment with TCDD.

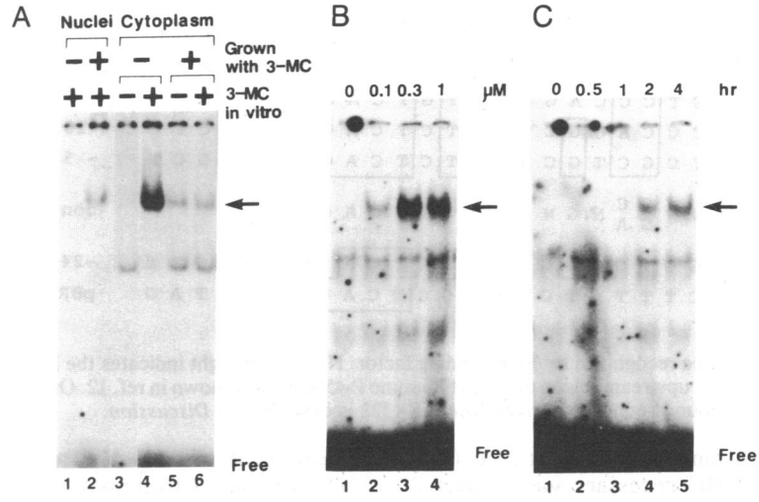

FIG. 3. (*A*) Induction of XRE-binding factor *in vitro*. Nuclear extracts (lanes 1 and 2) or cytoplasmic fractions (lanes 3–6) prepared from Hepa-1 cells grown in the absence (lanes 1, 3, and 4) or presence (lanes 2, 5, and 6) of 3-MC were incubated with (lanes 1, 2, 4, and 6) or without (lanes 3 and 5) 3-MC *in vitro*. Arrow indicates inducible band. (*B*) Time course of induction of XRE-binding factor *in vitro*. Cytoplasmic fraction prepared from nontreated Hepa-1 cells was incubated with 3-MC *in vitro* for 0–4 hr and then the binding reaction was carried out with probe 1 for 20 min. Arrow indicates inducible band. (*C*) Concentration of 3-MC required for induction of XRE-binding factor *in vitro*. Cytoplasmic fraction prepared from nontreated Hepa-1 cells was incubated for 2 hr with 0 to 1 μM 3-MC. Arrow indicates inducible band.

bound to one XRE probe molecule and that the content of the active XRE-binding factor is ≈1000 molecules per nucleus on the assumption that 1 molecule of ligand is bound to 1 molecule of the factor. However, these numbers are very tentative, because they are liable to vary depending on the estimation of the background level.

Induction of XRE-Binding Factor *in Vitro* and Its Possible Intracellular Translocation. From the results suggesting that the XRE-binding factor is associated with xenobiotics in the treated nuclei, it is reasonable to suppose that induction of the XRE-binding factor in nuclei is triggered by binding of the ligands to the receptor. In this context, we have investigated the induction of the XRE-binding factor in the cell-free system. Either the cytoplasmic fraction or nuclear extract prepared from nontreated Hepa-1 cells was incubated with 3-MC to examine the generation of their binding activity to the XRE. Although the XRE-binding activity was absent in the nontreated cytoplasmic fraction *per se*, the incubation of the cytoplasmic fraction with 3-MC resulted in induction of the factor that gave the retarded band of the XRE with the same mobility as the nuclear factor from the inducer-treated cells (Fig. 3*A*, lanes 3 and 4). Similarly, 1–10 nM TCDD could also generate the XRE-binding activity in the cytoplasmic fraction (data not shown). More than 2 hr of incubation was

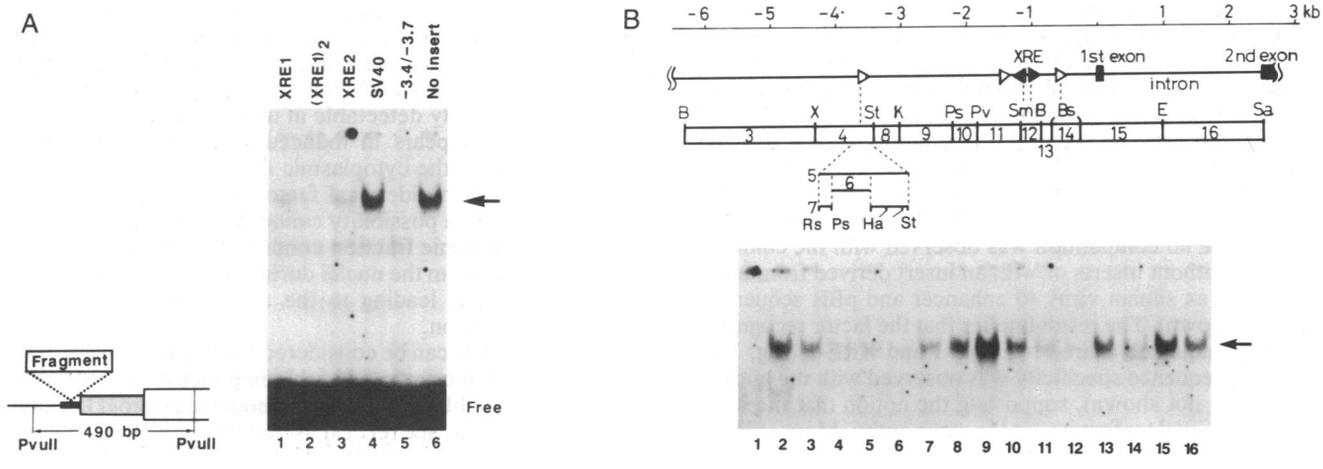

FIG. 4. (*A*) Competition of various DNA sequences with probe 1 in gel mobility shift assay. Structure of *Pvu* II/*Pvu* II fragments used as competitors is shown on the left. All the competitors share a 490-bp sequence [derived from a vector plasmid pUH (2)], which consists of a portion of pUC (thin and thick lines), a promoter of simian virus 40 (shaded box), and the structural gene for chloramphenicol acetyltransferase (open box). They contain different fragments inserted in polylinker cloning sites (thick line). The binding reaction was performed with probe 1 in the presence of 50-fold molar excess of competitors. Inserted fragments are as follows: lane 1, XRE-1; lane 2, a dimer of XRE-1 in tandem arrangement; lane 3, XRE-2; lane 4, simian virus 40 enhancer; lane 5, DNA fragment spanning from −3454 to −3719 of the P-450c gene; lane 6, no insert. In the case of lane 2, 25-fold molar excess of the competitor was used in the binding reaction. (*B*) Competition of various DNA fragments derived from the 5′ upstream region (−6.3 kb to +2.6 kb) of the P-450c gene with probe 1. Diagram of 5′ upstream sequence of the P-450c gene and its restriction endonuclease map are shown (*Upper*). Initiation codon starts at +2567. Solid and open triangles represent the locations of two XREs and three putative factor-binding sites identified in this study, respectively. In the restriction maps, dissected fragments used as competitors were shown. The number of each fragment corresponds to the lane number in the autoradiogram shown below. B, *Bam*HI; BS, *Bst*EII; E, *Eco*RI; Ha, *Hae* III; K, *Kpn* I; Pv, *Pvu* II; Ps, *Pst* I; Rs, *Rsa* I; Sa, *Sau*3AI; Sm, *Sma* I; St, *Stu* I; X, *Xho* I. In the autoradiogram, the cytoplasmic fraction prepared from Hepa-1 was preincubated with (lanes 2–16) or without (lane 1) 3-MC, and then the binding reaction was performed with probe 1 in the presence of 25-fold molar excess of competitor DNAs (lanes 3–16).

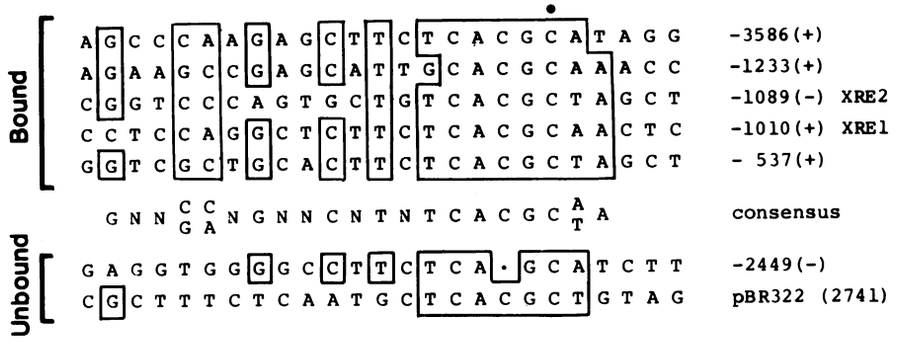

FIG. 5. Consensus DNA sequence recognized by XRE-binding factor. Number on right indicates the location of the invariant C with a dot above it. The entire sequence of the 5′ upstream region of the cytochrome P-450c gene is shown in ref. 12. Orientations of the represented strands are shown in parentheses. The unbound sequence derived from pBR322 is described in *Discussion*.

necessary for maximal DNA-binding activity at 25°C (Fig. 3*B*). The concentration of 3-MC necessary for a maximal level of the induction was 1 μM (Fig. 3*C*), more or less the same as the optimal concentration for the transcriptional activation of P-450c gene in Hepa-1 cells. The band migrating faster than the inducible one is occasionally observed, depending on the concentration of the probe used for the assay. It could be nonspecific because its intensity can be diminished when salmon sperm DNA was added as carrier DNA. On the other hand, the XRE-binding factor was absent in nuclei from nontreated cells, because the band was undetectable even after incubation with 3-MC (Fig. 3*A*, lane 1). In contrast to the case of the nontreated cells, interestingly, the factor was dramatically decreased in concentration from the cytoplasmic fraction upon treatment of the cells with 3-MC, with its concomitant appearance in the nuclear extract. The XRE-binding factor, which remained in a small amount in the cytoplasmic fraction of the treated cells, was not increased at all by incubation with 3-MC (lanes 5 and 6). Thus, the results show that 3-MC induces the XRE-binding activity in the preexisting cryptic factor and suggest that, at the same time, the activated factor–ligand complex is translocated from cytoplasm to nucleus.

Consensus DNA Sequence Recognized by XRE-Binding Factor. To examine the DNA sequences recognized by the factor, two kinds of competition experiments were performed. One group of competitors contain XRE-1, XRE-2, or other sequences as an insert in the common sequence (≈500 bp) as shown in Fig. 4*A*. The labeled probe 1 was efficiently blocked by competition with 50-fold molar excess of unlabeled XRE-1 or XRE-2 for binding to the activated cytoplasmic factor, while no competition was observed with the common sequence without inserts or with an insert derived from other genes such as simian virus 40 enhancer and pBR sequences (data not shown). The result implies that the factor recognizes the DNA sequence shared by XRE-1 and XRE-2 (Fig. 1*A*). The same sequence specificity was observed with the nuclear factor (data not shown), supporting the notion that the cytoplasmic and nuclear factors are the same entity. More efficient competition was observed reproducibly with 25 molar excess of the XRE-1 dimer. This observation may be related to the previous result that the XRE dimer shows 4 times as strong enhancer activity as the monomer in Hepa-1 cells (2).

The fragment from −3454 to −3719 of the P-450c gene, which contains the DNA sequence very similar to XRE-1 (12), also competed efficiently with the probe. This result encouraged us to carry out a second kind of competition experiment in which various restriction fragments from −6.3 kb to +2.6 kb of the P-450c gene were used as competitors, since several sequences similar to the XREs were interspersed in that region (12). As shown in Fig. 4*B*, in addition to the fragments from −3567 to −3686 (lane 6) and from −847 to −1140 containing both XRE-1 and XRE-2 (lane 12), the fragments from −1140 to −1790 (lane 11) and from −237 to −683 (lane 14) competed with the probe for binding to the factor. These fragments were found to contain the sequences homologous to the XRE. These 5 sequences revealed the consensus nucleotides, some of which are considered to be critical for binding to the factor (Fig. 5).

DISCUSSION

Using the gel mobility shift assay, we have demonstrated the presence of a factor that specifically interacted with the XRE in Hepa-1 nuclear extract only when the cells were treated with 3-MC or TCDD. Since the factor is undetectable in nuclear extracts from mutant cells (C4) of Hepa-1 and L929 cells treated with the inducer, in both of which XREs are inactive as enhancers (2), it is reasonable to consider that the interaction of the factor with XREs is a process leading to the transcriptional activation of the P-450c gene. The successful induction of the XRE-binding activity *in vitro* clearly shows the existence of a preactivated form of the XRE-binding factor in cytoplasm and its conversion into an active form by the xenobiotics. The nuclear and cytoplasmic factors are considered to be the same entity by the following criteria. First, the mobilities in the gel shift assay of these two factors are the same. Second, the sequence specificities recognized by them are also the same. Third, the fact that the cytoplasmic factor is mainly detectable in uninduced cells, while the nuclear factor appears in induced cells with concomitant disappearance of the cytoplasmic one strongly suggests the translocation of the identical factor from cytoplasm to nucleus. However, a possibility cannot be rigorously ruled out that the cytoplasmic fraction contains the nuclear materials that leaked out from the nuclei during cell fractionation in the hypotonic buffer, leading to the disturbance of their true cellular localization.

Several models can be considered for the mechanism of the activation of the cryptic DNA-binding factor by 3-MC. The simplest model, like a well-known model for steroid hormone action (for review, see ref. 13), is that the xenobiotic receptor transforms itself to an XRE-binding protein by ligand binding. Alternatively, the activation of the DNA-binding ability of the receptor requires the association or the dissociation of a cofactor(s) that is triggered by ligand binding. The experiment shown in Fig. 2*B* suggests that the receptor with a ligand(s) is included in the nuclear XRE-binding factor. However, further investigation is necessary to clarify whether the receptor binds to the XRE directly or some other factor mediates the binding of the receptor to the XRE.

Recently, the genes or cDNAs encoding a variety of steroid/thyroid hormone receptors and a retinoic acid receptor have been cloned. These studies have led to identification of discrete domains of proteins involved in DNA- and ligand-binding functions and demonstrated that the binding of

the receptor–ligand complex to the responsive DNA elements results in transcriptional activation (for review, see ref. 14). However, the molecular mechanism of activation of these receptors by ligand binding remains to be clarified. Although the genomic "footprint" suggested that steroid hormone receptors require the ligands for interacting with the responsive DNA elements *in vivo* (15), they were able to interact with their DNA-binding sites *in vitro*, even without ligands, when they were purified (16, 17). Bailly *et al.* (17) assumed that some protein(s) or structure prevents those receptors from accessing their DNA-binding sites *in vivo*. Binding of the ligands to the receptors would induce the dissociation of such complexes, which unmasks the DNA-binding domains of the receptors (16, 17). If a similar mechanism exists in xenobiotic induction, the activation of the XRE-binding factor *in vitro* described here will provide a powerful system to elucidate the mechanism for induction of the DNA-binding activity by ligands.

From two kinds of competition experiments, we have deduced the consensus sequence probably recognized by the XRE-binding factor (Fig. 5). The sequence CACGC$\begin{matrix}A\\T\end{matrix}$ appears to be critical for binding to the factor, as it is commonly found in all five sequences. On the other hand, this sequence alone does not seem to be sufficient, because a DNA fragment of pBR322, which happens to possess that sequence, did not work as a competitor (data not shown). The recognition or the binding of the factor may require the flanking sequence of 5′-CACGC$\begin{matrix}A\\T\end{matrix}$-3′, possibly a few more nucleotides shared by XRE-1 and XRE-2.

Probably all or some of these binding sites in addition to XRE-1 and XRE-2 are actually involved in induction of the gene, because the location of these sites roughly corresponds to the regions whose deletion lowered the level of the induced activity of various deletion mutants (10, 12). These multiple XREs upstream of the P-450c gene should work cooperatively to activate its transcription in response to the external stimuli.

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