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NUCLEASE HYPERSENSITIVITY OF THE RAT CYTOCHROME P450IA1 GENE

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Abstract—The bovine pancreatic deoxyribonuclease I (DNAase I) hypersensitivity of the rat cytochrome P450IA1 gene was investigated. A nuclease-hypersensitive region was observed at approximately 3.2 to 5.1 kilobase pairs upstream of exon 1 in adult and fetal rat liver. This region did not necessarily correlate with gene expression following 3-methylcholanthrene induction, although it may determine the potential for inducibility of this gene.

DNAase I¶ was first used to differentiate between "active" and "inactive" chromosomal regions by Billing and Bonner [1]. Southern blot analysis of new gene fragments that result from specific and discrete nuclease-generated "cuts", i.e. hypersensitive sites, has revealed much information about chromatin structure of that gene. The great majority of these sites are located in 5′-flanking sequences and are tissue specific, but do not necessarily correlate with transcriptional activity. Consequently, a nuclease-hypersensitive site at or near the 5′ end of a gene may be necessary, but not sufficient, for transcription by RNA polymerase II *in vivo* [2].

The Sprague–Dawley rat hepatic cytochrome P450IA1 gene has been successfully cloned and sequenced by our laboratory [3] as well as that of Sogawa *et al.* [4]. The "induction" of this cytochrome P-450 after administration of 3-methylcholanthrene (MC) has been shown to be at least partially regulated by developmental factors [5, 6]. In the present report, we further characterized the cytochrome P450IA1 gene with regard to nuclease hypersensitivity in fetal and adult livers. After this work was completed, a report appeared in which DNAase I-hypersensitive sites were described in the chromosomal domain of the cytochrome P450IA1 gene in control and MC-treated adult rat liver [7]. Our results describe an extension of these studies to include fetal rat liver and to encompass more distal 5′ regions of the cytochrome P450IA1 gene.

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¶ Abbreviations: DNAase I, bovine pancreatic deoxyribonuclease I (EC 3.1.4.5); MC, 3-methylcholanthrene; 1× SSC, 150 mM NaCl/15 mM sodium citrate, pH 7.0; 1× Denhardt's solution, 0.04% (w/v) Ficol/0.04% polyvinylpyrrolidone/0.04% bovine serum albumin (BSA); SDS, sodium dodecyl sulfate; and kbp, kilobase pairs.

METHODS

The reagents and materials were purchased from the following companies: Eco RI and Hind III-digested bacteriophage lambda DNA, New England Biolabs, Inc. (Beverly, MA); DNAase I (DPFF grade, 1600–2333 units/mg, or ribonuclease-free DPRF grade, 2040 units/mg), Worthington/Cooper Biomedical (Freehold, NJ); and nylon 66 membranes (Magnagraph, 0.45 μm), Micron Separations Inc. (Honeoye Falls, NY). The sources of all other materials have been described previously [8, 9].

Animal experiments. Four-week-old male Sprague–Dawley rats (Sasco Inc., Omaha, NE) were injected i.p. with MC at a dose of 25 or 40 mg/kg body weight. Control animals were either not treated or injected i.p. with an equivalent volume of the vehicle, corn oil. The animals were provided food (Lab Chow, Ralston Purina Co.) and water *ad lib.* and were killed at various times after treatment.

In the fetal liver experiments, 18-day pregnant Sprague–Dawley rats were injected i.p. with MC (in corn oil) or the polycyclic hydrocarbon was administered directly into each amniotic sac, at a final dose of 25–40 mg/kg body weight, as previously described [10, 11]. Control animals were treated similarly with corn oil. The animals were killed on day 19 of pregnancy (approximately 15–18 hr after the treatment), and the fetuses were removed. Fetal liver was removed and rapidly frozen on dry ice.

Isolation of nuclei, DNAase I digestion, and isolation of high molecular weight genomic DNA were essentially as described by Burch and Weintraub [12]. The genomic DNA samples were dialyzed extensively at 4° under sterile conditions in a microdialysis unit (Bethesda Research Laboratories, Gaithersburg, MD) against 10 mM Tris–HCl, pH 7.5/2 mM EDTA. All digestions with restriction endonucleases were performed according to the specifications recommended by the enzyme manufacturer.

Southern blot analysis of genomic DNA. Restriction endonuclease-digested genomic DNA, and Hind III-digested bacteriophage lambda DNA standards were size-fractionated by electrophoresis on horizontal 1% (w/v) agarose gels. The agarose gels were incubated twice for 1 hr at room temperature in

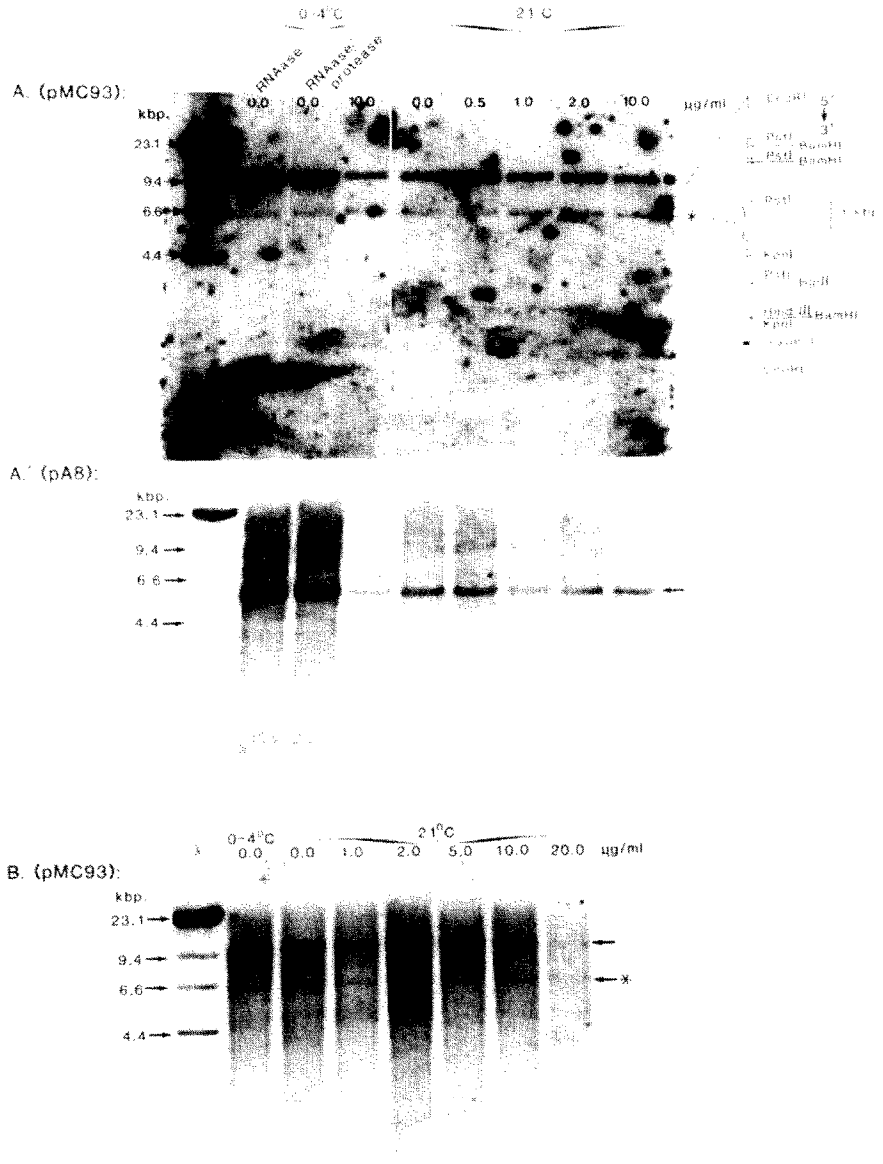

Fig. 1. Nuclease-hypersensitivity in the 5′ regions of the adult hepatic cytochrome P450IA1 gene. Nuclei isolated from untreated adult liver (A and A′) ($[DNA]_{nuclei}$ = 2.4 mg/ml) or from corn oil-treated adult liver (B) ($[DNA]_{nuclei}$ = 2.8 mg/ml) were incubated at 0–4° or 21° with various concentrations of DNAase I (as indicated). Isolated genomic DNA (30 µg) was digested with Eco RI (5 units/µg DNA), size-fractionated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized to radiolabeled pMC93 (A and B). The nylon membrane depicted in A was rehybridized to radiolabeled pA8 (sp. act. = 6.8×10^7 dpm/µg), and this is depicted in A′. The migration of Hind III-digested bacteriophage lambda DNA standards is indicated. The asterisk implicates an Eco RI fragment generated by nuclease-"hypersensitivity", and it 5′ location relative to exon 1 of the cytochrome P450IA1 gene is shown by the bracket.

0.5 M NaOH/0.8 M NaCl, and then incubated twice for 1 hr each time at room temperature in 0.5 M Tris–HCl, pH 7.6/1.5 M NaCl. The genomic DNA was then transferred overnight from the agarose gel to a nylon membrane, in 10× SSC. The genomic DNA was immobilized on the nylon membrane by exposure to long wavelength ultraviolet light for 2–3 min, and by baking at 80° for 2–2.5 hr.

The membranes were prehybridized at 42° for at least 4 hr in 5× SSC/50% (v/v) deionized formamide/1.5× Denhardt's/1% (w/v) SDS/10 mM EDTA, pH 8.0/sonicated and denatured salmon sperm DNA (100 µg/ml). The membranes were hybridized in the same solution in the presence of denatured radiolabeled probe at 42% for 24–48 hr and were then washed twice at room temperature, for 30 min each time, in 1× SSC/0.1% (w/v) SDS, and once for various times at 55°, in 0.1× SSC/0.1% (w/v) SDS.

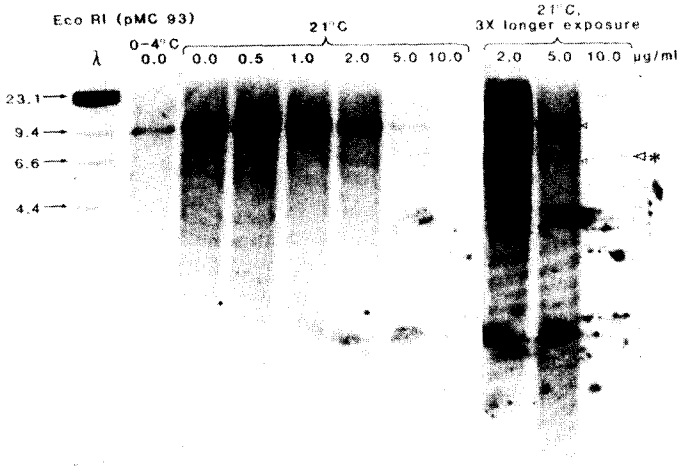

Fig. 2. DNAase I-hypersensitivity in the 5′ regions of the cytochrome P450IA1 gene in MC-treated fetal liver. Nuclei isolated from MC-treated fetal liver were incubated at 0–4° or 21° in the presence of various concentrations of DNAase I (as indicated and expressed in μg/ml). Eco RI-digested genomic DNA (30 μg) was size-fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized to radiolabeled pMC93. The autoradiograph exposure times were 1 or 3 days as indicated. The migration of Hind III-digested bacteriophage lambda DNA standards is shown (all sizes are expressed in kbp). The asterisk refers to an Eco RI fragment generated by the action of DNAase I.

For the purposes of rehybridization, the membranes were boiled for 10 min in distilled water or in 0.1% (w/v) SDS, pH 7.2. Removal of the probe was confirmed by autoradiography.

RESULTS AND DISCUSSION

To examine 5′-upstream sequences of the cytochrome P450IA1 gene for DNAase I-hypersensitive regions, a cloned 200 bp Bam HI/Kpn I genomic DNA fragment immediately 5′ of exon 1 was employed; this fragment is referred to as pMC93. It is apparent from the experiments depicted in Figs. 1A, 1B and 2 that pMC93 hybridized to two genomic Eco RI fragments of 11.2 ± 0.8 and 6.5 ± 0.3 kbp.* The 6.5 kbp fragment was observed in genomic DNA samples obtained from undigested adult (Fig. 1, A and B) but not fetal hepatic nuclei (Fig. 2) (possibly as a result of differential endogenous nuclease activities), and its relative intensity with regard to the "parental" 11.2 kbp Eco RI fragment increased in a concentration-dependent fashion following DNAase I-digestion. This was confirmed by laser densitometry (data not shown). Visualization of the Eco RI-digested genomic DNA of Fig. 1, A and B, by ethidium bromide staining established that similar amounts of DNA had been loaded in each lane. The presence of the 6.5 kbp pMC93-hybridizable fragment depended upon prior digestion of the genomic DNA with Eco RI (data not shown). Therefore, this fragment was apparently juxtaposed on one end by an Eco RI recognition site and, on the other end, by a nuclease (endogenous or DNAase I)-hypersensitive site. The genomic region encompassing this "nuclease-hypersensitive site", as deduced by comparison of the sizes of the "parental" and "nuclease-generated" pMC93-hybridizable genomic DNA fragments, is depicted on the right side of Fig. 1. No additional nuclease-hypersensitive regions were observed in a 16.5 kbp chromosomal domain encompassing the cytochrome P450IA1 gene (data not shown).

In control experiments, plasmid pA8 [3, 8], which encompasses approximately 80% of the cytochrome P450IA1 gene, hybridized to a single genomic Eco RI fragment of 5.7 ± 0.3 kbp (Fig. 1A′). No additional genomic DNA fragments generated by nuclease digestion were observed even on longer exposures of the autoradiograph or when similar experiments employing Bam HI-, Bgl II-, or Pst I-digested genomic DNA were conducted using pA8 as a probe (data not shown).

The sequences encompassed by the nuclease-hypersensitive region, at 3.2–5.1 kbp 5′ of exon 1 of the cytochrome P450IA1 gene (see right side of Fig. 1), were similar in adult and fetal liver. This region appears to be involved in the regulation of cytochrome P450IA1 gene expression by MC since its insertion 5′ of a reported chloramphenicol acetyltransferase (CAT) gene and the resultant transfection into a mouse hepatoma cell line led to an MC-determined expression of CAT; deletion of this region from these constructs was accompanied by a 2-fold loss in "inducibility" of the reporter [13, 14]. More precise mapping of this nuclease-hypersensitive region was unsuccessful because of the repetitive nature of the DNA sequences found in the 5′ regions of the cytochrome P450IA1 gene (data not shown).

We have also compared the 5′ genomic regions of the cytochrome P450IA1 gene in liver obtained from control and MC-treated adult rats to assess whether new DNAase I-hypersensitive sites would be generated as a result of the treatment. However, *no*

* In all instances the values are means ± standard deviation; the number of observations is a minimum of 3 and a maximum of 8.

additional genomic fragments generated by DNAase I digestion were observed (data not shown). Therefore, we were unable to see those DNAase I-hypersensitive sites in the chromosomal domain of the cytochrome P450IA1 gene that were reported by Einck *et al.* [7]. This inconsistency can be partially explained by the use of different probes by their laboratory and our laboratory. Probe pR5-1.1 used by Einck *et al.* [7] recognizes both the cytochrome P450IA1 and IA2 genes, whereas our probe only interacts with the former.

Additional research is required to further define the nature of alterations in liver chromatin after treatment of rats with MC, and to decipher the relationship of these changes to cytochrome P450IA1 gene expression.

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