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In Situ Protein-DNA Interactions at a Dioxin-Responsive Enhancer Associated with the Cytochrome P_1-450 Gene

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We used an in situ exonuclease III protection technique (C. Wu, Nature [London] 309:229, 1984) to analyze protein-DNA interactions at a dioxin-responsive enhancer. Our results imply that the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-receptor complex interacts with the dioxin-responsive enhancer to activate transcription of the cytochrome P_1-450 gene.

Halogenated aromatic hydrocarbons are environmental contaminants that produce diverse biological effects (11, 12). In mouse hepatoma cells, the prototypical compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) activates transcription of the cytochrome P_1-450 gene (3, 4, 16). Studies of receptor-defective cells reveal that this response requires the formation of a TCDD-receptor complex and an interaction between the inducer-receptor complex and the nucleus (3, 4). A *cis*-acting dioxin-responsive element (DRE) located upstream of the cytochrome P_1-450 gene mediates the action of the TCDD-receptor complex (2, 5, 7). The DRE contains two TCDD-responsive domains, which have properties typical of transcriptional enhancers and require TCDD-receptor complexes for their function (5–7). Here, we use an exonuclease protection technique (18, 19) to show that both the DRE and the TCDD-receptor complex participate in the formation of a stable nucleoprotein complex within the cell nucleus. These results imply that the TCDD-receptor complex interacts with the DRE in vivo.

Figure 1 shows the relevant restriction sites in the DNA upstream of the mouse cytochrome P_1-450 gene. The hatched boxes indicate the position of the two DREs (8). To examine this region for resistance to exonuclease III (Exo III), nuclei were prepared by Dounce homogenization and centrifugation (1) and were digested with *Bam*HI (Bethesda Research Laboratories, Inc.) (300 U/ml, 30 min, 30°C) to nick the DNA downstream of the proximal DRE. Next, chromatin (at a DNA concentration of about 1 mg/ml) was digested for 30 min with various concentrations of Exo III (BRL) as previously described (9). The *Bam*HI-Exo III-digested DNA was purified by phenol-chloroform extraction, digested with S1 nuclease (BRL) (4,000 U/ml, 15 min, 30°C) as previously described (18), and then digested to completion with *Hin*dIII (BRL). The repurified and ethanol-precipitated DNA was fractionated by agarose gel electrophoresis, transferred to a nylon membrane (15), and hybridized to a nick-translated (13) probe complementary to the 5′ end of the *Hin*dIII-*Hin*dIII fragment. The ^{32}P-labeled bands were detected by autoradiography, with Kodak XAR-5 film and an intensifying screen.

The results in Fig. 2 reveal that *Bam*HI digested the cytochrome P_1-450 regulatory region in nuclei from TCDD-induced cells (Fig. 2B, lane b) to a greater extent than in nuclei from uninduced cells (Fig. 2A, lane b; see also Fig. 3B and C). These findings suggest that TCDD induces a more open chromatin structure, which may reflect the interaction of regulatory molecules with the nucleoprotein. Digestion of nuclei from uninduced cells produced the expected 2.65-kilobase *Hin*dIII-*Hin*dIII and 0.81-kilobase *Hin*dIII-*Bam*HI bands (Fig. 2A, lane b). However, digestion with Exo III (Fig. 2A, lanes c to g) failed to generate a smaller *Hin*dIII-Exo III subband(s). In contrast, Exo III digestion of nuclei from TCDD-induced cells generated a 0.67-kilobase *Hin*dIII-Exo III subband (Fig. 2B, lanes c to g) that maps to a site within the proximal DRE (8). Even at high concentrations, Exo III failed to digest beyond this site, suggesting the existence of a relatively stable protein-DNA complex in this region of chromatin. Generation of the *Hin*dIII-Exo III

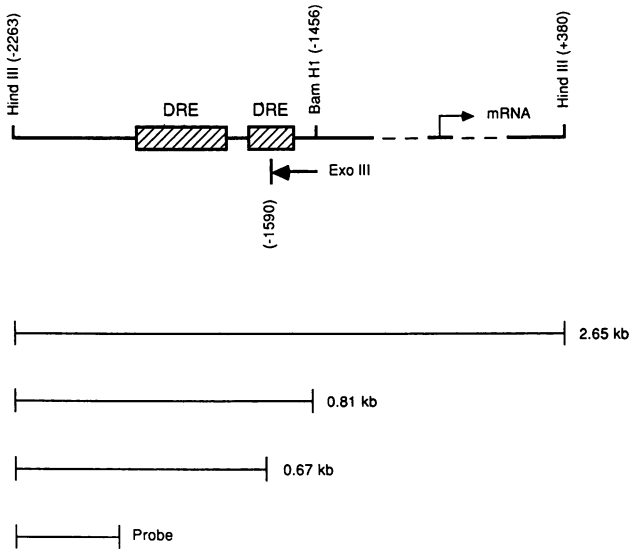

FIG. 1. Restriction map of the cytochrome P_1-450 gene regulatory region. The horizontal line represents the cytochrome P_1-450 gene. Unique restriction endonuclease sites are shown around the TCDD-responsive domains of the cytochrome P_1-450 gene. The start site of transcription is designated by a bent arrow showing the direction of mRNA synthesis. Two DREs, identified previously (5), are indicated by the hatched boxes. Exo III digestion from the *Bam*HI site (position −1456) is indicated by a horizontal arrow below the map, and the approximate 3′ boundary of Exo III protection is shown at position −1590. Below the map of the cytochrome P_1-450 gene, horizontal lines illustrate the sizes of the *Hin*dIII-*Hin*dIII, *Hin*dIII-*Bam*HI, and *Hin*dIII-Exo III DNA fragments. The fragment of cytochrome P_1-450 DNA that was purified, labeled with ^{32}P, and used as a hybridization probe is shown at the bottom of the figure.

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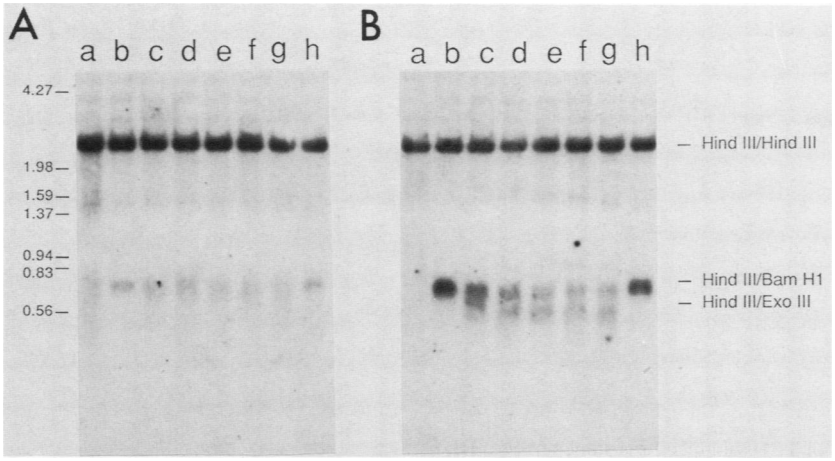

FIG. 2. Induction of Exo III resistance by TCDD in wild-type cells. Wild-type mouse hepatoma cells were treated with 0.1% dimethyl sulfoxide alone (A) or with 1 nM TCDD in 0.1% dimethyl sulfoxide (B) for approximately 18 h. Nuclei were digested for 30 min with 300 U of *Bam*HI per ml (except for lane a) and subsequently digested with Exo III for 30 min at the following concentrations (in units per milliliter): 0 (lanes b and h); 500 (lane c); 1,000 (lane d); 2,000 (lane e); 5,000 (lane f); and 10,000 (lane g). Purified DNA in lanes c to h was digested for 15 min with 4,000 U of S1 nuclease per ml at 30°C. After purification, the DNA was digested to completion with *Hin*dIII, fractionated on a 1.5% agarose gel, and transferred to a nylon membrane. Immobilized DNA was hybridized to the ^{32}P-labeled 5′-end probe (Fig. 1). Molecular weight markers (left of figure) were lambda DNA digested with *Hin*dIII and *Eco*RI, mixed with an amount of cellular DNA equivalent to that in the experimental lanes, and fractionated in an adjacent lane. DNA fragments, described in the legend to Fig. 1, are shown to the left of the figure.

subband did not result from either an endogenous endonuclease (Fig. 2A and B, lane b) or S1 nuclease (Fig. 2A and B, lane h). Nor is the Exo III stop due to DNA primary or secondary structure, because it did not occur in uninduced cells (Fig. 2A). Also, absence of the Exo III boundary is not the result of decreased access of the enzyme to chromosomal DNA. The *Hin*dIII-*Bam*HI fragment in uninduced cells disappeared at higher concentrations of Exo III (Fig. 2A, lanes c to g; see also Fig. 3A and B). These results suggest that resistance to Exo III in TCDD-induced cells may reflect the binding of the TCDD-receptor complex (or some other protein) to the DRE.

The results in Fig. 3A show that TCDD failed to induce a *Hin*dIII-Exo III subband in variant cells in which the TCDD-receptor complex fails to interact properly with the nucleus (10, 17). Therefore, resistance to Exo III requires functional TCDD-receptor complexes. This finding suggests that nuclease resistance reflects the binding of the TCDD-receptor complex to the DRE. Alternatively, the Exo III resistance could result from the binding of some other protein (e.g., a transcription factor) to the DRE, in association with the activation of the cytochrome P_1-450 gene by the TCDD-receptor complex. To address this possibility, we studied a high-activity variant (HAV) cell line, which contains normal TCDD receptors but which transcribes the cytochrome P_1-450 gene, even in the absence of TCDD (8). The uninduced rate of cytochrome P_1-450 gene transcription in HAV cells is approximately one-third the rate in TCDD-induced

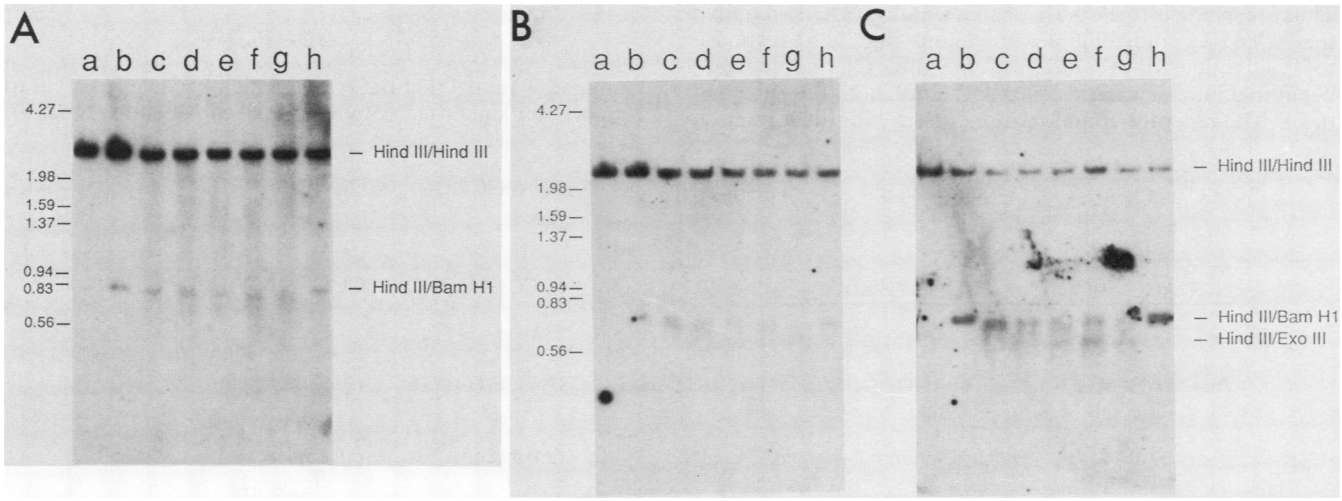

FIG. 3. Exo III resistance in variant cells. Class II variant cells (A) or HAV cells (B and C) were treated with 1 nM TCDD in 0.1% dimethyl sulfoxide (A and C) or with 0.1% dimethyl sulfoxide alone (B) for approximately 18 h. Nuclei were prepared from the cells and digested with *Bam*HI (except for lane a) for 30 min as described in the legend to Fig. 2. Exo III was used at the following concentrations (in units per milliliter): 0 (lanes b and h); 500 (lane c); 1,000 (lane d); 2,000 (lane e); 5,000 (lane f); and 10,000 (lane g). Purified DNA was digested with S1 nuclease and then with *Hin*dIII and further analyzed as described in the legend to Fig. 2.

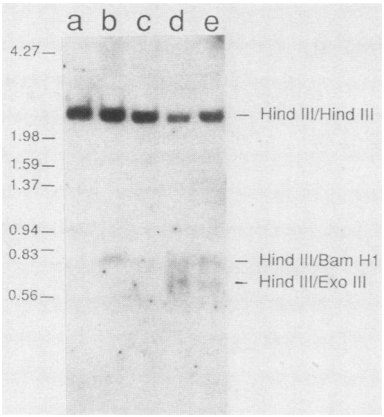

FIG. 4. Temporal onset of TCDD-induced resistance to Exo III. Wild-type cells were treated for 18 h with 0.1% dimethyl sulfoxide only (lanes a to c) or with 1 nM TCDD in 0.1% dimethyl sulfoxide for 1 h (lane d) or 18 h (lane e). Nuclei were digested with 300 U of *Bam*HI per ml (except for lane a), followed by 5,000 U of Exo III per ml (except for lanes a and b). Purified DNA in lanes c to e was digested with S1 nuclease and, after repurification, with *Hin*dIII. DNA was further analyzed as described in the legend to Fig. 2.

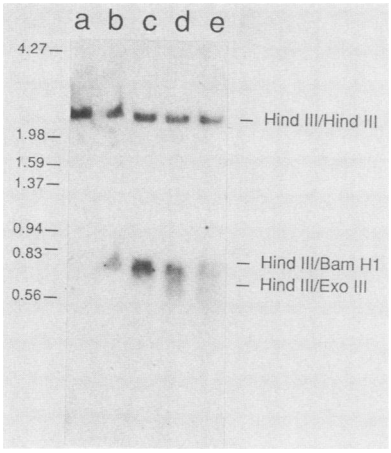

FIG. 5. Resistance to Exo III in the absence of ongoing protein synthesis. Wild-type cells were treated for 2 h with 1 nM TCDD plus 10 μg of cycloheximide per ml. Nuclei were digested with 300 U of *Bam*HI per ml (except for lane a), followed by Exo III at the following concentrations: (in units per milliliter): 0 (lane b); 500 (lane c); 2,000 (lane d); and 5,000 (lane e). Purified DNA in lanes c to e was digested with S1 nuclease and, after repurification, with *Hin*dIII. DNA was further analyzed as described in the legend to Fig. 2.

wild-type cells (4, 8). If Exo III resistance reflects gene transcription per se, rather than the binding of the TCDD-receptor complex to the DRE, it should be detectable in nuclei from uninduced HAV cells. However, even in HAV cells, resistance to Exo III required exposure to TCDD (Fig. 3B and C). These findings do not support the idea that an unknown transcription factor confers Exo III resistance upon the DRE.

The results in Fig. 4 indicate that maximal levels of Exo III resistance occur within 1 h of exposure to TCDD. Thus, resistance develops rapidly in response to TCDD and correlates temporally with the activation of cytochrome P_1-450 gene transcription (4). We also observed that Exo III resistance occurs in cells in which protein synthesis is inhibited 95 to 97% by cycloheximide (Fig. 5). These findings argue against the idea that TCDD induces the synthesis of a new protein(s) that is responsible for the resistance to Exo III. Thus, resistance to Exo III occurs within a DRE, requires the TCDD-receptor complex, develops rapidly in response to TCDD, does not require protein synthesis, and does not occur in uninduced HAV cells. Therefore, both the DRE and the TCDD-receptor complex are necessary for the formation of an Exo III-resistant chromatin structure. We interpret these results to mean that the TCDD-receptor complex binds to the DRE in situ. However, we cannot formally rule out the possibility that the TCDD-receptor complex causes some other preexisting protein to bind to the DRE, producing resistance to Exo III.

We have made several attempts to map the upstream boundary of the Exo III-resistant region and to establish whether TCDD-dependent resistance to Exo III digestion exists in the distal DRE. Probably for technical reasons, we have observed no major sites of nuclease resistance. It is possible that a weaker site(s) of Exo III resistance is present in the distal DRE, but our assay is not sensitive enough to reveal it. Sogawa et al. (14) have suggested that the DRE contains the consensus sequence

$$5'\text{-}\overset{G}{C}N\overset{TA}{GG}GCTGGG\text{-}3'.$$

Our studies reveal that the region which became resistant to Exo III contained one copy of this decanucleotide (Fig. 6).

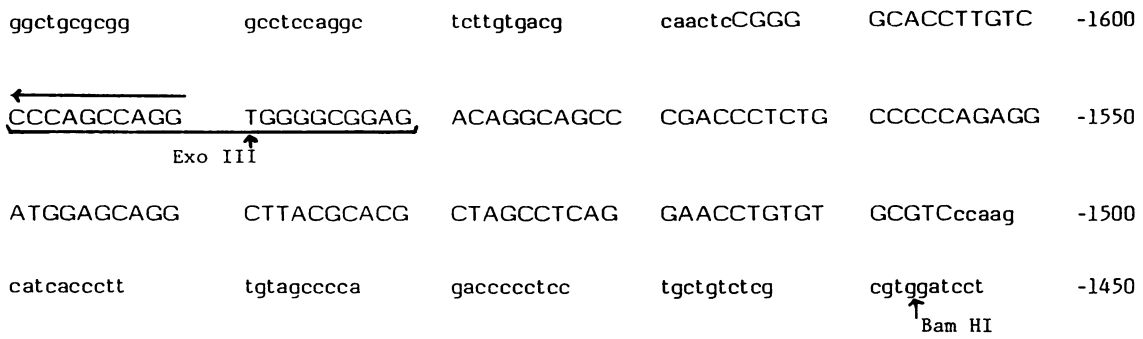

FIG. 6. Sequence of the proximal DRE associated with the cytochrome P_1-450 gene. The proximal DRE (5) is represented by upper case letters (position −1505 to −1614), and the surrounding sequences by lower case letters. The *Bam*HI restriction endonuclease cutting site is indicated by a vertical arrow at its recognition site. The Exo III stop at −1590 ± 10 base pairs is shown by a vertical arrow, and the surrounding region is underlined to indicate the potential error in the measurement. A 10-base-pair DRE consensus sequence (14) is indicated by a horizontal arrow above the sequence.

Thus, this DNA sequence may be a component of the recognition site for the TCDD-receptor complex. On the other hand, some copies of the sequence appear to be nonfunctional (14). Therefore, additional interactions (possibly between the TCDD-receptor complex and other proteins) may be required for the activation of cytochrome P_1-450 gene transcription. Our observations imply that the binding of TCDD to its protein receptor generates a complex with a high affinity for a specific site(s) in chromatin. Understanding the mechanistic role of this inducer-receptor complex in the transcriptional activation of the cytochrome P_1-450 gene is a challenging issue for the future.

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