MOLECULAR AND CELLULAR BIOLOGY, July 1993, p. 4365–4373
0270-7306/93/074365-09$02.00/0

Vol. 13, No. 7

Dioxin Receptor and C/EBP Regulate the Function of the Glutathione *S*-Transferase Ya Gene Xenobiotic Response Element

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Received 12 January 1993/Returned for modification 5 February 1993/Accepted 12 April 1993

The rat glutathione *S*-transferase Ya gene xenobiotic response element (XRE) has both constitutive and xenobiotic-inducible activity. We present evidence that the XRE is regulated by both the constitutive C/EBP transcription factor and the xenobiotic-activated dioxin receptor. A ligand-activated XRE-binding protein was shown to be dioxin receptor by specific antibody immunodepletion and binding of highly purified receptor. Identification of C/EBPα as the constitutive binding protein was demonstrated by competition with a C/EBP binding site, protein-DNA cross-linking to determine the molecular weight of the constitutive protein(s), specific antibody immunodepletion, and binding of purified bacterially expressed C/EBPα. Mutational analysis of the XRE revealed that the constitutive factor (C/EBPα) shares a nearly identical overlapping binding site with the dioxin receptor. In functional testing of the putative C/EBP-XRE interaction, cotransfected C/EBPα activated an XRE test promoter in the non-xenobiotic-responsive HeLa cell line. Unexpectedly, cotransfected C/EBPα had no effect on basal activity but significantly increased the xenobiotic response of the XRE test promoter in the xenobiotic-responsive, C/EBP-positive HepG2 cell line. Furthermore, inhibition of C/EBP-binding protein(s) in HepG2 cells by transfection of C/EBP oligonucleotides suppressed the xenobiotic response. These results suggest that C/EBPα and dioxin receptor recognize the same DNA sequence element and that transcriptional regulation can occur by cooperative interactions between these two transcription factors.

The rat glutathione *S*-transferase (GST) Ya gene is one of a set of genes (including several P450 genes) that are coordinately induced by planar aromatic compounds such as dioxin or 3-methylcholanthrene (3-MC). The induced proteins provide a number of enzymatic reactions which are thought to be involved in either the detoxification or carcinogenic activation of these xenobiotic compounds (32, 49). This coordinate regulation is mediated by a xenobiotic response element (XRE) located in single or multiple copies upstream of the responsive genes (30, 50).

Xenobiotic transcriptional regulation by the XREs is conferred through the intracellular aryl hydrocarbon receptor, or dioxin receptor (DR). DR exists in a latent form in the cytoplasm as a complex with hsp90 (50). Upon binding xenobiotic ligand, DR becomes activated (8, 29) and is thought to dissociate from hsp90 and translocate to the nucleus, where it binds to XRE target sequences (14). Thus, xenobiotics transduce extracellular signals to the nucleus directly through the mediation of DR. Recently it has been shown that DR is likely a heteromeric complex consisting of 100- and 110-kDa components (11). One of those components is the ARNT protein, which contains a basic helix-loop-helix motif (18, 35). ARNT by itself does not specifically bind XRE target sequences but is part of the binding complex (35). Its DNA-binding partner is the ligand-binding protein, which also contains a basic helix-loop-helix motif and has been shown to be part of the DNA-binding complex (2, 4, 12). Interestingly, it appears that phosphorylation plays an important role in regulation of DR function because specific inhibition of protein kinase C also inhibits xenobiotic activation of target sequences (6).

We and others have shown that in addition to the inducible DR, there is a constitutive protein(s) in hepatoma and liver extracts that interacts with XRE sequences (16, 30, 31, 38) which we called cXREBP (constitutive GST Ya XRE-binding protein). The identity and function of cXREBP have not previously been determined. The interaction of cXREBP with the XRE appears to be weak because dimethyl sulfate footprinting in vivo does not clearly identify the interaction (46, 47, 52). However, it has recently been demonstrated that the XREs on the P450IA1 gene are always in an open conformation independent of xenobiotic stimulation, as assessed by DNase hypersensitivity (16). The differences between these results are likely due to the difference in sensitivity of the methods. Therefore, it was concluded that the XRE is always occupied, either by cXREBP or by DR. Thus, the regulation of the XRE is likely to be highly complex, making the identification of cXREBP of importance in understanding the mechanism of regulation via the XRE.

In this report, we show that C/EBPα is, or is part of, cXREBP, using competition analysis, immunodepletion, protein-DNA cross-linking, and direct binding by bacterially expressed C/EBPα. Interestingly, mutagenesis of the XRE revealed that DR and cXREBP (C/EBPα) share overlapping DNA sequence requirements. C/EBPα is a member of a larger family of DNA-binding proteins (including β, δ, and others), all containing closely related bZIP motifs (5, 7, 9, 22–24). C/EBP proteins are important in the expression of a

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number of genes in the liver (13, 25, 33, 34, 48). In functional tests of C/EBPα, expression in HeLa cells activates an XRE test plasmid, but unexpectedly, expression in HepG2 cells enhances the xenobiotic induction mediated by DR. These results suggest that two different classes of regulatory factors can regulate the XRE by cooperative interactions.

MATERIALS AND METHODS

Cells. HepG2 cells were maintained as monolayer cultures and grown in Ham F12 medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal calf serum (HyClone), 0.5× minimal essential amino acids (GIBCO), garamycin (Schering Corp.) at 25 μg/ml, and insulin (Eli Lilly & Co.) at 0.5 U/ml. HeLa cells were maintained as monolayer cultures and grown in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and garamycin at 25 μg/ml.

GST Ya XRE plasmid constructions and transfections. Site-directed mutants of the XRE were constructed by synthesizing an oligonucleotide (Tufts Protein Chemistry Facility) complementary to one strand of the wild-type GST Ya XRE oligonucleotide (31, 37) but containing the mutation required. Mutant and wild-type oligonucleotides were hybridized and cloned into the *Eco*RI site of the GST Ya deletion construct at −190 bp (31). A- and B-site complementary oligonucleotides were also synthesized, hybridized, and cloned as described above. After transformation of *Escherichia coli* MC1061, clones were analyzed by restriction analysis and DNA sequencing (42).

Cultured cells were transfected at 40 to 50% confluency in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 1× nonessential amino acids, and insulin at 0.5 U/ml, using the calcium phosphate coprecipitation method as previously described (31). Expression levels of the GST Ya constructs were determined by RNase T_2 digestion of hybrids between transfected RNA (20 μg) and ^{32}P-labeled antisense RNA probes specific to the GST Ya first exon as described previously (31). The RNase-resistant hybrids were analyzed by using 8 M urea–6% polyacrylamide gels and autoradiography. The SV-βglobin control plasmid (31) was included in the transfections to monitor transfection efficiency. Quantitation of expression was determined with a Molecular Dynamics PhosphorImager.

GST-C/EBP plasmid fusion constructs. The cDNA sequence of C/EBPα was cloned into pGEX2T (19) to obtain in-frame fusions with bacterial GST. GST-C/EBPα was prepared by digesting MSV-C/EBPα (13) with *Nco*I, repairing ends with the Klenow fragment of DNA polymerase I, ligating *Bam*HI linkers, and cloning the 1.8-kbp cDNA fragment into the *Bam*HI site of pGEX2T, resulting in a fusion at amino acid 1.

Protein-DNA binding assays. Extracts were prepared from cultured cells as described previously (31). Bacterially expressed GST-C/EBP fusion proteins were prepared according to Kaelin et al. (20). The ligand-activated DR was purified from rat liver extracts as described elsewhere (24b). Protein-DNA binding was carried out in the presence or absence of 50-fold excess specific competitor as described by Cuthill et al. (8) and analyzed by electrophoresis on a 6% polyacrylamide–0.25× Tris-borate-EDTA gel and autoradiography.

Protein-DNA cross-linking. ^{32}P-labeled probes containing bromodeoxyuridine (BrdU) were prepared by annealing 100 pmol of the pBluescript T3 sequencing primer to 1 pmol of a pBluescript clone containing three tandem GST Ya XREs (the full sequence is shown in Fig. 5A) and subjecting the mixture to primer extension with 6 U of Klenow enzyme at 25°C for 1 h in 1× Klenow buffer (40) containing 1 mM bromo-dUTP–1 mM dGTP–1 mM dCTP–100 μCi of [^{32}P]dATP (3,000 Ci/mmol). Primer-extended product was digested with *Eco*RI to release three BrdU-substituted, ^{32}P-labeled XREs, which were isolated on a 1.5% agarose gel in the dark and visualized on X-ray film. The BrdU-substituted, ^{32}P-labeled XRE was then used in protein-DNA binding assays carried out as described above. The gel was exposed to UV light for 15 to 30 min in a Stratagene Stratalinker 1800. The gel was exposed to X-ray film for 4 h, and ^{32}P-labeled bands were cut out of the gel and digested in situ with 40 U of DNase I overnight at 4°C. The gel bands were then denatured in sodium dodecyl sulfate (SDS)–β-mercaptoethanol sample buffer and directly electrophoresed into an SDS-polyacrylamide gel or eluted overnight in SDS–β-mercaptoethanol sample buffer prior to gel electrophoresis (21).

RESULTS

Ligand-inducible DR binds to the GST Ya XRE. As discussed above and previously (31, 37), the GST Ya XRE has high sequence homology to other XRE sequences and is bound by a xenobiotic-inducible binding protein which interacts with the GST Ya XRE. We have proposed that this binding protein is likely DR. To test this possibility, we determined that highly purified ligand-activated DR binds to the GST Ya XRE (Fig. 1A). As demonstrated by the specific binding complex, the receptor was purified from the constitutive factor. In addition, purified receptor complex comigrates with the receptor-dependent band in cruder preparations. Figure 1B demonstrates that ligand is required to activate receptor binding to the P450 and GST Ya XREs (compare lanes 2 and 3 and lanes 7 and 8). Moreover, anti-DR serum specifically inhibits binding to XRE sequences (lanes 4 and 9). Incubation with preimmune serum does not affect receptor binding (lanes 5 and 10). These results clearly show that DR binds the GST Ya XRE in a ligand-dependent manner.

Identification of cXREBP as C/EBP. Our initial clue to the identity of cXREBP came from an examination of the XRE DNA sequence. As we described previously (31), the xenobiotic-induced footprint of the GST Ya XRE encompasses the XRE core homology and contains a region of dyad symmetry. Interestingly, both the highly divergent C/EBP consensus DNA binding sequence and the optimal high-affinity binding site defined by Shuman et al. (42) are homologous to the GST Ya XRE (Fig. 2A). To test whether the C/EBP high-affinity binding site could abolish the cXREBP gel shift band, we did a competition binding series using human HepG2 nuclear extracts. As shown in Fig. 2B, the C/EBP oligonucleotide competes for the cXREBP band, although at a somewhat reduced affinity compared with XRE self-competition. Similar results were obtained when a C/EBP oligonucleotide probe was used in competition binding assays (data not shown). These results indicated cXREBP binds to C/EBP DNA binding sequences. However, we could not be certain whether cXREBP was a C/EBP family member. Therefore, we performed several additional experiments to confirm the identity of cXREBP.

To determine the number and molecular weights of the protein components in cXREBP, we performed protein-DNA cross-linking. We prepared a BrdU-substituted, ^{32}P-

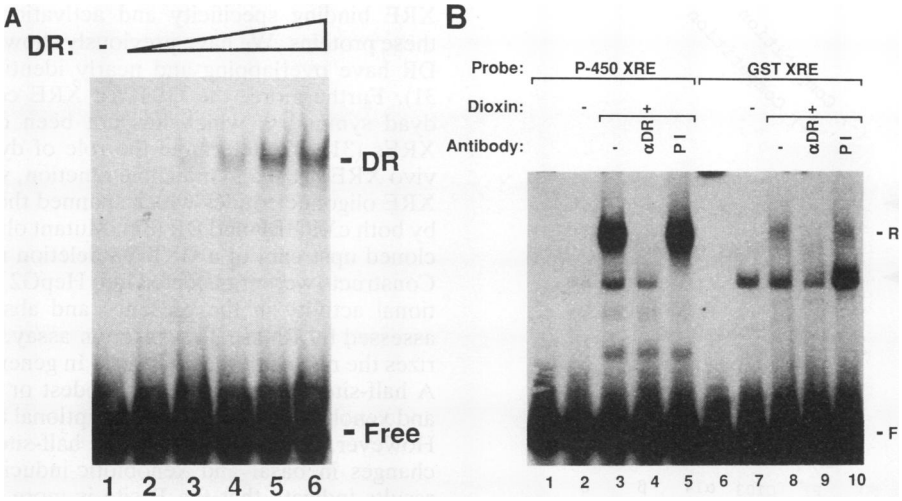

FIG. 1. Analysis of DR-XRE interaction. (A) Increasing amounts of purified DR (consisting of DR and ARNT heterodimer subunits) were added to a binding reaction mixture containing ^{32}P-labeled GST Ya XRE as indicated by the ramp. The binding reaction was analyzed by a gel retardation assay in which protein-DNA complexes are separated from free DNA on a low-ionic-strength polyacrylamide gel. (B) Depletion of DR gel shift by anti-DR antibodies. Cytoplasmic extracts were induced with dioxin as described previously (6). Ligand-dependent binding to the XRE is indicated by R. A specific DR antiserum (1/100; αDR) or preimmune serum (PI) was added following DR-XRE complex formation. The DR antiserum specifically inhibits the ligand-dependent gel shift complex. GST XRE, GST Ya XRE probe; P-450 XRE, P450 XRE probe.

labeled XRE oligonucleotide. This probe was then used in a gel shift assay, and the cXREBP-DNA complex was cross-linked under UV light. Cross-linked proteins were excised from the gel, digested with DNase in situ, and run on a 10% polyacrylamide–SDS gel (Fig. 3A). Two small proteins of molecular masses 37 and 43 kDa were identified. Larger complexes of 72 and 150 kDa were also observed when longer UV light exposures were used. Longer exposure times give rise to first the 72-kDa complex and then the 150-kDa complex (data not shown). Therefore, the two smaller polypeptides probably represent the primary binding components of cXREBP. We believe that the larger complexes are likely the result of increased cross-linking between protein-DNA complexes because of the nearly additive nature and order of appearance of the larger complexes. However, we cannot rule out the possibility that the larger complexes result from cross-linking of accessory proteins of cXREBP. Interestingly, the 37- and 43-kDa proteins identified by cross-linking have molecular weights similar to those of C/EBP proteins, which again suggests that cXREBP may be composed of C/EBP proteins. Although C/EBPα and -β are both present in liver and hepatoma cells, it is unclear from these experiments which C/EBP family member(s) could comprise cXREBP.

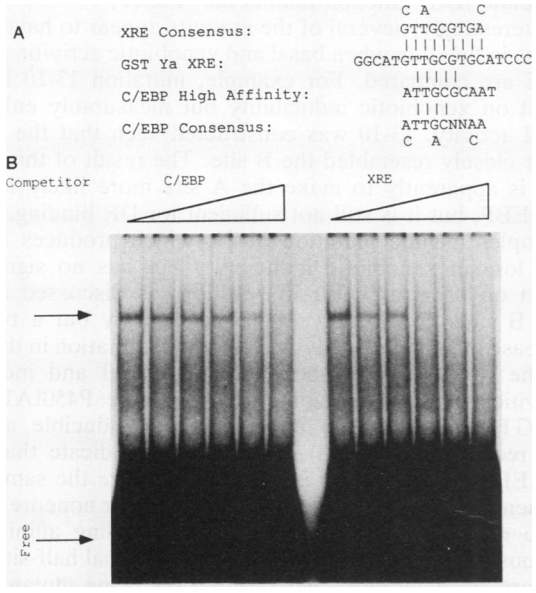

FIG. 2. (A) Comparison of XRE sequences and C/EBP binding sites. The XRE consensus, GST Ya XRE, C/EBP high-affinity site, and C/EBP consensus sequences are aligned to show overlapping homology. (B) Competition for cXREBP by the C/EBP binding site. Crude nuclear uninduced HepG2 extracts containing the constitutive factor were bound to an XRE probe (0.5 ng) in the presence of increasing amounts of cold XRE or C/EBP oligonucleotide (0.5 to 20 ng) as indicated by the ramp. The binding reaction was assayed by gel shift as described for Fig. 1. The constitutive complex (upper arrow) is competed for by both XRE and C/EBP oligonucleotides.

To test whether C/EBP proteins are part of the cXREBP complex, two approaches were used. In the first approach, we immunodepleted untreated rat liver nuclear extracts by using antibodies to the rat C/EBP isoforms α, β, and δ (5, 21; generously provided by Zhaodan Cao and Steven McKnight). As shown in Fig. 3B, two different C/EBPα antibodies specifically eliminated the cXREBP gel shift bands, while the preimmune serum and the β and δ antibodies had no effect. Note that the major band and the two higher bands are consistently present in various nuclear extract preparations, while two lower bands are not consistently present. Second, we determined that C/EBPα protein binds directly to the XRE with high affinity. To this end, C/EBPα was expressed as a fusion protein with GST in bacteria. As shown in Fig. 4, purified bacterially expressed GST-C/EBPα protein binds the XRE with an apparent K_d of 1.64 nM. These results demonstrate that C/EBPα is a component of the cXREBP binding complex and that C/EBPα alone is able to bind the XRE.

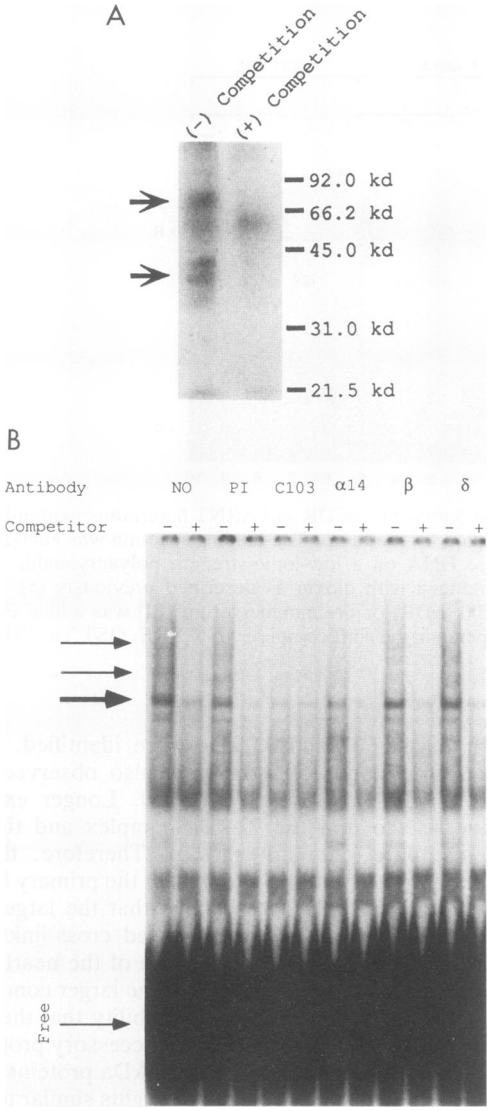

FIG. 3. Identification of C/EBPα as cXREBP. (A) UV cross-linking identifies XRE-binding proteins of molecular weights consistent with those of C/EBPs. A BrdU-substituted XRE probe was synthesized (see Materials and Methods) and used in a gel shift with uninduced HepG2 extract. The protein-XRE band shift (see Fig. 2A) was cross-linked for 20 min in a Stratagene Stratalinker. The protein-XRE band shift was cut out of the gel, DNase treated in situ, and run on an SDS–10% polyacrylamide gel (18). The lower two bands (lower arrow; 43 and 35 kDa) appear first when cross-linked for shorter times. The higher band (upper arrow; 72 kDa) appears after longer cross-linking times. (B) Depletion of constitutive factor gel shift by anti-C/EBPα antibodies. Crude rat nuclear extracts were incubated with 1/100 dilutions of anti-C/EBPα (C103 and α14), anti-C/EBPβ (β), anti-C/EBPδ (δ), and preimmune (PI) sera. Antibody complexes were removed from the extract by incubation with protein A-Sepharose (17). Depleted extracts were incubated with XRE probe and assayed by gel shift. Specific complexes are shown by competition with 20-fold excess cold XRE oligonucleotide (indicated by arrows).

Interactions of cXREBP (C/EBPα) and DR proteins with the XRE. To examine the functional significance of interactions of both cXREBP (C/EBPα) and DR proteins with the XRE, we used a DNA mutagenesis approach to investigate the XRE binding specificity and activation characteristics of these proteins. We have previously shown that cXREBP and DR have overlapping and nearly identical footprints (30a, 31). Furthermore, the GST Ya XRE contains a region of dyad symmetry, which has not been described for other XREs (31). To determine the role of dyad symmetry in in vivo XRE basal and inducible function, we prepared mutant XRE oligonucleotides which spanned the region footprinted by both cXREBP and DR (31). Mutant oligonucleotides were cloned upstream of a GST Ya deletion mutant at −190 bp. Constructs were transfected into HepG2 cells, and transcriptional activity in the presence and absence of 3-MC was assessed by RNase T_2 protection assay. Figure 5A summarizes the results of these studies. In general, mutations in the A half-site resulted in either modest or no effects on basal and xenobiotic-inducible transcriptional activity of the XRE. However, mutations in the B half-site resulted in large changes in basal and xenobiotic-inducible activity. These results indicate that the B site is more important for XRE function than is the A site and that dyad symmetry is unlikely to be required for XRE function.

To confirm these results, we determined whether individual half-sites could confer XRE function. Independent A and B sites were cloned at −190 bp and analyzed for basal and inducible function. As shown in Fig. 5A, two B sites (B1) confer twice the inducible activity of the wild-type XRE, while one B site (B4) has no inducible activity. Interestingly, reduced basal activity was seen with both B-site clones. Multiple A sites (AB6 and 56I) had much less effect on transcription. Only the presence of eight A sites (56I) resulted in partially restored basal expression. However, the significance of eight sites seems questionable. Taken together with the studies of XRE point mutants, these results indicate that the B site is more important for basal and inducible XRE function than is the A site.

Interestingly, several of the mutants appear to have alterations in function when basal and xenobiotic activities of the XRE are compared. For example, mutation 13-10 has no effect on xenobiotic inducibility but measurably enhances basal activity. 13-10 was constructed such that the A site more closely resembled the B site. The result of this mutation is apparently to make the A site more likely to bind cXREBP, but it is still not sufficient for DR binding. Other examples include mutation 11-24, which produces a twofold loss in xenobiotic inducibility but has no significant effect on basal activity. In addition, as discussed above, two B sites confer only low basal activity but a twofold increase in inducible activity. However, mutation in the core of the XRE (12A28) abolishes both basal and inducible activities. A similar point mutation in the P450IA1 XRE (GCGTGA→GCATGA) also abrogates inducible activity and receptor binding (8). These results indicate that both cXREBP (C/EBPα) and DR likely recognize the same core sequence but have different requirements for noncore bases.

To determine the relative in vitro binding affinities of various XRE point mutants and the individual half-sites, we performed quantitative gel shift assays using mutant XRE oligonucleotides as competitors and crude nuclear protein extracts. As shown in Fig. 5B, the in vivo down mutants were less effective competitors of XRE-protein complexes, and the results correlated with the observed loss of in vivo function. For example, mutant 9-10 is not an effective competitor of either the cXREBP or DR gel shift. As another example, there was relatively little competition with single B sites (approximately 30-fold lower affinity) and none detectable with single A sites (data not shown). We also examined

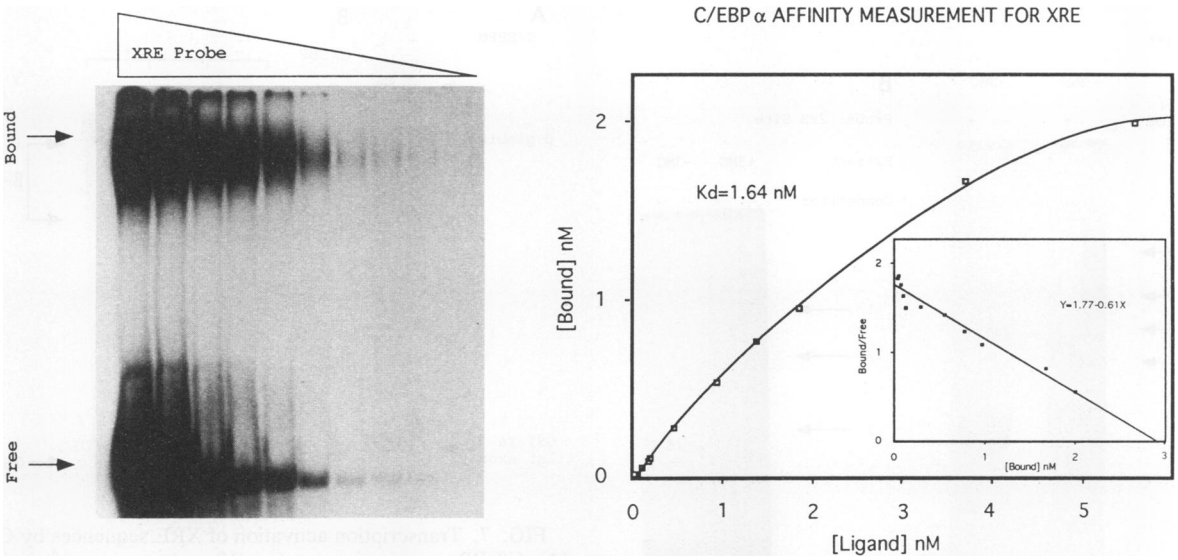

FIG. 4. Affinity measurement of C/EBPα for the GST Ya XRE. Purified, bacterially expressed GST-C/EBPα was incubated with increasing amounts of XRE probe as indicated by the ramp. Arrows indicate bound C/EBPα and free probe. Bound and free complexes were quantitated with a Molecular Dynamics PhosphorImager. The K_d for C/EBPα (1.64 nM) was determined by Scatchard analysis.

the direct binding of DR and cXREBP (C/EBPα) to the A and B half-sites. A single A or B site produced no detectable gel shift band. However, two tandem B sites (2×B) gave a strong gel shift with the three characteristic xenobiotic-inducible bands in crude nuclear extracts (Fig. 6). Interestingly, cXREBP (C/EBPα) did not bind to the 2×B probe, unlike the wild-type XRE (Fig. 6). This result corresponds to the observations obtained in vivo, in which clone B1 (con-

A

		In Vivo Activity -3MC	In Vivo Activity +3MC
WT	GAATTCGTCAGGCATGTTGCGTGCATCCCTGAGGAATTC	1.0±.1	4.5±.6
13-10	GAATTCGTCACGCATGTTGCGTGCATCCCTGAGGAATTC	1.5±.1	4.3±.5
3B10	GAATTCGTCAGACATGTTGCGTGCATCCCTGAGGAATTC	0.9±.3	2.5±.3
9-10	GAATTCGTCAGGCATTTTGCGTGCATCCCTGAGGAATTC	0.3±.1	1.1±.3
12A28	GAATTCGTCAGGCATGTTGTGTGCATCCCTGAGGAATTC	0.3±.1	0.5±.2
11-24	GAATTCGTCAGGCATGTTGCGTG-ATCCCTGAGGAATTC	0.8±.1	2.1±.1
B4	TGTTGCGTGCATCC	0.3±.1	0.6±.1
B1	2xB Site	0.4±.2	8.3±.9
AB6	CGTCAGGCATGTTG	0.2±.1	0.4±.1
56I	8xA Site	0.6±.2	0.7±.2

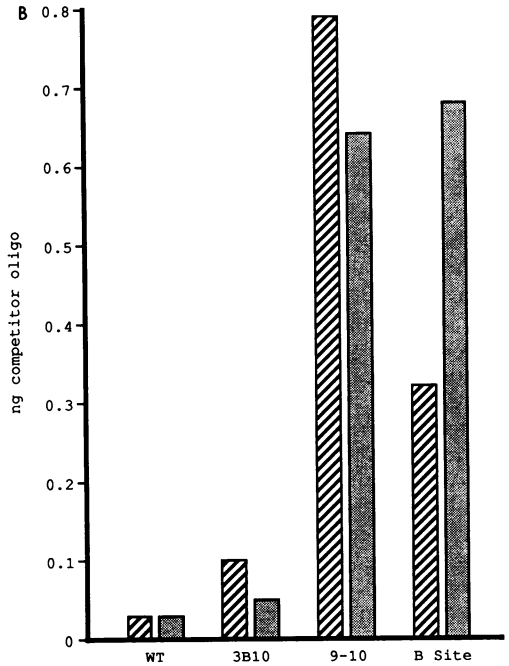

FIG. 5. Evidence that cXREBP (C/EBP) and DR have overlapping binding sites on the XRE. (A) Mutational analysis of constitutive and xenobiotic-inducible activities of the XRE. Mutations in the XRE were generated and cloned in front of the minimal GST Ya promoter at −190 bp. HepG2 cells were transfected with these constructs and treated with or without the xenobiotic inducer 3-MC. Expression was assayed by RNase T_2 protection of the GST Ya first exon. Gel lanes were normalized for transfection efficiency. Expression was quantitated with a Molecular Dynamics PhosphorImager. The results of at least three experiments ± standard deviations are presented. wt, wild type. (B) Mutational analysis of constitutive and inducible protein-DNA interactions. Oligonucleotides containing mutations generated in panel A were used as competitors of constitutive or inducible protein-XRE interactions in gel shift assays. Quantitation of the complexes was determined with a Molecular Dynamics PhosphorImager. The bar graph shows the amount of mutant or wild-type competitor required to achieve 50% inhibition of a gel shift, using 0.02 ng of wild-type GST Ya XRE probe. The striped bars represent DR competition; the stippled bars represent cXREBP competition.

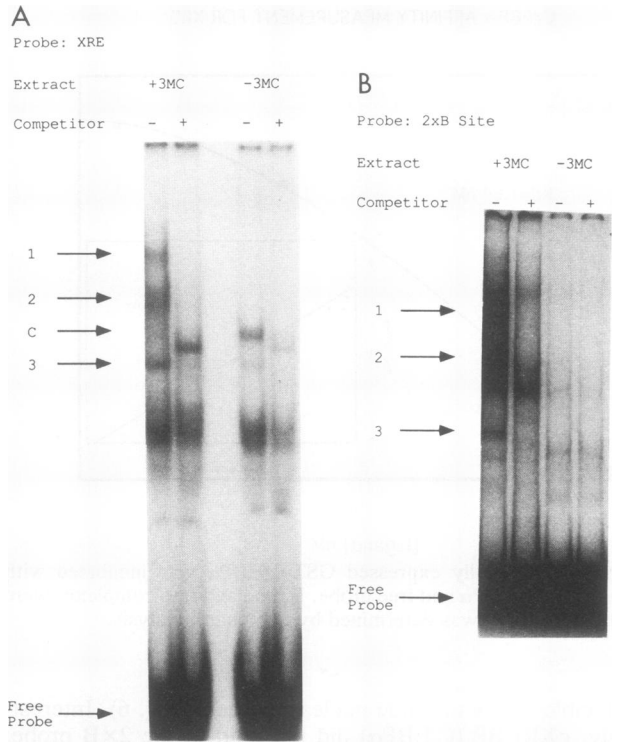

FIG. 6. Evidence that two B sites bind DR but not C/EBP. (A) Gel shift of 3-MC-induced and uninduced HepG2 extracts, using wild-type XRE probe. Arrows 1, 2, and 3 indicate induced gel shift bands; arrow C indicates the constitutive complex. (B) Gel shift of 3-MC-induced and uninduced HepG2 extracts, using 2×B site probe. Arrows 1, 2, and 3 indicate induced gel shift bands. No constitutive complex is observed.

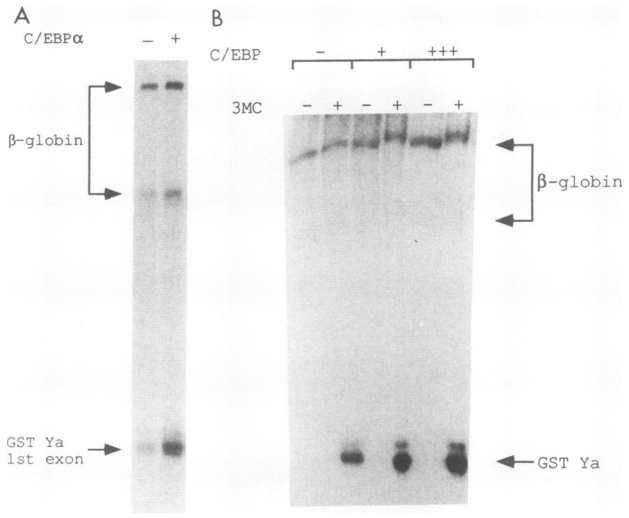

FIG. 7. Transcription activation of XRE sequences by C/EBPα. (A) C/EBPα expression vector (10 μg) was transfected into a C/EBP-negative cell line (HeLa) together with a test plasmid containing three XRE sequences ligated at −190 bp in the GST Ya promoter (40 μg) along with the SV-βglobin control plasmid (2.5 μg). Expression was assayed by RNase T_2 protection assay of the GST Ya first exon (arrow). Relative transfection efficiency is shown by the β-globin signal (bracketed arrows). (B) C/EBPα expression vector (5 μg [+] or 15 μg [+++]) was transfected into a C/EBP-expressing line (HepG2) together with a test plasmid containing three XRE sequences ligated at −190 bp in the GST Ya promoter (40 μg) along with the SV-βglobin control plasmid (2.5 μg). Expression with or without 3-MC induction was assayed by RNase T_2 protection assay of the GST Ya first exon (arrow). Relative transfection efficiency is shown by the β-globin signal (bracketed arrows).

taining two tandem B sites) gave a strong xenobiotic response but low basal activity. This finding provides additional evidence that the B site is necessary and possibly sufficient for DR binding and xenobiotic-inducible activity. In line with this model, we did not observe cooperative binding of purified DR to the wild-type GST Ya XRE probe (50a). Furthermore, our results confirm that although the B site is necessary for cXREBP (C/EBPα) binding and basal function, it does not contain all of the DNA sequences required.

Functional analysis of C/EBPα binding on XRE function. To determine the functional significance of C/EBPα expression on XRE function, we initially coexpressed C/EBPα with an XRE test plasmid in the nominally C/EBP-negative background HeLa cell line (51). As shown in Fig. 7A, C/EBPα was able to activate the XRE test plasmid by a factor of approximately 4.1 (average result of two experiments). While this result was clearly expected from our binding data and observation that C/EBPα is a component of cXREBP, it was unclear how C/EBPα might act in the presence of DR. Therefore, the consequences of C/EBPα overexpression on the basal activity and xenobiotic response was examined by cotransfecting C/EBPα and the XRE test plasmid into HepG2 cells. As shown in Fig. 7B, C/EBPα had no significant effect on basal activity, but surprisingly, C/EBPα enhanced the inducible response by a factor of approximately 3.0 (average result of two experiments). These results suggested that dependent on the host cell environment, C/EBPα not only was important in the basal activity of the XRE but was involved in the xenobiotic response as well.

We further explored the role of C/EBP protein in the xenobiotic response by suppressing C/EBP-XRE binding in vivo. The strategy for this experiment was to inhibit endogenous C/EBP-XRE interactions by the addition of excess C/EBP binding sites but to leave DR binding intact. DR does not bind C/EBP binding sites in vitro, as assessed by DNA binding competition assays with up to 40-fold excess competitor as well as by direct DR-C/EBP binding site experiments (data not shown). As shown in Fig. 8, an XRE test plasmid was cotransfected with a pBluescript plasmid containing multiple C/EBP binding sites at several different molar ratios (the amount of DNA in the transfection was held constant by the addition of pBluescript). At higher molar ratios, the C/EBP binding site was almost completely able to suppress the xenobiotic response. This result further confirms that C/EBPα is involved in the xenobiotic response of the wild-type XRE.

DISCUSSION

In our previous analysis of the GST Ya enhancer, we reported that the GST Ya XRE is bound by a xenobiotic-inducible protein which was likely to be DR (31). Xenobiotic transcriptional activation via the XRE correlated with the appearance of the inducible protein in the nucleus and had kinetics similar to those for DR nuclear localization. In this report, we demonstrated direct DR-GST Ya XRE interaction. Therefore, we concluded that ligand-activated DR does bind the GST Ya XRE and that binding is required for

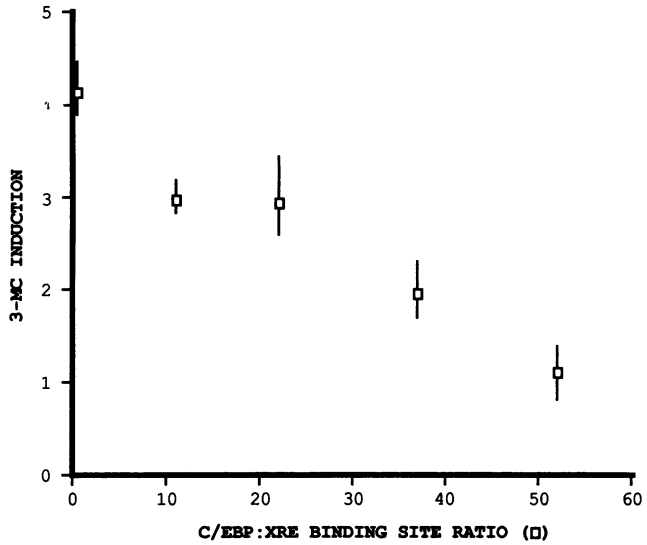

FIG. 8. Effect of C/EBP binding site competition on XRE 3-MC induction. Four C/EBP binding sites ligated into pBluescript KS (0 to 40 μg) were transfected into a C/EBP-expressing line (HepG2) together with a test plasmid containing one XRE sequence ligated at −190 bp in the GST Ya promoter (5 μg) and the SV-βglobin control plasmid (1 μg). The total amount of DNA was kept constant by adding a variable amount of pBluescript to compensate for the C/EBP binding site. Expression with or without 3-MC induction was assayed by RNase T_2 protection assay of the GST Ya first exon. The level of expression was quantitated with a Molecular Dynamics PhosphorImager and corrected for transfection efficiency by reference to the β-globin signal. The level of 3-MC inducibility is graphed relative to the molar ratio of C/EBP to XRE binding sites.

xenobiotic transcriptional activation. In untreated cells, we and others have reported that there is a cXREBP which has been neither identified nor functionally characterized (16, 30, 31, 38). In this report, we conclude that C/EBPα is likely part of the cXREBP complex. This conclusion is supported by both direct binding and immunological data. However, it should be noted that potential C/EBPα antibody cross-reactivities could indicate that other C/EBP family members bind to the GST Ya XRE as well.

Although C/EBPα can bind the XRE with high affinity, the functional significance of that binding was initially unclear. At first, we thought that C/EBPα and DR would compete for the XRE and that this competition would be controlled by simple differences in affinity. Although we have not obtained a direct affinity measurement for DR-GST Ya XRE interactions (because of a lack of large amounts of purified DR), our evidence from competition data suggests no large differences in affinity between DR and C/EBPα. We propose instead that C/EBPα is important for DR binding to the XRE. This view is supported by our mutagenesis analysis indicating that (i) the binding sequences for the two factors closely overlap and (ii) no mutations which decreased basal activity only were obtained. Clearly, C/EBPα can directly activate an XRE target vector in HeLa cells, which have very low levels of C/EBPs. Furthermore, we have shown that C/EBPα can enhance the xenobiotic response of an XRE target vector. The importance of this result was confirmed by suppressing the xenobiotic response of the XRE by abolishing C/EBP binding in vivo.

Interestingly, when C/EBPα was transfected into the hepatoma cell line HepG2, we did not observe an enhancement of the basal level of expression from the XRE target vector (Fig. 7B and data not shown). Likely this is because there is already a large concentration of C/EBPα protein in HepG2 cells. Thus, additional C/EBP would not affect an already saturated basal rate of transcription. We tested this possibility by measuring C/EBPα levels in transfected cells on Western immunoblots. We found that the total amount of specific C/EBPα protein increased by only twofold in transfected cells (data not shown).

The final question that we are left with is the mechanism of the C/EBP and DR regulation of the GST Ya XRE. There are several reports of studies in which different transcription factors recognize the same or closely overlapping sequence elements. In some cases, the binding of one factor is required for or facilitates the binding of another factor (3, 10, 27, 34, 45, 53). Other examples show that binding between two factors is antagonistic, resulting in inactivation or suppression of specific genes, depending on which factor is dominant (1, 7, 15, 26, 28, 36, 41, 44). Our data show that C/EBPα acts as a positive regulator of the XRE. In interpreting our data, we propose that C/EBP facilitates binding of DR. While this interpretation explains our XRE point mutation data, it also sheds light on the data for one B site versus two B sites. While one B site is nonfunctional, two B sites are additive for inducible activity only and do not bind C/EBP. This finding suggests that in the absence of C/EBP, more than one DR is required for function, as if binding were cooperative. Thus, in the context of the wild-type GST Ya XRE, C/EBPα would be the facilitative binding partner. To test this proposal, we have examined DR and C/EBP binding in gel shift assays. We have never observed any evidence of C/EBP-DR complexes. However, DR binding to the XRE does appear to be stimulated in the presence of C/EBP (24a). We are currently pursuing additional evidence that C/EBP facilitates the binding of DR as well as examining possible suppression and activation mechanisms.

Is C/EBP binding a general mechanism of XRE function? Other XREs which have been examined by footprint and mutagenesis analysis show a similar pattern of close overlap between constitutive factors and DR (38, 39). However, these constitutive factors have not been positively identified. Furthermore, the GST Ya gene has a relatively high basal rate of transcription and is transcriptionally induced by xenobiotics by a factor of 4 to 5 (30, 31), whereas the P450IA1 gene has a low basal rate of transcription and is transcriptionally induced at least 10-fold from a single XRE (43). These data suggest that the GST Ya gene may have a higher basal transcription rate because of the presence of C/EBP. Interestingly, C/EBPα does not appear to bind the P450IA1 XRE with high affinity (1a). Therefore, the P450IA1 gene may not use C/EBP in the xenobiotic response and thus may have a lower basal transcription rate and a relatively higher level of induction by xenobiotics. Finally, given the prevalence of cross-coupling regulatory mechanisms in other systems, it is possible that other DNA-binding proteins also bind to XRE sequences. We are currently examining additional XRE sequences to determine whether there are different classes of elements which have different responses dependent on their constitutive factor coupling.

ACKNOWLEDGMENTS

We thank A. S. Yee and E. Cochary for helpful discussions and critical review of the manuscript. We are very grateful to Steven L. McKnight and Zhaodan Cao for generously providing C/EBPα, -β, and -δ antisera and mammalian expression vectors.

This project was funded by a grant from the Swedish Cancer Society (L.P.) and at least in part with federal funds from the Agricultural Research Service, U.S. Department of Agriculture, under contract 53-3K06-0-1 (K.E.P.).

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