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Role of the Ligand in Intracellular Receptor Function: Receptor Affinity Determines Activation In Vitro of the Latent Dioxin Receptor to a DNA-Binding Form

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To reconstitute the molecular mechanisms underlying the cellular response to soluble receptor ligands, we have exploited a cell-free system that exhibits signal- (dioxin-)induced activation of the latent cytosolic dioxin receptor to an active DNA-binding species. The DNA-binding properties of the in vitro-activated form were qualitatively indistinguishable from those of in vivo-activated nuclear receptor extracted from dioxin-treated cells. In vitro activation of the receptor by dioxin was dose dependent and was mimicked by other dioxin receptor ligands in a manner that followed the rank order of their relative affinities for the receptor in vitro and their relative potencies to induce target gene transcription in vivo. Thus, in addition to triggering the initial release of inhibition of DNA binding and presumably allowing nuclear translocation, the ligand appears to play a crucial role in the direct control of the level of functional activity of a given ligand-receptor complex.

The dioxin receptor regulates transcription of the cytochrome P-450IA1 gene and several other genes encoding drug-metabolizing enzymes in response to the environmental contaminant dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) or related compounds. This process is mediated by the binding of dioxin to an intracellular receptor protein presumably in the cytosolic compartment of target cells (for reviews, see references 20 and 29). Upon ligand binding, the dioxin-receptor complex becomes activated by a poorly understood structural alteration to a form which is retained on DNA cellulose in vitro (references 5 and 34 and references therein). In wild-type hepatoma cells, ligand-induced receptor activation results in the apparent translocation of the dioxin receptor from the cytosolic to the nuclear compartment (17). Transcription of the cytochrome P-450IA1 target gene requires the activated, nuclear dioxin-receptor complex, since it is not observed in mutant Hepa 1 cells (nt^-) which form normal ligand-receptor complexes but fail to accumulate these complexes in the nucleus (reviewed in reference 20). More recently, the in vivo-activated wild-type Hepa 1 but not the mutant nt^- dioxin receptor has been implicated in recognition of specific DNA sequences, termed xenobiotic response elements (XREs), which modulate the activity of linked promoters, thereby serving as dioxin-inducible enhancers (10–12, 14, 22). Thus, the mechanism of action of dioxin is similar to that of steroid hormones in that an intracellular soluble receptor protein transduces the extracellular signal to the transcriptional unit it regulates. More importantly, the dioxin receptor together with most, if not all, steroid receptors belongs to a class of gene regulatory proteins that require activation for function.

In the case of steroid hormone receptors, the mechanism of activation to a functional species is not yet understood, and a number of different regulatory mechanisms have been proposed to control this process. These models include, among others, intramolecular alterations of receptor conformation or intermolecular interactions with distinct proteins as plausible mechanisms of control of receptor activity (reviewed in reference 2). For instance, hormone has been shown to induce dimerization of the estrogen receptor, a critical step which determines high-affinity interaction with the estrogen response element (16). Importantly, several steroid receptors have been shown to be associated in vitro with the 90-kDa heat shock protein, hsp90 (reviewed in reference 8). Furthermore, hsp90 appears to repress the DNA-binding activity of the glucocorticoid receptor, since interaction of the receptor with target DNA sequences is observed only following dissociation of hsp90 (9, 30).

In particular, the role of hormone in steroid receptor-DNA interaction has been strongly debated, given that in vivo and in vitro studies have yielded conflicting results (3, 31, 36). The most likely explanation for this controversy is the fact that the nonactivated, heteromeric glucocorticoid receptor complex represents a very labile complex which readily breaks down in vitro (9, 21). However, under conditions exposing the heteromeric glucocorticoid receptor complex to as few in vitro manipulations as possible, we have shown that hormone was indeed required for activation of the DNA-binding activity of the receptor and concomitant release of hsp90 (9).

In similarity to the glucocorticoid receptor, the dioxin receptor can be recovered in cellular extracts in a form associated with hsp90 (7, 25, 35). Moreover, we have recently demonstrated that hsp90 modulates the DNA-binding activity of the dioxin receptor in vitro in a manner analogous to that of steroid receptors (35). However, the nonactivated dioxin receptor represents a much more stable complex in vitro than the corresponding form of the glucocorticoid receptor (21) and therefore represents a suitable system with which to study the activation process of soluble receptors.

Here we have exploited an in vitro activation system derived from Hepa 1c1c7 wild-type and nt^- mutant cell cytosol to identify and characterize functional steps during the activation process of the dioxin receptor. We demonstrate that activation of the dioxin receptor in vitro is ligand and dose dependent and that the DNA-binding properties of the in vitro-activated dioxin receptor form are indistinguish-

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able from those of the in vivo-activated species. The biological potency and affinity of a variety of ligands for the receptor protein were correlated with their potency to induce receptor activation in vitro. Moreover, activation could not be achieved in cytosol from nt$^-$ hepatoma cells which contain mutant dioxin receptors. We conclude that this in vitro activation model will be a useful tool for the study of molecular mechanisms determining the conversion of receptors from a cryptic form to a functional species.

MATERIALS AND METHODS

Cells and extract preparation. The wild-type Hepa 1c1c7 cell line and the nt$^-$ mutant cell line c4 derived from it (13) were grown in minimum essential medium as previously described (6, 35). Cytosol was prepared by homogenization of wild-type or mutant cells in 1 volume of 20 mM Tris hydrochloride (pH 7.4)–10% (wt/vol) glycerol–1 mM EDTA–2 mM 2-mercaptoethanol and centrifugation at 120,000 × *g* for 45 min. The resulting supernatant was taken as the cytosolic fraction and either used immediately or frozen in small aliquots at −70°C. For in vitro experiments, the cytosol was labeled with either [1,6-^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]dioxin; Chemsyn, Lenexa, Kans.), [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin ([^{125}I]dioxin; generously supplied by A. Poland), [^{3}H]methylcholanthrene, or [^{3}H]benzo(a)pyrene (Amersham), routinely at a final concentration of 10 nM, and incubated for the stated period of time at 25°C or, in indicated cases, at 0 to 4°C. Cells were treated in vivo with dioxin, and nuclear extracts were prepared as described previously (35). Receptors were analyzed on linear 10 to 40% (wt/vol) sucrose density gradients as described previously (5). The gradients were centrifuged at 300,000 × *g* to a cumulative centrifugal effect of 1.7 × 10^{12} rad^2/s in a Beckman L8-60 ultracentrifuge.

DNA-binding assay. DNA-binding activities were monitored by a gel retardation assay performed essentially as described previously (14). DNA-binding reactions were carried out in a final volume of 50 μl and a final concentration of 60 mM NaCl together with the protein samples described in the figure legends. ^{32}P-3′-end-labeled synthetic oligonucleotide probes (2 to 4 fmol, 10,000 cpm; Fig. 1A) were added to the reaction mixtures after preincubation of the proteins with nonspecific DNA [1 to 2 μg of poly(dI-dC)] at 0 to 4°C for 15 min. After addition of specific probe, the reaction mixtures were incubated for 15 min at 25°C. Protein-DNA complexes were resolved on 4% (acrylamide/bisacrylamide ratio of 30:1) low-ionic-strength native polyacrylamide gels at 30 mA at 0 to 4°C, using a Tris-glycine buffer (14). The conditions for competition experiments were exactly as described above except that different concentrations of unlabeled competitor oligonucleotides (as detailed in the figure legends) were added to the mixture prior to addition of the specific probe. In some experiments, generated protein-DNA complexes were excised from the vacuum dried gel and assayed for radioactivity for quantitation.

Methylation interference experiments. A 38-bp oligonucleotide spanning the XRE1 motif of the rat cytochrome P-450IA1 upstream/promoter region (14) was subcloned into the *Bam*HI site of the polylinker of pUC19 and excised as an *Eco*RI-*Hin*dIII fragment of 86 bp which was used in methylation interference studies. The DNA probe was ^{32}P end labeled at either the *Eco*RI or *Hin*dIII site with the Klenow fragment of DNA polymerase I and partially methylated at guanine residues with dimethyl sulfate as detailed by Maxam and Gilbert (19). DNA-binding reactions were assembled

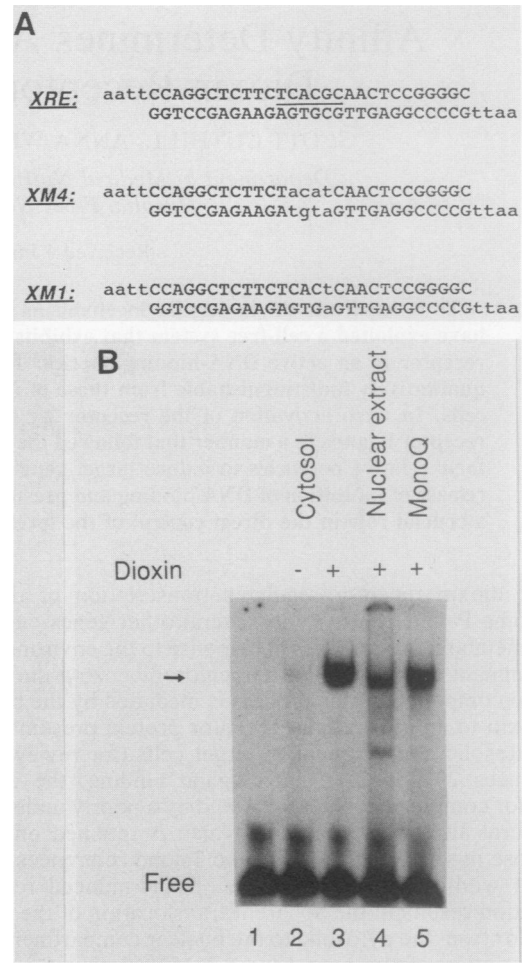

FIG. 1. Dioxin-induced in vitro activation of cytosolic dioxin receptor. (A) Nucleotide sequences of the double-stranded wild-type and point-mutated oligonucleotide probes used. Probe XRE represents the dioxin-responsive element XRE1 that corresponds to nucleotides −1026 to −999 of the promoter/upstream element of the rat cytochrome P-450IA1 gene. A hexanucleotide motif which is conserved between the rat cytochrome P-450IA1 XRE1 and XRE2 (positions −1092 to −1069) dioxin-responsive elements (11) is underlined. The XM4 oligonucleotide carries clustered point mutations in this motif, whereas oligonucleotide XM1 contains one single point mutation of the hexanucleotide core sequence. Lowercase letters indicate linker sequences and point-mutated nucleotides. (B) Results of an electrophoretic gel retardation assay performed with a ^{32}P-labeled XRE fragment and cytosol from Hepa 1c1c7 wild-type cells. For in vitro activation, 100 μg of cytosolic protein was incubated in vitro in the absence (lane 2) or presence (lane 3) of 10 nM [^{3}H]dioxin for 3 h at 25°C prior to the DNA-binding assay. The relative mobility of the dioxin-induced XRE complex (indicated by an arrow) was compared with that of crude nuclear extract prepared from wild-type cells treated with 1 nM [^{3}H]dioxin for 1 h at 37°C in vivo (lane 4) or with a partially purified preparation of dioxin receptor obtained by chromatographic fractionation (Mono Q high-performance liquid chromatography) of nuclear extract from dioxin-treated cells (lane 5). The mobility of the probe in the absence of added protein (designated Free) is shown in lane 1.

with the lightly methylated probe and in vitro dioxin-activated Hepa 1c1c7 cytosol under conditions described above. Protein-DNA complexes were separated from unbound probe by preparative gel electrophoresis and isolated from the gel by electric transfer to NA45 paper (Schleicher & Schuell). Complexes visualized by autoradiography of the paper were salt eluted, extracted sequentially with phenol and chloroform, and cleaved at modified guanine residues with piperidine as described previously (19). The products were analyzed by electrophoresis through an 8% polyacrylamide gel containing 8 M urea.

Safety precautions. Since the analyzed dioxin receptor ligands are extremely toxic or carcinogenic (29), their use required special handling procedures as outlined previously (reference 5 and references therein). Contaminated disposable materials were sent away for high-temperature incineration.

RESULTS

Identification of a dioxin-inducible cytosolic DNA-binding activity as the dioxin receptor. It has previously been demonstrated (12, 14) that treatment of wild-type Hepa 1c1c7 cells with dioxin results in induction of a nuclear factor that specifically interacts with the XRE1 motif (Fig. 1A) found upstream of the rat cytochrome P-450IA1 gene (11). Similarly, addition of the carcinogen 3-methylcholanthrene has recently been reported to convert a cryptic cytosolic factor to an XRE-specific DNA-binding species in vitro (12). 3-Methylcholanthrene is known to interact with the dioxin receptor with high affinity (27). To determine whether the in vivo- and in vitro-induced XRE-specific DNA-binding activities are related, we first investigated the effect of treatment of wild-type Hepa 1c1c7 cytosol with dioxin in vitro. Incubation of cytosol with 10 nM dioxin at 25°C for 3 h resulted in the generation of an XRE complex which comigrated in a gel retardation assay with the in vivo-induced nuclear binding activity (Fig. 1B; compare lanes 3 and 4). In addition, the dioxin-induced cytosolic and nuclear DNA-binding activities were compared qualitatively to a preparation of partially purified dioxin receptor. The receptor was partially purified by Mono Q high-performance liquid chromatography of the in vivo dioxin-activated receptor form (35). During purification, the specific DNA-binding activity cochromatographed with the ligand-binding activity. Importantly, the partially purified receptor preparation yielded one major protein-DNA complex which was indistinguishable in mobility from the binding activities in crude cytosolic or nuclear extracts (Fig. 1B; compare lanes 3 to 5). Additional evidence that the dioxin-induced XRE-specific complex contained the dioxin receptor was obtained by labeling crude cytosol from wild-type hepatoma cells with the high-affinity (4) ligand [^{125}I]dioxin. The high specific activity of this ligand enabled us to directly visualize the ligand-receptor complex by autoradiography following nondenaturing polyacrylamide gel electrophoresis in the presence of an unlabeled XRE probe. Under standard gel retardation assay conditions, the dioxin-induced cytosolic XRE-specific protein-DNA complex (as visualized by a ^{32}P-labeled probe) comigrated with the [^{125}I]dioxin ligand-binding activity (data not shown). Furthermore, formation of the [^{125}I]dioxin-protein complex could be inhibited by coincubation with an excess of the unlabeled dioxin analog 2,3,7,8-tetrachlorodibenzofuran, establishing that the observed ligand-binding activity was attributable to specific receptor interaction. Taken together, these data strongly suggest that dioxin-dependent in vitro or in vivo induction of XRE-specific DNA-binding activity directly involves the genuine dioxin receptor.

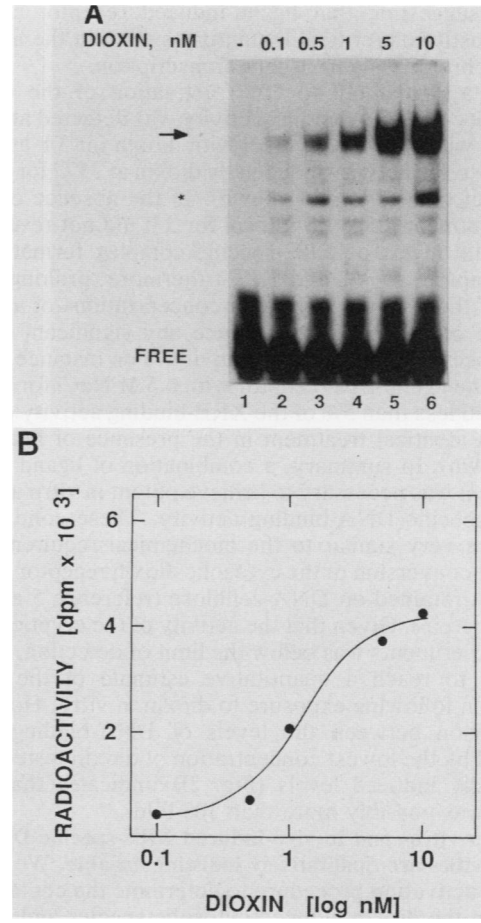

FIG. 2. Dose dependency for in vitro induction by dioxin of XRE-specific DNA-binding activity in wild-type cell cytosol. (A) Cytosol (4 mg of protein per ml) from wild-type hepatoma cells was incubated with 0.1 to 10 nM dioxin at 25°C for 3 h. The DNA-binding activity was monitored by gel retardation assays, using the ^{32}P-labeled XRE oligonucleotide as a specific probe. Lane 1 shows a binding reaction performed with cytosolic protein in the absence of dioxin. The major dioxin-induced complex is indicated by an arrow. The faster-migrating band indicated by a star is also dioxin inducible and appears to represent a distinct receptor degradation product occasionally seen after exposure of cytosol to 25°C. (B) The bands representing the dioxin-induced XRE complexes were excised from vacuum-dried gels and quantitated by liquid scintillation counting.

In vitro induction of the cytosolic XRE-specific complex by dioxin was fully dose dependent. Incubation of wild-type Hepa 1c1c7 cytosol for 3 h at 25°C with increasing concentrations of [^{3}H]dioxin ranging from 0.1 to 10 nM showed clearly detectable induction of formation of the specific complex already at the lowest concentration of [^{3}H]dioxin tested (0.1 nM) (Fig. 2A, lane 2). Maximal levels of complex formation were induced in vitro after treatment of the cytosolic material with 5 to 10 nM [^{3}H]dioxin (Fig. 2B). The concentrations of [^{3}H]dioxin (5 to 10 nM) producing the most potent in vitro response are in excellent agreement with the concentrations required for maximal in vitro occupation of the dioxin receptor in cytosolic extracts (reference 27 and references therein). Thus, the relative increase in XRE binding was correlated with the concentration of the dioxin

signal, suggesting that ligand-induced receptor activation may constitute a critical regulatory switch in the activation of cytochrome P-450IA1 gene transcription.

Only a partial (10 to 20%) activation of the cytosolic XRE-specific DNA-binding activity was detected after treatment of wild-type cell cytosol with dioxin for 3 h at 0 to 4°C relative to the activity induced by dioxin at 25°C for the same period of time (data not shown). In the absence of dioxin, heat treatment alone of cytosol for 3 h did not result in any significant levels of XRE-specific complex formation (Fig. 1B; compare lanes 2 and 3). Furthermore, prolonged treatment of the receptor with high concentrations of salt in the absence of ligand did not induce any significant levels of XRE-specific DNA-binding activity. For instance, incubation of the ligand-free receptor with 0.5 M NaCl for 16 h only produced less than 5% of the XRE-binding activity observed after an identical treatment in the presence of ligand (data not shown). In summary, a combination of ligand and heat treatment was necessary to achieve potent in vitro activation of the specific DNA-binding activity. These conditions, in turn, are very similar to the biochemical requirements for efficient conversion of the cytosolic dioxin receptor to a form which is retained on DNA-cellulose (reference 5 and references therein). Given that the activity of the cryptic factor in most experiments was below the limit of detection, it is very difficult to reach a quantitative estimate of the level of induction following exposure to dioxin in vitro. However, a comparison between the levels of DNA-binding activity induced by the lowest concentration of dioxin tested and the maximally induced levels (Fig. 2B) indicates that the increase was possibly more than 100-fold.

The in vitro- and in vivo-induced XRE-specific DNA-binding activities are qualitatively indistinguishable. We used the in vitro activation procedure to determine the contact points between the dioxin-induced cytosolic species and the XRE target sequence by methylation interference analysis. A DNA fragment containing the XRE1 sequence motif (Fig. 1A) was partially methylated, ^{32}P labeled on either the upper or lower strand, and used in a preparative gel retardation assay with dioxin-treated cytosol from wild-type Hepa 1c1c7 cells. Subsequent analysis of dioxin-induced protein-bound DNA produced a methylation interference pattern (Fig. 3A) centered over the hexanucleotide 5′-TCACGC-3′, which is conserved between the two dioxin response elements XRE1 and XRE2 of the rat cytochrome P-450IA1 gene (11). The pattern (summarized in Fig. 3B) is identical to that obtained with in vivo-activated dioxin receptor from nuclear extracts of dioxin-treated wild-type Hepa 1c1c7 cells (22). Thus, the in vitro-activated species in wild-type cytosol faithfully reproduced the very characteristic methylation interference pattern of the bona fide dioxin-induced nuclear factor, suggesting that the mode of DNA recognition is the same for both the cytosolic and nuclear proteins.

To further characterize the DNA-binding properties of the cytosolic XRE-specific factor, oligonucleotides with point mutations within the hexameric 5′-TCACGC-3′ motif were synthesized. First, a mutant oligonucleotide (XM4; Fig. 1A) was synthesized which contained a cluster of four transversions in the center of the hexanucleotide sequence. This mutant encompasses, on both strands, transversions of all three guanine nucleotides which interfered with the protein-DNA interaction when methylated (Fig. 3B). On the upper strand of the XRE1 sequence, modification of only one guanine residue (at position -1011 of the rat cytochrome P-450IA1 promoter/upstream element) interfered with dioxin-inducible binding (Fig. 3B). It has been reported that

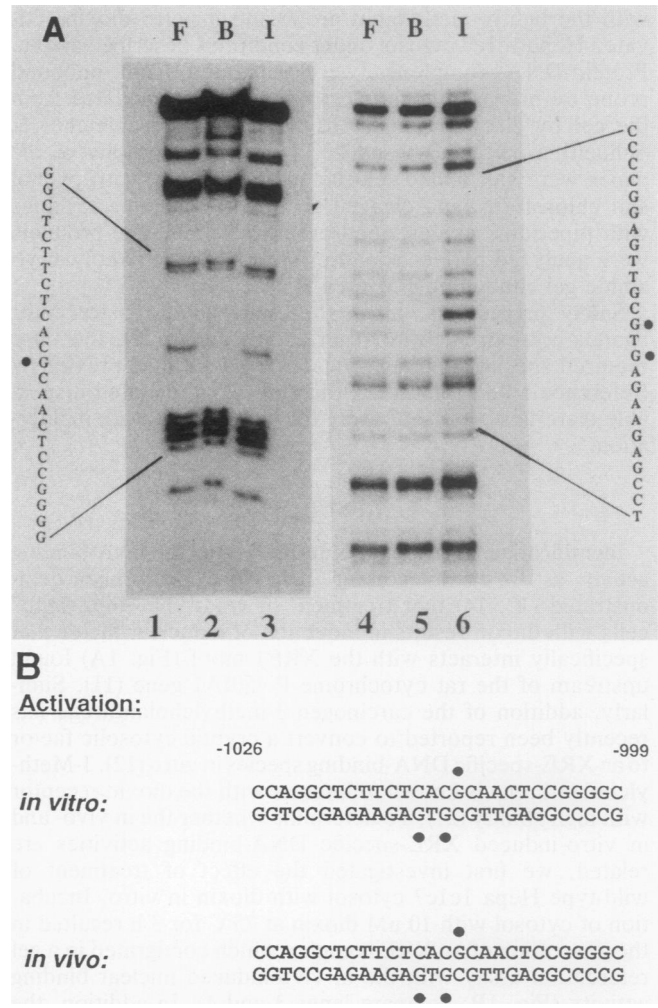

FIG. 3. Methylation interference analysis of the in vitro-induced XRE-specific complex. (A) Methylation interference assays were performed with in vitro-activated cytosol from wild-type Hepa 1c1c7 cells on the coding (lanes 1 to 3) and noncoding (lanes 4 to 6) strands to the cytochrome P-450IA1 XRE1 element. Cytosol was treated with 10 nM [^3H]dioxin for 3 h at 25°C and used for a preparative gel retardation experiment with partially methylated probes. Input DNA (I), DNA present in the bound band (B) representing the dioxin-induced complex, and free DNA probe (F) were isolated, cleaved at the modified guanine residues, and subjected to denaturing polyacrylamide gel electrophoresis. (B) Summary of the guanosine interactions obtained on the XRE sequence with hepatoma cell cytosol treated with dioxin in vitro or with nuclear extracts from hepatoma cells treated with dioxin in vivo (22). Guanosines whose methylation interfered with dioxin-inducible binding are indicated (●).

incorporation of 5-methylcytosine immediately adjacent to this residue abolishes the dioxin-inducible function of the XRE element when introduced into wild-type hepatoma cells (32). Thus, a second mutant (XM1; Fig. 1A) containing a single transversion of this guanine residue was also synthesized. The mutant oligonucleotides XM1 and XM4, together with the wild-type XRE sequence and the nonrelated octamer motif from the BCL1 immunoglobulin heavy-chain promoter, were used in gel retardation competition experiments (28). Formation of either the in vitro or in vivo dioxin-induced XRE complex showed a very similar, if not

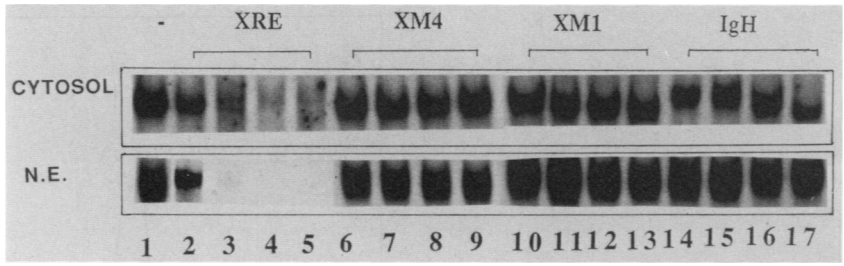

FIG. 4. Demonstration that single-base substitution abolishes receptor recognition of the XRE sequence. The effect of mutagenesis of the XRE sequence on interaction with the dioxin receptor was assessed by gel retardation competition assays. The experiments were carried out with the XRE oligonucleotide as a labeled probe, in vitro (upper panel)- or in vivo (lower panel)-activated dioxin receptor, and increasing concentrations of the following unlabeled competitor oligonucleotides: wild-type XRE (lanes 2 to 5), mutant XM4 (lanes 6 to 9), mutant XM1 (lanes 10 to 13), or an unrelated sequence of similar length spanning the octamer motif of an immunoglobulin heavy-chain promoter (IgH; lanes 14 to 17). As shown in Fig. 1A, the mutant oligonucleotides XM4 and XM1 contain transversions of the dioxin receptor contact points identified by methylation interference analysis. The receptor was in vitro activated by treatment of wild-type hepatoma cell cytosol (4 mg of protein per ml) with 10 nM dioxin for 3 h at 25°C. To activate the receptor in vivo, wild-type cells were exposed to 1 nM dioxin for 1 h, and nuclear extracts (N.E.) were prepared. Shown are autoradiographs of the dioxin-induced XRE complex. Lanes: 1, no competitor DNA; 2, 6, 10, and 14, 5-fold molar excess of competitor; 3, 7, 11, and 15, 25-fold molar excess of competitor; 4, 8, 12, and 16, 100-fold molar excess of competitor; lanes 5, 9, 13, and 17, 200-fold molar excess of competitor.

identical, sensitivity to competition by increasing concentrations of the wild-type XRE sequence (Fig. 4; compare lanes 2 to 5). In contrast, no competition for complex formation was seen with either of the mutant oligonucleotides, XM4 or XM1, in the concentration range tested (compare lanes 6 to 9 with lanes 10 to 13). In fact, at the maximal concentration of competitor (a 200-fold molar excess), the mutant sequences and the unrelated DNA fragment from the immunoglobulin heavy-chain promoter were equally inactive as competitors (compare lanes 9, 13, and 17). In conclusion, no differences were observed in the relative affinities of either the cytosolic or nuclear dioxin-induced factor toward the XRE motif.

These findings were corroborated by direct binding experiments in which the wild-type XRE and the mutant XM4 and XM1 sequences were used as probes. Specific XRE complex formation was observed only with the wild-type probe following incubation with either in vitro-activated cytosol or in vivo-activated nuclear extract. Use of the two mutant oligonucleotides as probes did not show any detectable formation of the specific complex in the presence of either the cytosolic material or nuclear extract (data not shown). Thus, several independent assays demonstrated that the in vivo- and in vitro-induced XRE-specific DNA-binding activities were qualitatively indistinguishable.

A mutant cytosolic dioxin receptor deficient in nuclear translocation is not activated in vitro. XREs fail to function when introduced into nt^- mutant hepatoma cells (11), which have been reported to express levels of dioxin receptor similar to those expressed by parental Hepa 1c1c7 cells (17). Moreover, the native nt^- receptor has the same calculated mass (about 300 kDa) as the nonactivated wild-type receptor (5). In vivo, dioxin does not induce any detectable nuclear XRE-specific DNA-binding activity in nt^- mutant cells (14). In the case of the NFκB transcription factor, it is known that in vitro manipulation can unmask the previously cryptic DNA-binding activity of a form of the factor which is detected in cytosolic but not nuclear extracts from nonlymphoid cells (reviewed in reference 18). We therefore wanted to examine whether XRE-specific DNA-binding activity could be induced in vitro in cytosol from nt^- cells by exposing the nt^- receptor to 10 nM [^{3}H]dioxin for increasing periods of time at 25°C (Fig. 5A). After 5 h of incubation, nt^- cytosol showed a level of specific binding of [^{3}H]dioxin similar to the level observed in wild-type Hepa 1 cytosol (data not shown). At all time points tested, dioxin failed to induce any specific interaction with the XRE target sequence in cytosol from the nt^- mutant cells, although nonspecific complexes of faster mobility and identical to those present in wild-type cell cytosol were detected (Fig. 5A; compare lanes 2 to 5 with lane 6). Moreover, no XRE-binding activity of the nt^- receptor was detected following exposure of the receptor to high ionic strength or increasing concentrations of deoxycholate or Nonidet P-40 (data not shown). Sucrose gradient centrifugation of [^{3}H]dioxin-labeled cytosols from wild type or nt^- mutant hepatoma cells showed no differences in sedimentation properties of the wild-type or nt^- receptor (Fig. 5B), indicating that both receptor proteins had remained intact during the labeling and activation procedure. To support this conclusion, the wild-type and nt^- mutant receptors were labeled with [^{125}I]dioxin in the absence or presence of an excess of radioinert ligand (2,3,7,8-tetrachlorodibenzofuran) and qualitatively compared with each other by nondenaturing polyacrylamide gel electrophoresis. Although a significant amount of radioactivity in cytosolic extracts from both cell lines was detected in the application slots of the gel, this activity was not competed for by an excess of the unlabeled ligand (Fig. 5C, lanes 1 to 4), whereas specific [^{125}I]dioxin-binding activity (lanes 3 and 4; specific ligand complex indicated by an arrow) was detected in a region of the gel corresponding to the migration position of the wild-type receptor-XRE complex (not shown). More importantly, the specific [^{125}I]dioxin-receptor complex in nt^- cytosol had a relative mobility on nondenaturing polyacrylamide gels identical to that of the corresponding complex present in wild-type cytosol (Fig. 5C; compare lanes 1 and 3). Thus, we believe that proteolysis of the nt^- mutant dioxin receptor does not account for the absence of ligand-inducible XRE-specific DNA-binding activity in the mutant cell cytosol.

The affinities of different ligands for the dioxin receptor are proportional to their potencies to induce XRE-specific DNA-binding activity in vitro. To further correlate dioxin receptor activation and in vitro induction of XRE-specific DNA-binding activity, crude cytosol from wild-type Hepa 1c1c7 cells was exposed to dioxin for increasing periods of time.

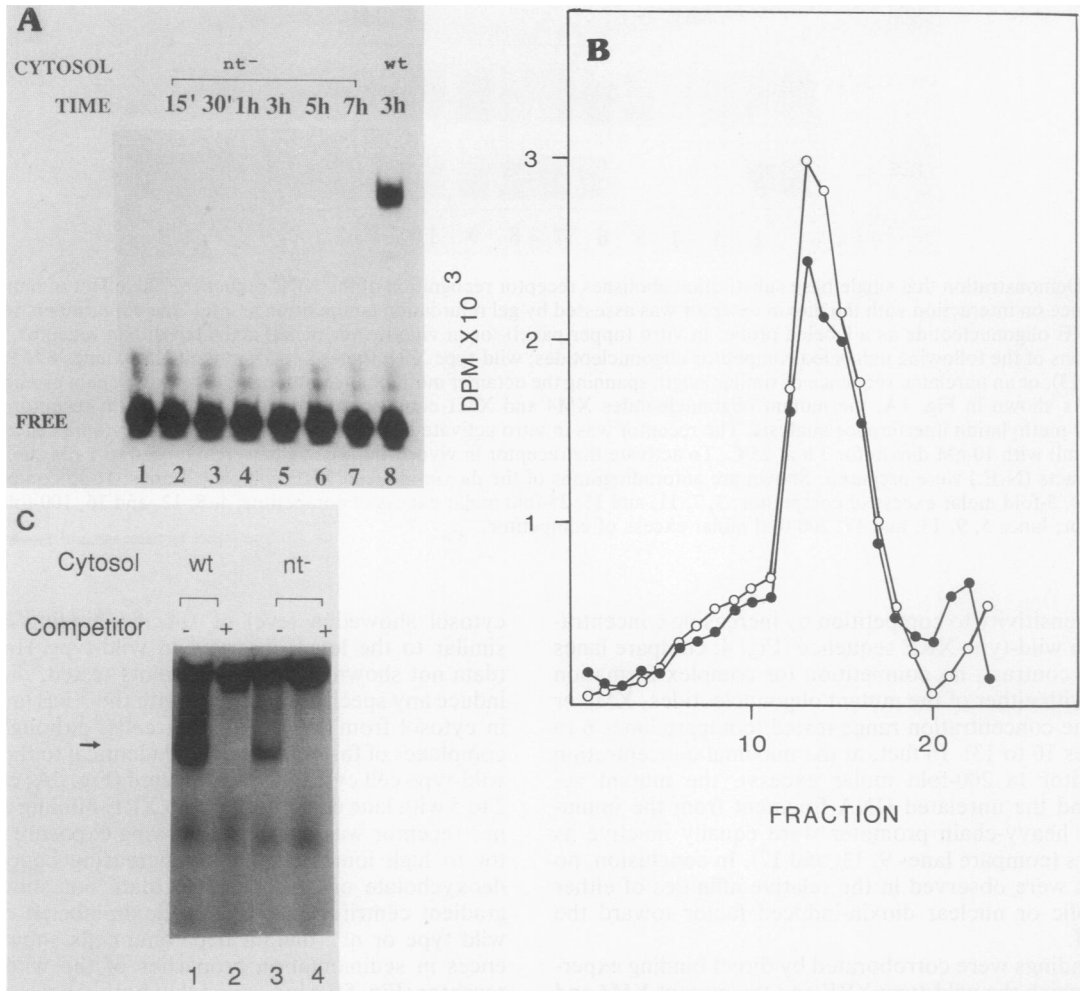

FIG. 5. Lack of inducibility of XRE-specific complex formation in nt$^-$ mutant hepatoma cell cytosol after dioxin treatment in vitro. (A) Cytosol (about 4 mg of protein per ml) from nt$^-$ mutant cells (lanes 2 to 7) was incubated with 10 nM dioxin at 25°C for the indicated periods of time. DNA-binding activities were analyzed by gel retardation assays, using a ^{32}P-labeled wild-type XRE oligonucleotide as a probe. As a control, cytosol (about 4 mg of protein per ml) from wild-type (wt) hepatoma cells was treated with 10 nM dioxin for 3 h at 25°C (lane 8). Lane 1 shows the mobility of the probe in the absence of any added protein. (B) The dioxin receptor present in either wild-type (●) or nt$^-$ mutant (○) hepatoma cell cytosol was labeled with [^{3}H]dioxin and analyzed by sucrose density centrifugation. The protein concentration in both cytosols was about 4 mg/ml. (C) To qualitatively compare the relative mobility of ligand-receptor complexes on nondenaturing polyacrylamide gels following exposure to in vitro activation conditions, the wild-type (wt; lanes 1 and 2) and mutant (lanes 3 and 4) dioxin receptors were labeled with 3 nM [^{125}I]dioxin in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 50-fold molar excess of radioinert 2,3,7,8-tetrachlorodibenzofuran. The specific [^{125}I]dioxin-receptor complexes are indicated by an arrow.

These experiments showed that induction of the XRE-specific complex was time dependent and maximal after about 5 h of incubation (Fig. 6A). Importantly, no DNA-binding activity toward the XRE sequence was detected in cytosol in the absence of dioxin treatment after 3 h of incubation at 25°C (Fig. 6A, lane 8). Incubation of cytosol for even longer periods of time (5 or 7 h) also failed to produce any detectable levels of specific DNA-binding activity (data not shown). Thus, under these conditions, induction of the XRE-specific DNA-binding activity in Hepa 1c1c7 wild-type cell cytosol was strictly dioxin dependent. This result is consistent with the pronounced stability of the non-DNA-binding form of the dioxin receptor, a property which distinguishes it from steroid receptors such as the glucocorticoid receptor (21). Moreover, the time dependency of this activation process is in good agreement with the time course for occupation of receptor by ligand in vitro (27) and the time course for dioxin-induced uptake of dioxin receptor into hepatoma cell nuclei in vivo (23).

In addition to dioxin, polyaromatic compounds such as 3-methylcholanthrene and benzo(a)pyrene bind to the dioxin receptor. Their relative affinities for the receptor protein in in vitro ligand-binding assays are summarized in Table 1. It is interesting to note that these affinities correlate well with the relative potencies of the ligands to induce cytochrome P-450IA1 gene expression and cytochrome P-450 IA1-dependent catalytic activities in vivo (Table 1). To address the mechanistically important issue of whether increasingly weaker ligands for the dioxin receptor (and agonists for the cytochrome P-450 IA1 induction response) exhibited any differences in their effects on activation of the dioxin receptor in vitro, Hepa 1c1c7 wild-type cell cytosol was incubated with a fixed concentration of dioxin, 3-methylcholanthrene, or benzo(a)pyrene. The level of XRE-specific DNA-binding

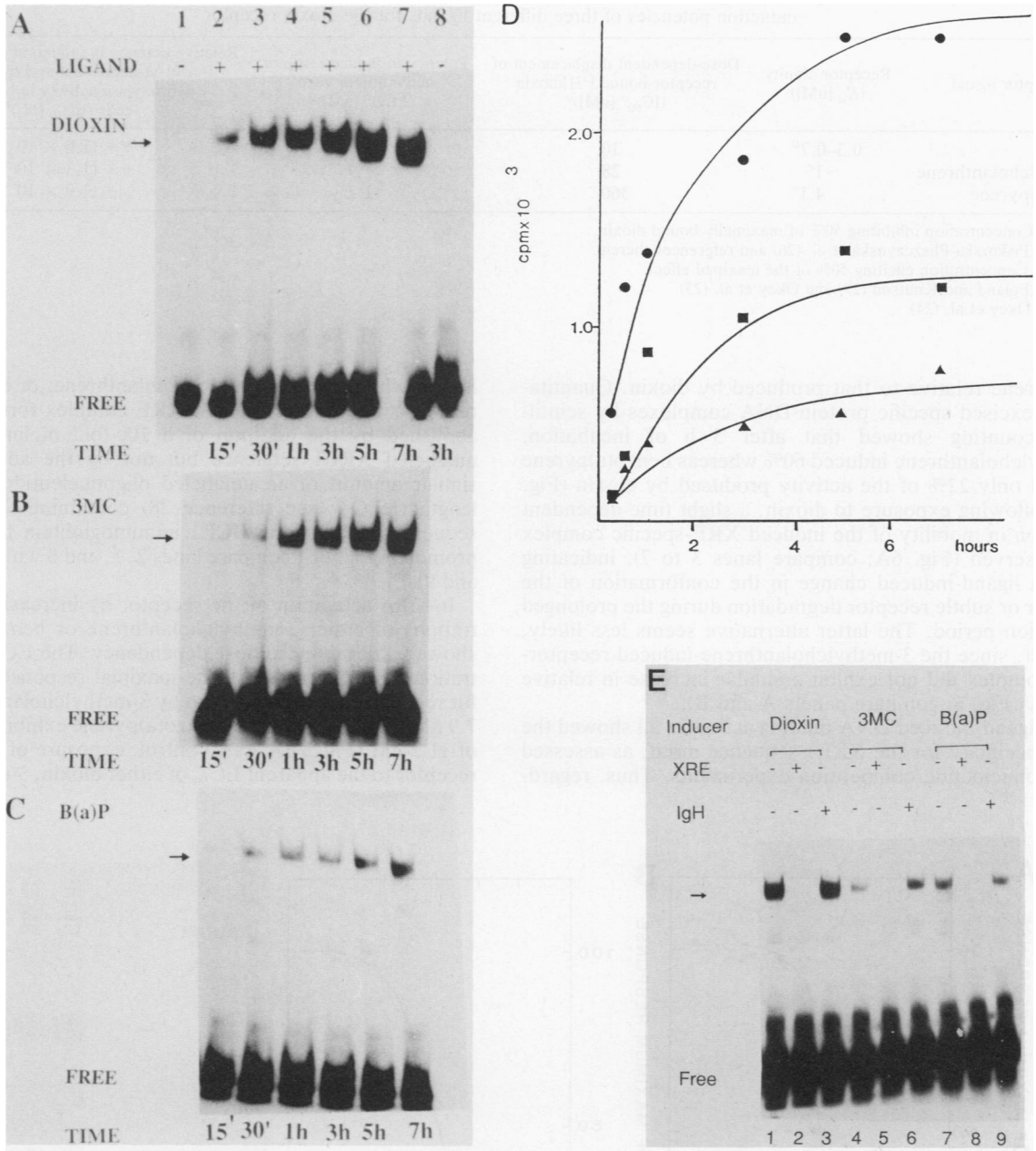

FIG. 6. Time and ligand dependency of activation of the cytosolic dioxin receptor in vitro. Wild-type Hepa 1c1c7 cytosol was incubated with various dioxin receptor ligands at 25°C for increasing times as indicated and analyzed in gel retardation assays. The wild-type XRE oligonucleotide was used as a specific probe. (A) Cytosol treated with 10 nM dioxin. Lanes: 1, mobility of unbound labeled probe with no added protein; 2 to 7, cytosol treated for increasing periods of time as indicated; 8, cytosol incubated at 25°C for 3 h in the absence of dioxin. (B) Cytosol treated with 10 nM 3-methylcholanthrene (3MC) for indicated time periods. (C) Cytosol incubated with 10 nM benzo(a)pyrene [B(a)P] for indicated time periods. Ligand-induced complexes are indicated by arrows. (D) Bands representing the XRE-specific complexes were excised from vacuum-dried gels and assayed by ligand scintillation counting. Symbols: ●, dioxin-treated cytosol; ■, 3-methylcholanthrene-treated cytosol; ▲, benzo(a)pyrene-treated cytosol. (E) Specificity of the induced XRE-binding activities. Cytosolic extracts prepared from untreated wild-type cells were incubated with the indicated ligands and radiolabeled XRE in the absence (lanes 1, 4, and 7) or presence of a 100-fold molar excess of unlabeled XRE (lanes 2, 5, and 8) or an identical excess of the unrelated octamer motif of immunoglobulin promoters (IgH; lanes 3, 6, and 9). Free indicates the mobility of unbound probe.

activity was monitored by gel retardation assays at different time points following incubation with ligand. As outlined above, dioxin induced maximal levels of DNA-binding activity after 5 h of incubation (Fig. 6A). The same pattern of induction kinetics was produced by both 3-methylcholanthrene and benzo(a)pyrene, showing specific DNA-binding maxima after 5 h of incubation (Fig; 6; compare panels A, B, and C). However, the magnitude of the in vitro induction response was considerably lower at all time points following incubation of cytosol with 3-methylcholanthrene or ben-

TABLE 1. Comparison of receptor-binding affinities, in vitro activation potencies, and cytochrome P-450IA1-dependent monooxygenase induction potencies of three different ligands for the dioxin receptor

Receptor ligand	Receptor affinity (K_d [nM])	Dose-dependent displacement of receptor-bound [^{3}H]dioxin (IC_{50}[a] [nM])[b]	Potency to induce receptor activation in vitro (EC_{50}[c] [nM])	Relative potency in induction of cytochrome P-450IA1-dependent aryl hydrocarbon hydroxylase activity in hepatoma cells (EC_{50} [M])[b]
Dioxin	0.3–0.7[d]	10	1.9	+++ (1.0×10^{-11})
3-Methylcholanthrene	~1[e]	28	7.9	++ (1.3×10^{-6})
Benzo(a)pyrene	4.1[e]	360	11.2	+ (1.0×10^{-4})

[a] IC_{50}, Concentration inhibiting 50% of maximally bound dioxin.
[b] From Piskorska-Pliszczynska et al. (26) and references therein.
[c] EC_{50}, Concentration eliciting 50% of the maximal effect.
[d] From Poland and Knutson (29) and Okey et al. (23).
[e] From Okey et al. (24).

zo(a)pyrene relative to that produced by dioxin. Quantitation of excised specific protein-DNA complexes by scintillation counting showed that after 5 h of incubation, 3-methylcholanthrene induced 60% whereas benzo(a)pyrene induced only 22% of the activity produced by dioxin (Fig. 6D). Following exposure to dioxin, a slight time-dependent alteration in mobility of the induced XRE-specific complex was observed (Fig. 6A; compare lanes 3 to 7), indicating either a ligand-induced change in the conformation of the receptor or subtle receptor degradation during the prolonged incubation period. The latter alternative seems less likely, however, since the 3-methylcholanthrene-induced receptor-XRE complex did not exhibit a similar increase in relative mobility (Fig. 6; compare panels A and B).

The ligand-induced DNA-binding activities all showed the same specificity for the XRE1 sequence motif, as assessed by oligonucleotide competition experiments. Thus, regardless of whether dioxin, methylcholanthrene, or benzo(a)pyrene was chosen as a ligand, XRE complex formation was abolished by the addition of a 100-fold molar excess of unlabeled XRE1 sequence but not by the addition of a similar amount of an unlabeled oligonucleotide of similar length (H^+O^+; see reference 28) containing the octamer sequence motif of the BCL1 immunoglobulin heavy-chain promoter (Fig. 6E; compare lanes 2, 5, and 8 with lanes 3, 6, and 9).

In vitro activation of the receptor by increasing concentrations of either 3-methylcholanthrene or benzo(a)pyrene showed a pronounced dose dependency. The EC_{50} (concentration that elicits 50% of the maximal response) observed for receptor activation in vitro by 3-methylcholanthrene was 7.9 nM (Fig. 7A), whereas benzo(a)pyrene exhibited an EC_{50} of 11.2 nM (Fig. 7B). As a control, exposure of the cryptic receptor to the apparent EC_{50} of either dioxin, 3-methylchol-

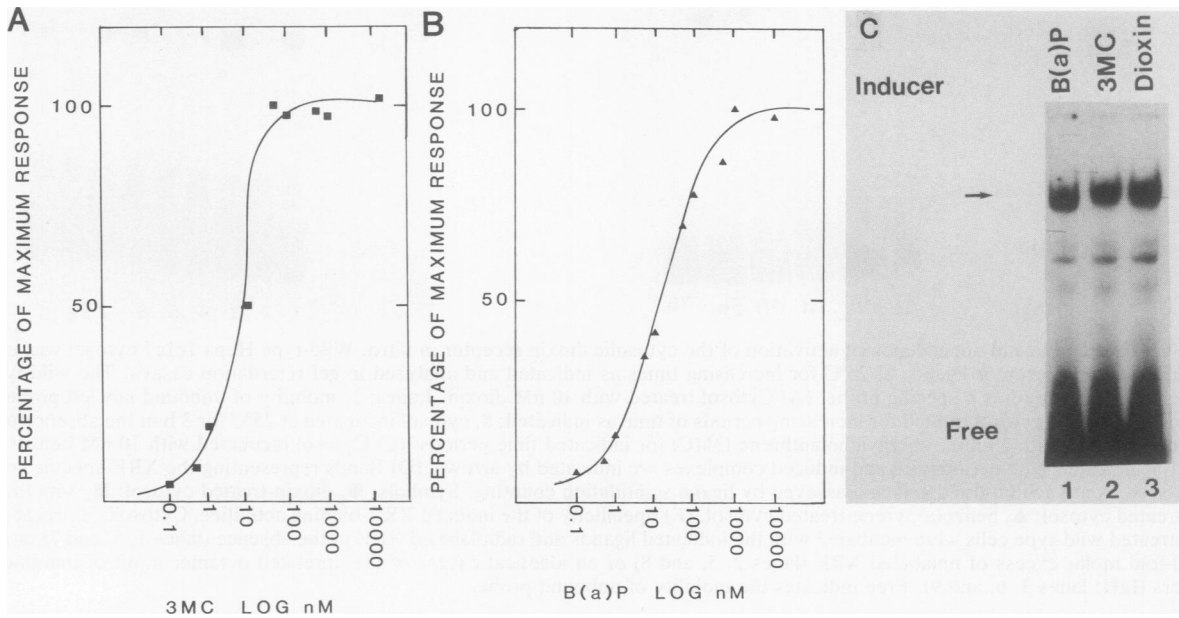

FIG. 7. Dose response to 3-methylcholanthrene or benzo(a)pyrene. Cytosolic extracts from untreated wild-type cells were incubated with increasing concentrations of 3-methylcholanthrene (3MC; A) or benzo(a)pyrene [B(a)P; B]. Ligand-induced XRE-binding activities were monitored by gel retardation analysis. XRE complexes were excised from vacuum-dried gels and quantitated by liquid scintillation counting. The levels of DNA-binding activity were plotted as percentages of the maximal response observed in a particular experiment. No specific XRE-binding activity was detected in the absence of ligand. (C) Gel retardation analysis showing in vitro activation of the cytosolic dioxin receptor following exposure to an EC_{50} dose of dioxin (1.9 nM; lane 1), 3-methylcholanthrene (7.9 nM; lane 2), or benzo(a)pyrene (11.2 nM; lane 3). The unbound XRE probe is indicated by Free. Arrow indicates ligand-induced XRE complexes.

anthrene, or benzo(a)pyrene (1.9, 7.9, or 11.2 nM, respectively) resulted in induction of very similar levels of XRE-binding activity (Fig. 7C). We conclude not only that induction in vitro of XRE-specific DNA-binding activity is dependent on the addition of dioxin receptor ligand to the cytosol but also that the in vitro activation response produced by various receptor ligands follows the rank order of in vivo potencies to induce cytochrome P-450IA1 gene expression. Equally important is the observation (summarized in Table 1) that there exists a correlation between the affinity of a ligand for the dioxin receptor in vitro and its ability to induce receptor activation in vitro.

DISCUSSION

Models of regulation of the DNA-binding activity of transcription factors. We demonstrate here that wild-type hepatoma cell cytosol contains a cryptic XRE-binding activity that can be induced by dioxin treatment in vitro. Our results strongly argue that this DNA-binding activity represents the authentic dioxin receptor. Thus, the inactive dioxin receptor form in cytosol can respond to the ligand and convert that signal to a change in function.

In several instances, regulation of inducible gene expression has been shown to involve posttranslational modification of a preexisting latent transcription factor to an active form which specifically binds to regulatory DNA elements in target genes (reviewed in reference 15). Here we show a similar mode of action for the dioxin receptor which requires ligand-dependent conversion in vivo from a non-DNA-binding to a DNA-binding form, a process which we can faithfully mimick in vitro. In many cases of inducible DNA-binding transactivators, the induced DNA-binding activity can be accounted for by covalent modification (such as phosphorylation) of a factor (reviewed in reference 15). Alternatively, activation of a cryptic transcription factor can be achieved by release from an inhibitor. A well-studied example of such a model is presented by the NFκB transcription factor, which is present in the cytosol of cells in an inactive form as a complex with an inhibitor protein termed IκB (for a review, see reference 18). Similarly, a presently prevailing model of glucocorticoid receptor function (see introduction) postulates that the receptor is initially present in an inactive form complexed with hsp90. The hormone then causes an as yet poorly understood alteration in receptor structure, allowing release of hsp90 and interaction with target DNA sequences.

Implications for dioxin receptor function. While obviously speculative, the model proposed for steroid receptor activation could account for how the cryptic dioxin receptor becomes activated. This suggestion is fully supported by the observation that the dioxin receptor, under a number of conditions, is recovered as a complex with hsp90 (7, 25, 35). Moreover, we have recently demonstrated that the heteromeric, hsp90-containing dioxin receptor complex represents a non-DNA-binding form (35).

The stability and relative insensitivity to in vitro manipulation distinguish the non-DNA-binding form of the dioxin receptor from the latent glucocorticoid receptor complex. Unlike the glucocorticoid receptor-hsp90 complex (9) but in similarity to the cytosolic NF-κB-IκB complex (1), the cryptic, ligand-free dioxin receptor form is remarkably resistant to dilution and exposure to an elevated temperature or high ionic strength (21). Similarly, exposure of wild-type cell cytosol to a variety of dissociating agents, including the detergents deoxycholate and Nonidet P-40, did not significantly unmask the DNA-binding activity of the ligand-free receptor (5a), emphasizing the requirement of ligand for efficient in vitro activation of the receptor. However, exposure of the ligand-free dioxin receptor to high ionic strength for a prolonged period of time results in the generation of an apparently intact receptor monomer and the unmasking of some, albeit low levels of XRE-binding activity (21). Thus, in analogy to the glucocorticoid receptor, it is likely that the ligand-free dioxin receptor is able to interact with its target DNA sequence, once released from hsp90 (35).

In contrast to the wild-type dioxin receptor, no in vitro activation of the nt^- receptor was achieved either by exposure to high ionic strength or by treatment with increasing concentrations of various detergents. Consequently, the present data do not allow us to speculate as to whether the nt^- receptor is primarily defective in its DNA-binding domain or other, as yet unidentified critical determinants for receptor activation.

Role of ligand in receptor activation. We envisage that cellular activation of the dioxin receptor involves primarily two events: translocation of the receptor to the nucleus and appearance of specific DNA-binding activity. Both of these events are under the control of the ligand in vivo. It is interesting that although ligand was required, it was not sufficient for potent in vitro activation of the dioxin receptor. To achieve maximal levels of activation, it was necessary to combine ligand treatment with exposure to the receptor to an elevated temperature. Thus, we believe that the latent dioxin receptor is modified in response to the ligand by at least two steps: interaction with the ligand (and a possible concomitant alteration in structure of the ligand-receptor complex), followed by a temperature-dependent step. It remains to be determined whether the temperature-dependent step solely accelerates subunit dissociation or also reflects a secondary modification and the use of enzymatic intermediaries. Moreover, it is not known whether the in vitro-activated dioxin receptor, although capable of binding the XRE motif, can activate transcription of target genes.

Our in vitro experiments demonstrate a relationship between the potency of different dioxin receptor ligands to induce XRE-specific DNA-binding activity in vitro, their affinity for the receptor protein in vitro, and their potency to induce target gene expression in vivo. It is therefore possible that receptor activation represents a key regulatory event controlling receptor function. This hypothesis is supported by the observations that (i) there exists a correlation between receptor affinity and potency to induce nuclear translocation of various receptor ligands (14) and (ii) intranuclear levels of dioxin receptor are proportional to the levels of induction of cytochrome P-450 target gene expression (33). To our knowledge, the dioxin receptor system represents the first model in which the activity of a latent gene regulatory factor can be very precisely modulated in vitro by an extracellular signal. In contrast, a discrete activation response has been observed both in vivo and in vitro after exposure of a number of latent steroid receptors to their corresponding ligands (9, 16). For instance, exposure of the estrogen receptor to either an agonist or a potent synthetic antisteroid results in maximal levels of in vitro activation of DNA binding of the receptor (16). Furthermore, it has been very difficult to establish an in vitro activation system for the glucocorticoid receptor (see introduction). Ligand-dependent in vitro activation of the dioxin receptor, in turn, seems to be unique in that it appears to be very delicately modulated by, among other parameters, ligand concentration and ligand affinity for the receptor protein, suggesting that signal

transduction might be primarily determined by the level of active receptor sequestered from the latent complex. Obviously, to ultimately understand how the receptor ligand controls this process requires reconstitution in vitro of the latent and active receptor forms with purified components and the establishment of a cell-free, receptor-dependent functional assay.

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