MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6408–6416
0270-7306/90/126408-09$02.00/0

Vol. 10, No. 12

Multiple DNA-Binding Factors Interact with Overlapping Specificities at the Aryl Hydrocarbon Response Element of the Cytochrome P450IA1 Gene

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Received 10 May 1990/Accepted 18 September 1990

Three nuclear factors, the Ah receptor, XF1, and XF2, bind sequence specifically to the Ah response elements or xenobiotic response elements (XREs) of the cytochrome P450IA1 (P450c) gene. The interactions of these factors with the Ah response element XRE1 were compared by three independent methods, methylation interference footprinting, orthophenanthroline-Cu^+ footprinting, and mobility shift competition experiments, using a series of synthetic oligonucleotides with systematic alterations in the XRE core sequence. These studies established the following (i) all three factors interact sequence specifically with the core sequence of XRE1; (ii) the pattern of contacts made with this sequence by the Ah receptor are different from those made by XF1 and XF2; and (iii) although XF1 and XF2 can be distinguished by the mobility shift assay, the sequence specificities of their interactions with XRE1 are indistinguishable. Further characterization revealed the following additional differences among these three factors: (i) XF1 and XF2 could be extracted from nuclei under conditions quite different from those required for extraction of the Ah receptor; (ii) XF1 and XF2 were present in the nuclei of untreated cells and did not respond to polycyclic compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β-napthoflavone, while nuclear Ah receptor was undetectable in untreated cells and rapidly increased in response to TCDD; (iii) inhibition of protein synthesis did not affect the TCDD-induced appearance of the Ah receptor but substantially decreased the constitutive activities of XF1 and XF2, suggesting that the Ah receptor must be present in untreated cells in an inactive form that can be rapidly activated by polycyclic compounds, while the constitutive expression of XF1 and XF2 depends on the continued synthesis of a relatively unstable protein; (iv) the receptor-deficient and nuclear translocation-defective mutants of the hepatoma cell line Hepa1, which are known to lack nuclear Ah receptor, expressed normal levels of XF1 and XF2, suggesting that the former factor is genetically distinct from the latter two; and (v) a divalent metal ion, probably Zn^{2+}, is known to be an essential cofactor for the Ah receptor but was not required for the DNA-binding activities of XF1 and XF2. Together, these findings indicate that the Ah receptor is distinct from XF1 and XF2, while the latter two activities may be related. Because the DNA-binding domains of these three factors overlap substantially, their binding to XREs is probably mutually exclusive, which suggests that the interplay of these factors at Ah response elements may be important to the regulation of *CYP1A1* gene transcription. The results of preliminary transfection experiments with constructs harboring XREs upstream of the chloramphenicol acetyltransferase gene driven by a minimal simian virus 40 promoter are presented that are consistent with this hypothesis.

Cytochrome P450IA1 (see Nebert et al. [34] for current P450 nomenclature) catalyzes the mixed-function oxidation of a wide variety of endogenous and exogenous polycyclic aromatic compounds. Many of these, including the potent environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the carcinogen benzo(*a*)pyrene, induce the expression of the cytochrome P450IA1 gene (*CYP1A1*) by both transcriptional and posttranscriptional mechanisms (9, 26, 27, 37, 46).

The transcriptional component of the response of the *CYP1A1* gene to polycyclic compounds has been shown to be mediated by a class of enhancer elements called aryl hydrocarbon (Ah) response elements or xenobiotic response elements (XREs) (11, 12, 16, 20, 23–25, 35, 43). It has been shown that the Ah receptor, when complexed with polycyclic ligands, is a DNA-binding protein that interacts specifically with the Ah response elements of the *CYP1A1* gene (5–7, 13, 18, 40). Those interactions have been mapped in detail by several independent methods (36, 40, 42). Zn^{2+} has also been shown to be an essential cofactor for the DNA-binding activity of the Ah receptor (40).

Multiple DNA-binding factors, taking part in complex DNA-protein and protein-protein interactions, have been found to be important to the regulation of a number of eucaryotic genes (4, 22, 31, 32, 38, 39, 49). Interactions involved in the regulation of liver-specific genes seem to be particularly complex (4, 31), possibly because of the complexity of liver functions. For example, the murine albumin promoter contains at least six distinct sites at which at least four different proteins interact (31). One feature of such complex interactions is that mutually exclusive binding of factors to the same (22) or adjacent (31, 49) sequences can be important in the regulation of transcription.

In this report, we describe two nuclear factors, in addition to the Ah receptor, that also interact with the XREs of the *CYP1A1* gene. Both factors, called XF1 and XF2, interact sequence specifically with XREs, yet their contacts within this element are different from those of the Ah receptor. The binding domains of these two factors overlap that of the Ah receptor quite substantially, indicating that they probably bind in a mutually exclusive manner. This observation

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suggests that the interplay of XF1, XF2, and the Ah receptor at XREs may be important to the regulation of transcription of the *CYP1A1* gene. We also present transfection experiments yielding preliminary evidence that XF1 and XF2 may function in this way.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extract A, containing XF1 and XF2, was prepared as described by Shapiro et al. (41). The procedure for one 850-cm^2 roller bottle containing 2×10^8 cells was as follows. Cells were harvested by being scraped into phosphate-buffered saline (without Ca^{2+} or Mg^{2+}), collected by centrifugation for 5 min at 800 $\times$ *g* at 4°C, and swollen for 10 min by resuspension in 5 ml 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.9)–10 mM KCl–0.75 mM spermidine–0.15 mM spermine–0.1 mM EDTA–0.1 mM [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA)–1 mM dithiothreitol. Cells were collected by centrifugation as described above, suspended in 1 ml of the same buffer, and broken with 10 strokes in a Dounce homogenizer, using a tight pestle. After addition of 0.2 ml 50 mM HEPES (pH 7.9)–0.75 mM spermidine–0.15 mM spermine–10 mM KCl–0.2 mM EDTA–1 mM dithiothreitol–67.5% sucrose, the nuclei were pelleted by centrifugation at 15,000 $\times$ *g* for 30 s in a microcentrifuge. The nuclei were lysed by resuspension in 0.9 ml of 20 mM HEPES (pH 7.9)–0.75 mM spermidine–0.15 mM spermine–0.2 mM EDTA–2.0 mM EGTA–2 mM dithiothreitol–25% glycerol, followed by the addition of 0.1 volume of saturated $(NH_4)_2SO_4$ at 4°C and incubation on a rotating mixer for 30 min at 4°C. Nuclear extract was obtained by centrifugation of the nuclear lysate at 15,000 $\times$ *g* in a Beckman 70 Ti rotor for 30 min at 4°C, followed by dialysis against 100 mM KCl–20 mM HEPES (pH 7.9)–20% glycerol–0.2 mM EDTA–2 mM dithiothreitol. Extracts were stored at −80°C in small aliquots and were found to retain activity for at least 1 year.

Nuclear extract B, containing the Ah receptor, was prepared as described by Whitlock and Galeazzi (47), with minor changes. Briefly, cells were harvested from tissue culture roller bottles by being scraped into phosphate-buffered saline (without Ca^{2+} or Mg^{2+}) and were centrifuged at 800 $\times$ *g* for 5 min at 4°C. Cells were resuspended in 5 volumes of 10 mM HEPES (pH 7.5) and swollen for 10 min on ice. Cells were collected by centrifugation as before and resuspended in 5 volumes of 3 mM $MgCl_2$–1 mM dithiothreitol–25 mM HEPES (pH 7.5). After centrifugation and resuspension in the same buffer at a concentration of 1×10^8 to 2.5×10^8 cells per ml, cells were broken with 15 strokes in a Dounce homogenizer, using a tight pestle. The cell homogenate was immediately centrifuged at 15,000 $\times$ *g* for 30 s in a microcentrifuge, and the crude nuclear pellet was resuspended in two times its volume of 0.1 M KCl–25 mM HEPES (pH 7.5)–1 mM dithiothreitol. Nuclei were lysed by adding 2 M KCl to a final concentration of 0.4 M and mixing gently at 4°C for 30 min. The lysate was adjusted to 20% glycerol and centrifuged at 150,000 $\times$ *g* for 30 min in a Beckman 70 Ti rotor. The transparent supernatant was stored at −80°C in small aliquots.

Cell culture and DNA transfections. We have previously shown that β-napthoflavone, TCDD, and other polycyclic compounds activate transcription of the *CYP1A1* gene in the rat hepatocyte-derived cell line LCS7, leading to the accumulation of cytochrome P450IA1 mRNA (45). LCS7 cells were obtained from J. Chou (National Institutes of Health, Bethesda, Md.). This line is a clonal derivative of line RALA255-qpG (2), which was generated by transformation of adult rat primary hepatocytes with the tsA255 strain of simian virus 40 (3), which is temperature sensitive for maintenance of transformation. These cells grow prolifically at the permissive temperature but regain many hepatocyte characteristics when shifted to the nonpermissive temperature. Cells from frozen stocks were maintained at 34°C (permissive temperature for transformation) in a 1:1 mixture of Dulbecco modified Eagle medium (4.5 mg of glucose per ml) and Ham F-12 medium (without hypoxanthine), with 4% fetal bovine serum (K. C. Biological, Kansas City, Mo.) and 1 μM cortisol (Sigma Chemical Co., St. Louis, Mo.). When confluent, the cells were transferred to 40°C (nonpermissive temperature for transformation) for 2 days before experiments were performed. Medium was changed every 3 days at 34°C and every day at 40°C. Gentamicin sulfate (50 μg/ml) and amphotericin B (1.5 μg/ml) (GIBCO, Grand Island, N.Y.) were present in the medium at all times. The murine hepatoma cell line Hepa 1c1c7 (Hepa1) and mutant derivatives B13NBii1 (a nuclear translocation defective, or C^-, mutant) and B1SECiiiO^{r}G^{r}4 (a receptor deficient, or B^-, mutant) were obtained from O. Hankinson (University of California at Los Angeles). The medium for Hepa1 cell lines was the same as for LCS7 cells except that it contained 5% fetal bovine serum and no cortisol. Hepa1 cell lines were maintained at 37°C at all times. All cultures were maintained in a humidified atmosphere containing 5% CO_2 and 95% air. Primary cultures of adult rat hepatocytes were prepared and maintained in culture as previously described (37). DNA-mediated gene transfer into these cells and assay of chloramphenicol acetyltransferase (CAT) activity were carried out as previously described (38).

Plasmid constructions. XRE-containing sequences were ligated into the *Bgl*II site of plasmid $pA_{10}CAT$ (29), which contains the bacterial CAT gene linked to an enhancerless simian virus 40 promoter. Three recombinants were constructed. X1,2(117)N carried, in native orientation, the 110-bp region from −1108 to −998, relative to the start site of *CYP1A1* transcription. This region included, from 5′ to 3′, XRE2, an intervening domain of 40 bp, and XRE1. The second construct, X2NX1N, lacked the intervening domain, containing only XRE2, bases −1098 to −1070, fused to XRE1, bases −1028 to −997. The third construct, X2NX1R, contained the same sequences as X2NX1N except that XRE1 was in reverse orientation. The structures of all plasmids were confirmed by DNA sequence analysis.

Gel electrophoresis DNA-binding assay. The mobility shift DNA-binding assay and the preparation of synthetic oligonucleotides and ^{32}P-labeled, double-stranded oligonucleotides were described previously (40). ^{32}P-labeled double-stranded XRE1-40 was used in all mobility shift and footprinting experiments. XRE1-40 is a 40-bp oligonucleotide corresponding in sequence to the region from −997 to −1028 from the transcription start site of the *CYP1A1* gene, with additional bases at each end to create *Bam*HI-ligatable ends. This sequence is 5′-CTCCAGGCTCTTCTCACGCA ACTCCGGGGCAC-3′ (coding strand) and spans the core region of the Ah response element XRE1, bases −1007 to −1021.

Methylation interference footprinting. Methylation interference footprinting was performed as described by Gilman et al. (15), with the minor modifications described previously (40).

Orthophenanthroline-copper footprinting. A preparative-scale mobility shift reaction, containing nuclear extract and

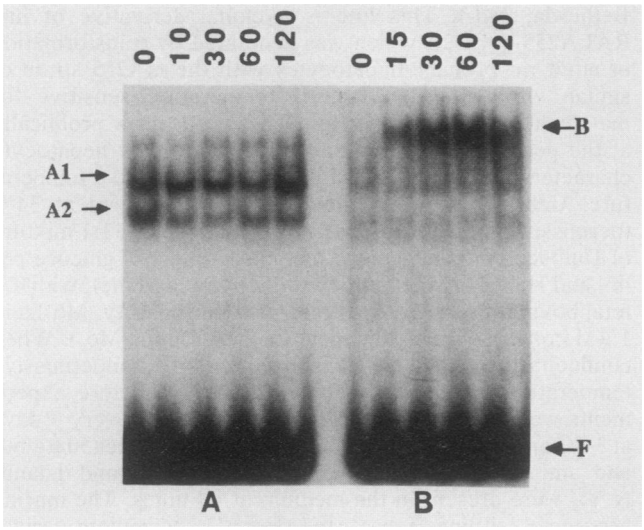

FIG. 1. Response to β-napthoflavone of XF1, XF2, and the Ah receptor from Hepa1 cells. Roller bottles (850 cm^2) containing approximately 2×10^8 Hepa1 cells were left untreated or were treated with β-napthoflavone (5 μg/ml) for 10 to 120 min, as specified above the lanes. Cells were harvested, and nuclear extract A (A) or nuclear extract B (B) was prepared as described in Materials and Methods. Specific binding to XRE1-40 was measured by the mobility shift assay. Each reaction mixture contained 5 μg (A) or 10 μg (B) of extract protein, 1.0 μg of poly(dI-dC) · poly(dI-dC), and 5,000 cpm ^{32}P-labeled XRE1-40 (0.1 ng) in a volume of 10 μl. A1, A2, and B, Specific DNA-protein complexes; F, free XRE1-40 DNA.

^{32}P-labeled XRE1-40, was carried out, and DNA-protein complexes were separated from free DNA by electrophoresis. The gel was then treated with orthophenanthroline-Cu^+ reagent, which cleaves the DNA while in the gel (28). DNA was electrotransferred to DE81 paper, isolated, and run on standard 10% polyacrylamide sequencing gels.

RESULTS

Three DNA-protein complexes form with the Ah response element. It has been demonstrated previously that the Ah receptor can be extracted by the procedure of Whitlock and Galeazzi (47) from the nuclei of β-napthoflavone- or TCDD-treated hepatocyte-derived cells and binds sequence specifically to Ah response elements (5–7, 13, 18, 36, 40, 42). We have used the mobility shift or gel electrophoresis DNA-binding assay (10, 14, 44) to show that under different extraction conditions (41), two other nuclear factors that also bind sequence specifically to XREs can be isolated. Proteinase K treatment destroyed these sequence-specific DNA-binding activities, suggesting that these factors were proteins (data not shown).

In the experiment presented in Fig. 1, nuclear extracts were prepared by either procedure A (41) or procedure B (47) from Hepa1 cells that had been treated for the specified periods of time with 5 μg of β-napthoflavone per ml. The presence of nuclear factors that bind specifically to the Ah response element XRE1 was then determined by the mobility shift procedure, using ^{32}P-labeled XRE1-40 as a probe. One major shifted band (B), corresponding to Ah receptor–XRE1-40 complexes, was observed with nuclear extracts prepared by procedure B (Fig. 1B). The activity giving rise to this band was absent in untreated cells, reached a maximum 1 h after exposure to β-napthoflavone, and then gradually decreased. This result confirmed previous reports (5, 6, 13, 18) that Ah receptor-related DNA-binding activity is undetectable in the nuclei of untreated Hepa1 cells but appears rapidly in response to inducer.

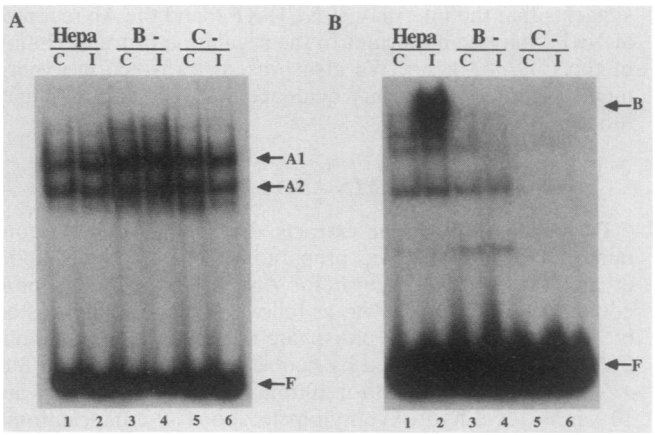

FIG. 2. XRE1-binding activity in wild-type cells and in B^- and C^- derivatives of Hepa1 cells. Nuclear extracts prepared by procedure A (A) or procedure B (B) from wild-type Hepa1 cells (lanes 1 and 2), mutant B^- (lanes 3 and 4), and mutant C^- (lanes 5 and 6) were tested for XRE1-binding activity as described for Fig. 1. Cells were left untreated (lanes C) or were treated with TCDD for 60 min (lanes I).

Surprisingly, extracts prepared by procedure A from the nuclei of Hepa1 cells contained DNA-binding activities that gave rise to a strikingly different banding pattern in the mobility shift assay using ^{32}P-labeled XRE1-40 as probe (Fig. 1A). Two bands, A1 and A2, were detected, whose electrophoretic mobilities were substantially greater than that of Ah receptor–XRE1-40 complexes (band B). Furthermore, the DNA-binding activity or activities responsible for bands A1 and A2 changed very little after treatment with β-napthoflavone for up to 2 h, while nuclear Ah receptor was induced by β-napthoflavone. Figures 5 to 7 demonstrate that bands A1 and A2 are due to sequence-specific DNA-protein interactions.

XF1, XF2, and the Ah Receptor in mutant Hepa1 cell lines. The experiment presented in Fig. 2 provides genetic evidence distinguishing XF1 and XF2 from the Ah receptor. Hankinson (17) has cloned mutants of the Hepa1 cell line that are defective in Ah receptor function. The B^- mutant is deficient in Ah receptor activity, as measured by binding of ^{3}H-TCDD. The C^- mutant is defective in the ability to translocate the receptor-ligand complex to the nucleus. On the basis of measurements of ^{3}H-TCDD-binding activity, nuclear extracts from both lines lack the Ah receptor (30). We examined extracts prepared from these mutants and from wild-type Hepa1 cells by both procedures A and B. XF1 and XF2 were present in nuclear extracts prepared from the B^- and C^- cell lines (Fig. 2A, lanes 3 to 6) at levels at least equal to those found in wild-type Hepa1 cells (Fig. 2A, lanes 1 and 2), while the Ah receptor was not detected in nuclear extracts prepared from either mutant cell line (Fig. 2B, lanes 3 to 6) but was present in nuclear extracts from β-napthoflavone-treated wild-type Hepa1 (Fig. 2B, lane 2). Since both B^- and C^- mutants are known to lack nuclear Ah receptor activity, the absence of band B in mobility shift reactions containing extracts from these two cell lines confirms that this band represents Ah receptor-XRE complexes. The fact that XF1 and XF2 were present in nuclear extracts from both variants demonstrated that the expression of XF1 and XF2 was not influenced by mutations that alter the

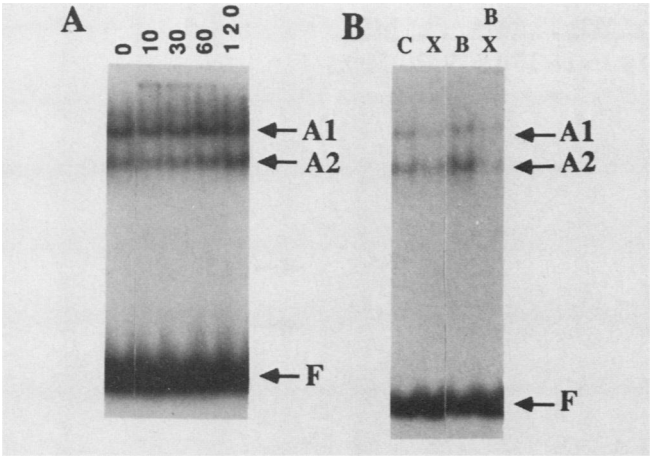

FIG. 3. Response of XF1 and XF2 from LCS7 cells to β-napthoflavone and cycloheximide. LCS7 cells were cultured and harvested, and mobility shift assays were performed as for Fig. 1. (A) Before harvesting, cells were treated for the specified times with β-napthoflavone. (B) Before harvesting cells were treated for 2 h with β-napthoflavone (lane B), for 2.5 h with cycloheximide (10 μg/ml; lane X), or with both (lane BX).

expression of nuclear Ah receptor-related DNA-binding activity.

Cycloheximide sensitivity of XF1 and XF2. We have previously reported that Ah receptor-related DNA-binding activity can be detected in the nuclei of β-napthoflavone-treated LCS7 cells (40). XF1 and XF2 could also be detected in LCS7 cells (Fig. 3); they were expressed constitutively and were not responsive to β-napthoflavone, just as was observed for Hepa1 cells (Fig. 1).

We previously reported that the protein synthesis inhibitor cycloheximide was unable to block the β-napthoflavone-induced accumulation of Ah receptor-related DNA-binding activity in the nuclei of LCS7 cells (40). In contrast, XF1 and XF2 were sensitive to cycloheximide (Fig. 3B). Treatment with cycloheximide for 2.5 h (lanes X and BX) caused both bands A1 and A2 to diminish compared with untreated cells (lane C) and cells treated with β-napthoflavone (lane B). This result suggests that both XF1 and XF2 must contain proteins of relatively short half-lives.

Mobility shift competition experiments. A series of mobility shift competition studies was performed to (i) determine whether bands A1 and A2 represent specific protein-DNA interactions and (ii) compare the interactions of XF1 and XF2 with XRE1 with the previously mapped (40) interactions of the Ah receptor with XRE1.

We tested the ability of a series of mutant double-stranded oligonucleotides to compete with ^{32}P-labeled XRE1-40 for binding to XF1 and XF2 (Fig. 4). XRE1-15 is a 15-bp oligonucleotide containing the core domain of the Ah response element XRE1 (Fig. 5). Each mutant oligonucleotide differed in sequence from XRE1-15 by three bases (Fig. 5). Bands A1 and A2 diminished in parallel when unlabeled XRE1-40 (Fig. 4, lanes 2 to 4) or XRE1-15 (lanes 5 to 7) was included in DNA-binding reactions at molar concentrations 5-, 25-, and 100-fold higher than that of ^{32}P-labeled XRE1-40. In contrast, these bands were undiminished in DNA-binding reactions carried out in the presence of the same concentrations of oligonucleotides M4 and M5 (lanes 17 to 22) and were decreased only slightly in reactions carried out in the presence of oligonucleotide M1 (lanes 8 to 10). Similar concentrations of oligonucleotides M2 and M3 competed almost as effectively as XRE1-15 for binding of ^{32}P-labeled XRE1-40 by XF1 or XF2.

These findings indicate that A1 and A2 represent sequence-specific DNA-protein complexes, since the ability of oligonucleotides to compete for binding with ^{32}P-labeled XRE1-40 was sequence dependent. These results also indicate those bases within the XRE1 core sequence that are most important for formation of complexes A1 and A2. For instance, in oligonucleotide M1, the triplet TCT, spanning base pairs −1020 to −1018 in XRE1, had been replaced with AGA. The greatly attenuated ability of this oligonucleotide to compete with XRE1-40 for binding to form complexes A1 and A2 indicated that at least one of these three base pairs was important in binding factors XF1 and XF2. Similarly, the inability of oligonucleotides M4 and M5 to compete indicated that at least one base pair in the triplet spanning positions −1011 to −1009 and at least one base pair in the triplet spanning positions −1008 to −1006 must have been important in the interaction of factors XF1 and XF2 with XRE1-40. Since oligonucleotides M2 and M3 competed effectively, we conclude that the positions at which these oligonucleotides differ from XRE1-15 must not have been essential for binding of XF1 and XF2. Competition was identical for bands A1 and A2, indicating that XF1 and XF2 interact very similarly with XRE1-40.

We previously carried out competition mobility shift experiments, using the same series of oligonucleotides to map the interactions between the Ah receptor and XRE1 (40). A different pattern of competition was observed in which M1, M3, and M4 failed to compete or competed very weakly with ^{32}P-labeled XRE1-40 for binding to form complex B, whereas M2 competed somewhat more strongly and XRE1-40, XRE1-15, and M5 competed strongly. Comparison of these findings with those presented in Fig. 4 identifies both similarities and distinct differences in the interactions of XF1, XF2, and the Ah receptor with XRE1-40. Some bases, those altered in oligonucleotides M1 and M4, were important to the binding of all three factors, while those bases altered in M3 were important only to the binding of the Ah receptor and those altered in M5 were important only to the binding of XF1 and XF2. It appears, therefore, that although XF1 and XF2 bind to the same sequence domain as does the Ah receptor, different bases within this domain are required for the binding of these proteins.

Methylation interference footprinting. To further compare the interactions of XF1, XF2, and the Ah receptor with XRE1, the methylation interference footprinting technique was used (15, 19). This procedure identifies guanine residues that, when methylated at the N-7 position in the major groove, prevent or weaken binding of a factor. End-labeled XRE1-40 was partially methylated with dimethyl sulfate (19) and used as probe in a preparative-scale mobility shift reaction. Free and bound DNAs were separated by electrophoresis, isolated from the acrylamide gel, cleaved with piperidine, and run on a standard 10% sequencing gel. When XRE1-40, 5′ end labeled on the coding strand, was used as a probe, one band corresponding to the guanine at position −1011 was dramatically reduced in intensity in complexes formed with XF1, XF2, and the Ah receptor, indicating that this guanine was important for the interactions of all three factors with XRE1 (Fig. 6). When the contacts on the noncoding strand were mapped, complexes formed with XF1 and XF2 gave identical interference patterns, while those formed with the Ah receptor gave a different but overlapping pattern; methylation of the guanines at positions −1007, −1010, and −1012 interfered with binding of both

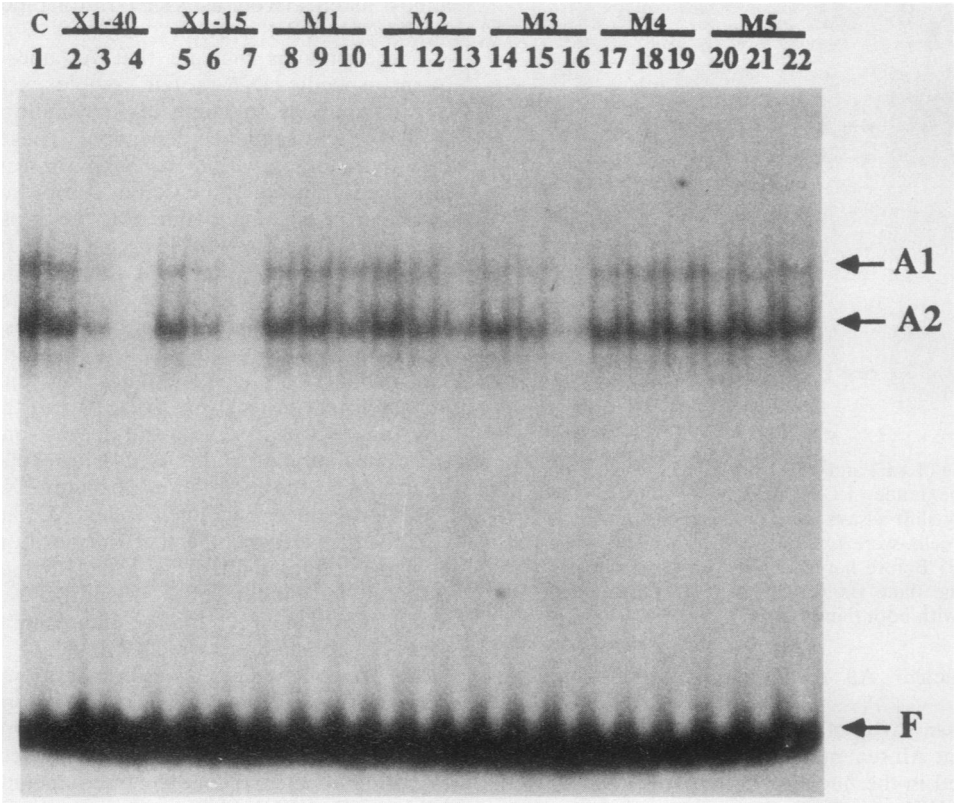

FIG. 4. Specific binding of XF1 and XF2 to the Ah response element of the *CYP1A1* gene. Nuclear extracts (5 μg), prepared from LCS7 cells as described by Shapiro et al. (41), were incubated with XRE1-40 (0.02 ng, 5,000 cpm) and 1 μg of poly(dI-dC) · poly(dI-dC), and the reactions were resolved by electrophoresis in a low-ionic-strength, 8% polyacrylamide gel. All lanes contained extract from β-napthoflavone-treated cells. Lane 1 contained no competitor DNA; each subsequent set of three lanes contained 0.1 ng, 0.5 ng, and 2.0 ng (5-, 25-, and 100-fold molar excess, respectively) of competitor: XRE1-40 (lanes 2 to 4), XRE1-15 (lanes 5 to 7), and XRE1-related oligonucleotides M1 (lanes 8 to 10), M2 (lanes 11 to 13), M3 (lanes 14 to 16), M4 (lanes 17 to 19), and M5 (lanes 20 to 22). Positions of the specific protein-DNA complexes (A1 and A2) and free probe (F) are indicated.

XF1 and XF2, whereas methylation of the guanines located at −1010, −1012, and −1014 suppressed binding of the Ah receptor. Thus, the guanine at position −1007 on the noncoding strand was involved only in formation of complexes A1 and A2 and that at position −1014 on the noncoding strand was involved only in formation of complex B. In contrast, the guanines at positions −1010 and −1012 on the noncoding strand and at −1011 on the coding strand were involved in formation of all three complexes.

Orthophenanthroline-copper footprinting. Footprinting by the orthophenanthroline-Cu^+ footprinting technique (28) was used as a third technique to map the interactions of XF1 and XF2 with XRE1. In this procedure, DNA-protein complexes were resolved from free DNA by electrophoresis and then subjected to cleavage by orthophenanthroline-Cu^+ reagent while still in the gel. This procedure takes advantage of the increased stability of DNA-protein complexes within the gel matrix. After cleavage, the DNA was eluted from the gel and run on a standard sequencing gel. XF1 and XF2 protected, and therefore interacted with, the same region of XRE1-40 (Fig. 7). Comparison of these footprints of XF1 and XF2 with the previously reported orthophenanthroline-Cu^+ footprint of the Ah receptor (40) shows that the receptor also interacted with this same domain within XRE1. It is apparent, however, that the boundaries of the protected domains were identical for XF1 and XF2 but somewhat different for the Ah receptor. For XF1 and XF2, the boundaries on the coding strand were at bases −1003 to −1004 and −1020 to −1018 and those on the noncoding strand were at bases −1003 to −1004 and −1020 to −1022. For the Ah receptor, the boundaries on the coding strand were at bases −1004 to −1005 and −1018 and those on the noncoding strand were at bases −1006 and −1022.

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WT    TCTTCTCACGCAACT
M1    AGA------------
M2    ---AGA---------
M3    ------GTG------
M4    ---------CGT---
M5    ------------TGA
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FIG. 5. Sequences of XRE1-related oligonucleotides used in competition studies. The complete XRE1 core sequence is specified. Only bases that differ in each mutant from the XRE1 sequence are indicated.

DNA-mediated gene transfer. In constructs X2NX1N and X2NX1R, tandem XRE domains placed upstream of a minimal promoter are sufficient to confer upon the CAT reporter gene responsiveness to TCDD and cycloheximide (Fig. 8; Table 1). Exposure of the cells to either one of these compounds caused a modest increase in CAT activity. Together, TCDD and cycloheximide strongly stimulated CAT expression. Similar responses were observed with a

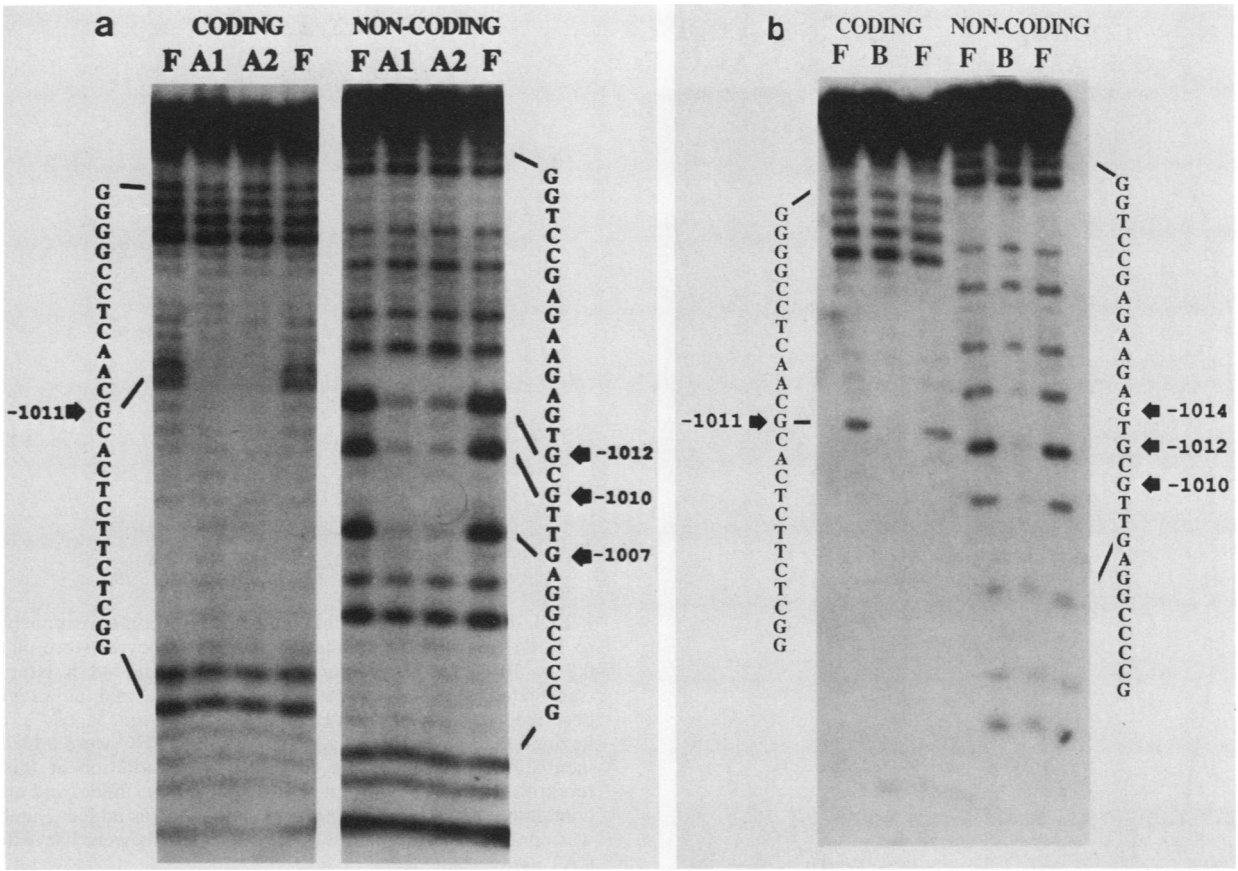

FIG. 6. Methylation interference analysis. (a) Analysis of contact sites with XF1 and XF2. XRE1-40, ^{32}P-labeled on either strand as indicated, was partially methylated and incubated with 150 μg of nuclear extract A from LCS7 cells. DNA-protein complexes were separated from free probe by gel electrophoresis, and both DNA fractions were recovered from the gel. The DNA was cleaved with piperidine and electrophoresed through a standard 10% acrylamide sequencing gel. Lanes: F, free probe, showing cleavage at all guanines; A1 and A2, DNA-protein complexes, as in Fig. 1 to 4. The nucleotide sequences corresponding to the regions of interest are presented in the margins. The guanines at which methylation blocks binding are indicated by arrows on the sequences. (b) Analysis of contact sites with the Ah receptor. The experiment was carried out as for panel a except that extract B, containing nuclear Ah receptor, was used. Lanes: F, free probe; B, the Ah receptor-XRE1 complex, as in Fig. 1 and 2.

third construct containing the native sequence domain that includes XRE1 and XRE2 [X1,2(117)N].

XF1, XF2, and the Ah receptor are the only nuclear proteins from hepatocyte-derived cells that bind specifically in vitro to the XRE sequences contained in plasmids X2NX1N and X2NX1R. Figures 1 to 7 and 9 demonstrate this for XRE1; similar data for XRE2 and for primary hepatocytes will be presented elsewhere. It is likely, therefore, that the effects of TCDD and cycloheximide (Fig. 8) are mediated by interactions of one or more of these proteins with the XREs contained in these constructs. Activation of gene expression by TCDD is probably mediated by interactions between the liganded Ah receptor and XREs, as has been proposed previously (5–7, 13, 18, 40). The effect of cycloheximide (Fig. 8) may be due to XF1 and XF2. This possibility is suggested by the results presented in Fig. 3 and by the observations of Saatcioglu et al. (40), showing that XF1 and XF2 decrease markedly following exposure to cycloheximide, while the Ah receptor is insensitive to this protein synthesis inhibitor. The synergistic activation of gene expression by cycloheximide and TCDD would therefore be due to the cycloheximide-dependent loss of XF1 and XF2, allowing increased binding of the Ah receptor to XREs and consequently increased activation of gene expression by the receptor.

DISCUSSION

A central focus of this research was to establish the existence of nuclear factors, in addition to the Ah receptor, that bind sequence specifically to Ah response elements. Neuhold et al. (36) have reported the sequence-specific binding of nuclear factors to domains near but not overlapping the Ah response elements of the *CYP1A1* gene. A careful examination of other work describing the interactions of the Ah receptor with Ah response elements reveals that bands other than that representing Ah receptor-XRE complexes are usually detected by the mobility shift DNA-binding assay using short XRE-containing probes (5–7, 13, 18). However, the results presented here are the first conclusive evidence that two of these other complexes are due to the sequence-specific binding by factors other than the Ah receptor.

Three independent approaches were used to establish that XF1 and XF2 interact specifically with Ah response elements. Figure 9 summarizes these analyses. It also compares the interactions of the Ah receptor with XRE1, reported by Saatcioglu et al. (40), with the interactions of XF1 and XF2 with XRE1. The following three conclusions can be drawn from these studies. (i) XF1, XF2, and the Ah receptor all interact in a sequence-specific manner with the XRE1 core

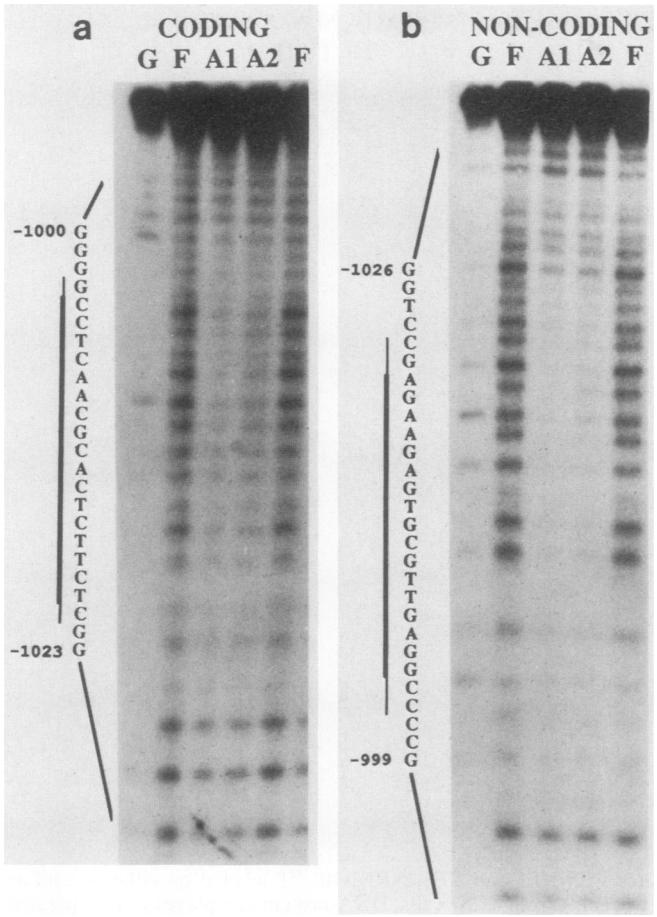

FIG. 7. Orthophenanthroline-Cu^+ chemical footprinting analysis. XRE1-40, end labeled on the coding (a) and noncoding (b) strands, was incubated with 100 μg of extract A isolated from LCS7 cells. DNA-protein complexes and free probe were separated by gel electrophoresis, and the intact gel was treated with orthophenanthroline-Cu^+. DNA was recovered as described in Materials and Methods and electrophoresed on standard 10% acrylamide sequencing gels. Lanes: A1 and A2, DNA isolated from bands corresponding to the similarly labeled bands in Fig. 1 to 4; F, DNA from free probe; G, guanine sequencing ladder. The nucleotide sequences corresponding to the footprints are shown at the left. Heavy and light bars indicate bases within the DNA sequence that were protected completely and partially, respectively, from orthophenanthroline-Cu^+ cleavage. Light exposures indicated that radioactivity in excess of that in other lanes had been loaded in lane A2 of panel a. This was taken into account when the boundaries of the footprints were defined.

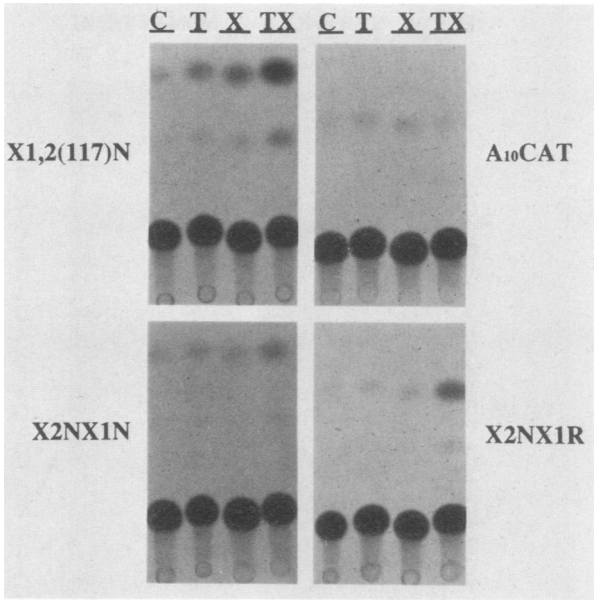

FIG. 8. Demonstration that tandem XREs confer responsiveness to TCDD and cycloheximide. Primary hepatocytes were plated at 0.7×10^6 cells per 60-mm-diameter dish and 48 h later were transfected with 12 μg of the specified plasmid as previously described (38). After another 40 h, TCDD (1 nm; lanes T), cycloheximide (10 μg/ml; lanes X), or both (lanes TX) were added, and incubation continued for 6 h. Lane C, no addition at this time (control). Cells were washed to remove cycloheximide, and medium containing the same concentration of TCDD was added. Incubation was continued for another 6 h, after which cells were harvested for CAT assay.

sequence. (ii) The pattern of contacts between XRE1 and the Ah receptor differs substantially from those between XRE1 and XF1 and XF2. Some bases are important to the binding of both factors, while others are uniquely involved in the binding of one or the other factor. (iii) XF1 and XF2 interact indistinguishably with XRE1.

All three mapping procedures supported the first conclusion. Mobility shift competition experiments using mutant oligonucleotide competitors as well as methylation interference footprinting, both of which yield precise information concerning the participation of specific bases in DNA-protein interactions, were the basis of the second and third conclusions. The inherently less precise information obtained by orthophenanthroline-Cu^+ footprinting was, however, consistent with these conclusions.

On the basis of results of all three mapping procedures, we conclude that the Ah receptor is distinct from XF1 and XF2, while the latter two activities may be related. The similarity of their interactions with XRE1 suggests that they might be generated either by the association of different proteins with a single DNA-binding subunit or by proteolytic or other modification of a single DNA-binding protein.

In addition to these footprinting data, we have presented several lines of evidence distinguishing the Ah receptor from XF1 and XF2. These are as follows: (i) these factors are extracted from nuclei under quite different conditions; (ii) XF1 and XF2 are present in the nuclei of untreated cells and are not influenced by exposure to β-napthoflavone or TCDD, while nuclear Ah receptor is undetectable in untreated cells and rapidly increases in response to inducer; (iii) XF1 and

TABLE 1. Responsiveness conferred by XREs to TCDD and cycloheximide

Plasmid	% Acetylation of [^{14}C]chloramphenicol[a]			
	Untreated	TCDD[b]	Cycloheximide[b]	TCDD + cycloheximide
X1,2(117)N	0.44	1.14	2.61	6.55
X2NX1N	0.36	0.48	0.55	2.11
X2NX1R	0.21	0.51	0.41	2.97
A_{10}CAT	0.29	0.29	0.28	0.25

[a] Averages of duplicates from two separate experiments.
[b] See legend to Fig. 8 for experimental conditions.

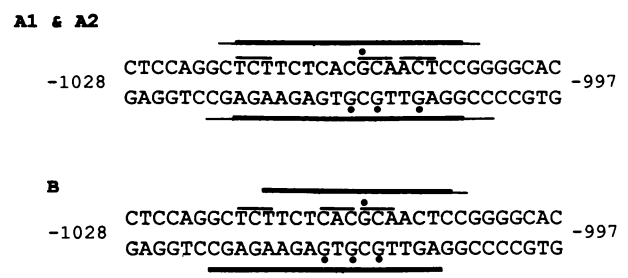

FIG. 9. Summary of the interactions between XRE1 and XF1, XF2, and the Ah receptor. *CYP1A1* sequences from −1028 to −997 are represented. Heavy and light lines indicate strong and weak protection, respectively, from orthophenanthroline-Cu^+ by XF1 and XF2 (A1 and A2) or the Ah receptor (B). The positions of apparent protein contacts determined by methylation interference are indicated by dots. Bases that were important for binding, as determined by competition experiments with mutant oligonucleotides, are indicated by short bars above the coding strand.

XF2 decrease following treatment with cycloheximide, while the Ah receptor is not affected; and (iv) the B^- and C^- mutants of Hepa1 both lack nuclear Ah receptor but have normal levels of XF1 and XF2, providing somatic cell genetic evidence that distinguishes the former factor from the latter two. However, since it has not been demonstrated that the locus of either the B^- or C^- mutation is actually the Ah receptor gene, this evidence is only suggestive.

A final line of evidence that distinguishes between the Ah receptor and XF1 and XF2 is that the DNA-binding activity of the Ah receptor is inhibited by the chelator 1,10-phenanthroline and by Cd^{2+} and Co^{2+} (40), while the activities of XF1 and XF2 are not (data not shown). This observation, along with other previously reported findings (40), suggests that a divalent metal ion, probably Zn^{2+}, is an essential cofactor for the Ah receptor but not for XF1 and XF2.

We found that the method of Shapiro et al. (41) (extraction procedure A) yielded nuclear extracts from both LCS7 and Hepa1 cells that were selectively enriched in XF1 and XF2, while the method of Whitlock and Galeazzi (47) (extraction procedure B) yielded extracts enriched in the Ah receptor. Other studies (data not shown) suggest that this difference in extractability is probably because the Ah receptor associates more weakly with the nucleus than do XF1 and XF2. In extraction procedure A, nuclei are prepared by homogenization of cells in a relatively large volume of buffer, probably resulting in leakage of the Ah receptor from the nucleus. Thus, nuclear extracts prepared from such nuclei contain predominantly XF1 and XF2. We do not quantitatively recover the Ah receptor in the cytoplasmic fraction obtained during extraction procedure A, probably because the homogenization buffer used lacks glycerol, which is required to stabilize the receptor. When extraction procedure A is modified by homogenizing a minimal volume and separating the nuclei very rapidly from the cytoplasmic fraction, the Ah receptor can be recovered along with XF1 and XF2. XF1 and XF2 are absent from extract B because the concentration of KCl used in extraction procedure B is sufficient for release of the receptor, but not for the release of XF1 and XF2, from the nucleus. We find that XF1 and XF2 can be recovered along with the receptor either by extraction with higher concentrations of KCl or by extraction with $(NH_4)_2SO_4$.

Modulation of transcription by competition between positive and negative regulatory factors for binding to overlapping recognition sequences has been proposed in other systems. For instance, the glucocorticoid receptor down-regulates transcription of the gene encoding the α subunit of the glycoprotein hormones by binding to sequences that overlap the cyclic AMP response element of that gene, thus competing with positive regulatory factors for binding to that element (1). A second example is the negative regulation of the pro-opiomelanocortin gene by glucocorticoids, in which it is thought that the activated glucocorticoid receptor-ligand complex competes for binding with the CAAT box transcription factor (8). The evidence presented here suggests that XF1 and XF2 may be negative regulatory proteins that act through XREs to modulate the positive regulatory action of the liganded Ah receptor. These observations also suggest a possible mechanism for at least some aspects of the previously reported superinduction of the *CYP1A1* gene by simultaneous treatment with polycyclic aromatic compounds and cycloheximide (21, 32, 48): in the absence of cycloheximide, XF1 and XF2 would be present in the nucleus and compete with the liganded Ah receptor for binding to XREs, attenuating the ability of the Ah receptor-ligand complex to activate transcription; in the presence of cycloheximide, the levels of XF1 and XF2 would be greatly reduced, leading to increased accessibility of XREs to the liganded Ah receptor and thereby to superinduction of *CYP1A1* gene transcription.

ACKNOWLEDGMENTS

This project was supported by Public Health Service research grant RO1-CA 38655 from the National Cancer Institute and by the Maharishi International University Research Fund. F.S. is the recipient of a predoctoral fellowship from the Maharishi International University Research Fund.

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