2,3,7,8-Tetrachlorodibenzo-\(p\)-dioxin-inducible, Ah Receptor-mediated Bending of Enhancer DNA*

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The environmental contaminant 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD, \(^1\) dioxin) is the prototype for a class of halogenated aromatic hydrocarbons that are widespread environmental contaminants, which pose a potential hazard to human health (1-4). In animals, TCDD elicits a variety of morphological, biochemical, immunologic, reproductive, and neoplastic effects (1, 2). Ligand-binding studies reveal that TCDD-responsive cells contain an intracellular protein, which binds the dioxin saturably and with high affinity and is known as the Ah receptor (5, 6). Receptor-defective tissues fail to respond to TCDD, a finding which implicates the protein in the mechanism of dioxin action (1, 10). The receptor has not been purified, and many of its properties remain unknown.

The induction of aryl hydrocarbon hydroxylase activity constitutes a useful model response for analyzing the mechanism of TCDD action. The cytochrome P-450IA1 enzyme catalyzes hydroxylase activity and is responsible for the oxygenation of polycyclic aromatic hydrocarbons, such as the environmental carcinogen, benzo(a)pyrene (11). The CYPIAl gene encodes the cytochrome P-450IA1 protein (12). Analyses of dioxin action in mouse hepatoma (Hepa 1-1-7) cells reveal that TCDD induces aryl hydrocarbon hydroxylase activity by increasing the transcription rate of the CYPIAl gene (18, 19).

In previous studies, injection of dioxin into intact animals (20) or administration of TCDD to cells in culture (18, 19) results in increased aryl hydrocarbon hydroxylase activity. In intact animals, the enzyme is induced in the liver, small intestine, and kidney, whereas in cell culture, it is induced in liver cell lines (21). The enzyme is induced in vivo and is also inducible in vitro by a number of structurally diverse compounds, including benz(a)pyrene, dibenzo-\(a,b\)-furan, and 3-methylcholanthrene (22).

*This work was supported by Research Grant CA 32786 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*the abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin; bp, base pair.

EXPERIMENTAL PROCEDURES

Materials

Molecular biological reagents were from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, and Promega Biotec. TCDD was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. \(^{[125]I}\)-Iodo-7,8-dibromodibenzo-\(p\)-dioxin was a gift from Dr. A. Poland (University of Wisconsin), and the plasmid pCY1 (23) was generously provided by Dr. D. Galas (University of Southern California).

Methods

Gel Retardation Assay—Preparation of nuclear extracts from uninduced and TCDD-induced cells and gel retardation assay were as previously described (18), with the following modifications: 1 \(\mu\)g of plasmid pBR322 DNA (containing a deletion between nucleotides 3609 and 3848 to remove an Ah receptor core recognition motif) was also included in the gel retardation assay, and gels were electrophoresed for 3.5-4 h.

Plasmid Construction—The circular permutation vector shown in Fig. 1 was generated using the pCY1 plasmid (23) and the 82-bp fragment of DNA polymerase I and ligated into the unique SmaI site of pCY1. The orientation of the insert was determined by Maxam and Gilbert sequencing (24).

Other Methods—Wild-type mouse hepatoma cells and their method of culture were as described (7). Protein concentration was measured by the method of Bradford (25) using bovine serum albumin as the standard.

RESULTS

Previous analyses in other systems reveal that a bent DNA fragment migrates more slowly during electrophoresis than unbent DNA of identical size and base composition (22). The
The degree of bending influences the electrophoretic mobility of the DNA fragment; the larger the bend, the slower the mobility (26). The position of the bend along the DNA fragment also affects its electrophoretic behavior; the more centrally located the bend, the slower the mobility (22, 27, 28). DNA bending may be intrinsic (i.e. a function of the specific nucleotide sequence) or dynamic (i.e. produced by the binding of a protein).

To determine whether the binding of the liganded Ah receptor to its recognition motif produces a bend in the DNA, we generated a series of five “circularly permuted” DNA fragments using the strategy outlined in Fig. 1. The DNA fragments are identical in size and base composition, contain a single recognition motif for the liganded Ah receptor, and differ only in the position of the recognition motif along the DNA. The five fragments were end labeled with $^{32}$P and were incubated with nuclear extracts from uninduced or TCDD-induced mouse hepatoma cells; the resulting protein-DNA complexes were analyzed by gel retardation and autoradiography. The autoradiogram reveals a TCDD-inducible band (designated $R_1$, in Fig. 2) for each of the five fragments. We have shown previously that the TCDD-inducible band reflects the binding of the receptor protein to enhancer DNA (19). The mobility of the TCDD-inducible protein-DNA complex varies substantially among the five permuted DNA fragments, implying that the receptor-DNA interaction produces a bend in the nucleic acid. The protein-DNA complex in which the recognition motif for the liganded Ah receptor is most centrally located (the sample designated RV) has a slower mobility than complexes in which the recognition motif is located toward an end of the DNA (the samples designated R1 and B). These findings suggest that the bend in the DNA occurs in the vicinity of the recognition motif. As a control, we note that the mobility of a second, constitutive protein-DNA complex (designated $R_2$ in Fig. 2) varies only slightly (and not in a pattern indicative of DNA bending) among the five DNA fragments. The results of competition studies imply that the constitutive band represents a specific protein-DNA interaction (18–20). Together, these observations indicate that the variation observed for the TCDD-inducible band is not simply a nonspecific effect due to the binding of any protein to the DNA. Finally, short autoradiographic exposures reveal that the mobilities of the five protein-free DNA fragments (designated $F$ in Fig. 2) are identical, demonstrating the absence of intrinsic DNA bending (data not shown).

To document that the liganded Ah receptor contributes to the bending of the DNA, we performed modified gel retardation experiments using nuclear extracts prepared from cells exposed to $[^{131}]2$-iodo-7,8-dibromodibenzo-p-dioxin, a high affinity agonist for the Ah receptor (29). In these studies, the liganded receptor protein contained the radioactive label, and the five permuted DNA fragments were unlabeled. Under these conditions, the gel retardation analyses detect only the protein-DNA complexes that contain the liganded Ah receptor. We have shown previously that the mobility of the protein-DNA complex observed when the protein is labeled is identical to the mobility of the complex observed when the DNA is labeled (19). The results shown in Fig. 3 reveal that the liganded Ah receptor participates in the formation of a protein-DNA complex with each of the five circularly per-
TCDD-inducible, Ah Receptor-dependent DNA Bending

5720

FIG. 13. Gel retardation analyses using $^{35}$S-labeled receptor protein. Crude nuclear extracts isolated from wild type cells induced with $[^{35}S]2$-iodo-7,8-dihromodibenzo-p-dioxin (0.3 nM, 60 min) were incubated with unlabeled circularly permuted fragments, and the protein-DNA complexes were analyzed by gel retardation. The band containing the liganded Ah receptor is designated $R_1$. H, EtoKl; H, HP/M; H, K 1, I:coRV; I, I+1, H, BssHl. The arrowheads indicate the position of the core recognition motif for the liganded Ah receptor.

FIG. 4. Determination of the position of the DNA bend. The mobilities of the circularly permuted fragments were plotted with respect to the position of the corresponding restriction sites on the reference fragment shown. $R_1$, EcoRI; H, HpaI; RV, EcoRV; F, FspI; B, BamHI. The position of minimum mobility was determined by polynomial regression analysis of the experimental data. Each point represents the average of three experiments. The brackets indicate the range of values. The arrowheads indicate the position of the core recognition motif for the liganded Ah receptor.

muted DNA fragments. The mobilities of the complexes vary with the position of the recognition motif, again implying that the protein-DNA interaction bends the DNA. The mobility pattern for the five protein-DNA complexes is similar to the pattern observed when the DNA fragments are labeled (compare the patterns designated $B_1$ in Figs. 2 and 3). Thus, our findings indicate that the liganded Ah receptor is a component of a nucleoprotein complex containing bent DNA. Our results do not exclude the possibility that the nucleoprotein complex also includes other proteins that contribute to DNA bending, although there is no reason to favor this idea. The issue of whether the liganded Ah receptor can by itself bend DNA requires receptor purification before it can be addressed.

To determine the location of the DNA bend, we plotted the mobilities of the five TCDD-inducible protein-DNA complexes as a function of the position of the five restriction sites used to generate the permuted DNA fragments. This procedure allows us to estimate directly the position of the bend with respect to the position of the recognition motif. The results (Fig. 4) indicate that, within experimental error (five to seven nucleotides), the DNA bend coincides with the recognition motif. We interpret these observations to mean that the binding of the liganded Ah receptor to its DNA recognition motif induces a bend in the DNA at the receptor-binding site.

DISCUSSION

Others have shown that protein-induced DNA bending occurs in several prokaryotic and eukaryotic systems (22, 27, 28, 30) and that the phenomenon may contribute to the processes of recombination (31), replication (32), and transcription (22, 33). The studies reported here imply that the binding of the liganded Ah receptor to its DNA recognition motif produces a bend in the DNA. We envision that the bending may stabilize the receptor-DNA complex by increasing the contacts between the protein and the nucleic acid. This idea is consistent with our previous observations that nucleotides beyond the core recognition motif appear to strengthen the interaction between the liganded receptor and DNA (19). Other experiments, analogous to those described above but utilizing permuted DNA fragments containing just the core motif, have been inconclusive, due to the decreased strength of the protein-DNA interaction, combined with the background noise associated with the use of crude nuclear extracts. Thus, we do not yet know whether the binding of the liganded receptor to just the core motif is sufficient to induce DNA bending or whether nucleotides that flank the core are also required. Purification of the Ah receptor may facilitate such studies.

The functional significance of our observation is unknown. The fact that the liganded receptor can bend DNA in vitro suggests that the receptor-enhancer interaction may produce a change in DNA structure in vivo. For example, the energy expended in DNA bending may facilitate base-pair opening, which should, in turn, increase the flexibility of the DNA in the vicinity of the bend (34). If increased DNA flexibility were to occur in vivo, we envision that it could facilitate the formation of other protein-DNA and protein-protein contacts required for the initiation of transcription. We note that the DNA upstream of the CYP1A1 gene contains six copies of the recognition motif for the liganded Ah receptor (20). Thus, the potential for TCDD-inducible, receptor-mediated DNA distortion in vivo appears substantial. These observations suggest that the analysis of the liganded receptor's effects on DNA structure in the intact cell (i.e. in chromatin) may be a productive area for future research.

Acknowledgment—We thank Shirley Kruk for typing the manuscript.

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TCDD-inducible, Ah Receptor-dependent DNA Bending

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J. Biol. Chem. 1990, 265:5718-5721.

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