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Chromatin Structure of the Cytochrome P-450c Gene Changes following Induction

Leo Einck, John Fagan, and Michael Bustin*
Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205
Received March 18, 1986; Revised Manuscript Received June 2, 1986

ABSTRACT: The chromatin structure of cytochrome P-450c and P-450d genes, which in the liver are highly inducible by 3-methylcholanthrene, was studied in normal and carcinogen-treated rats by using a cDNA probe specific for P-450c and a genomic probe that recognizes both genes. Digestion with micrococcal nuclease revealed that the active genes are not present in the typical 200 base pair nucleosomal structure. Gene induction is associated with a rearrangement of the nuclear organization of the genes. By use of indirect end-label hybridization, three DNase I hypersensitive sites were mapped, one in the 5′-terminal region and two in the 3′ region of the P-450c gene. Gene induction, by treatment with 3-methylcholanthrene, changes the location of the DNase I site present in the 5′ region without affecting the sites present in the 3′ region. Rat thymus chromatin does not contain these DNase I hypersensitive sites, suggesting that, in the liver, the chromatin structure is altered so as to allow tissue-specific expression of the P-450c gene. The chromatin structure of the highly inducible P-450c gene is compared to that of the P-450m gene, which is induced to a significantly smaller extent and is constitutively expressed.

The cytochrome P-450 family of proteins is involved in the metabolism of a variety of xenobiotic and endogenous compounds including drugs, carcinogens, and toxins (Conney, 1982; Boobis et al., 1985). Some of the metabolites produced are potent mutagens and carcinogens that bind covalently to cellular macromolecules such as proteins, RNA, and DNA (Gelboin, 1980). Synthesis of the various members of the cytochrome P-450 enzyme system are highly inducible in a very selective manner, and the isoenzymes generated have distinct yet overlapping substrate specificities (Lu & West, 1980; Adesnik & Atchison, 1985). The rapid, temporal induction of a specific set of genes in response to exposure to specific inducers is an important aspect of both the detoxification process and the production of harmful intermediates. The regulation of gene expression is therefore central to the coordinate control of this family of genes and to their biological role. Compared to total nuclear DNA, active or potentially active genes have an altered chromatin structure that can be

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examined with various nucleases [for a review of chromatin structure and nuclease sensitivity, see Igo-Kemenes et al. (1982) and Elgin (1981)]. Transcribable regions are preferentially digested with DNase I, presumably because of an altered chromatin structure. The relationship between gene expression and the DNase I sensitivity is observed not only in expressed genes but also in those genes that have been transcribed and those that retain the potential for transcription. Another feature of chromatin structure revealed by DNase I is the so-called hypersensitive site. These regions, which are found near active genes, are hypersensitive to digestion by the nuclease DNase I and, in some cases, change their pattern of sensitivity corresponding to changes in gene expression (Weintraub et al., 1981; McGhee et al., 1981; Wu, 1980, 1984; Stalder et al., 1980; Siebenlist et al., 1984; Becker et al., 1984; Wu & Simpson, 1985). The DNase I hypersensitive sites reflect specific protein–nucleic acid interactions (Emerson & Felsenfeld, 1984).

Treatment of rats with the chemical carcinogen 3-methylcholanthrene induces, in the liver, two major isoenzymes designated as P-450c and P-450d (Morville et al., 1983). Within 15–24 h after 3-MC administration, the level of their mRNA increases more than 100-fold (Bresnik et al., 1981). In addition to these two highly inducible isoenzymes, the liver contains other P-450 enzymes. Some of these are constitutively expressed at relatively low levels in the uninduced animal and are induced, to a much smaller degree, by treatment with carcinogens [for review see Adesnik and Atchinson (1985)]. We have reported (Einck et al., 1985) that gene induction does not cause detectable changes in the chromatin structure of P-450m, a P-450 of the latter class that is present constitutively and is induced approximately 4-fold after intraperitoneal injection of 3-MC (Fagan et al., 1982). In this paper, we examine the chromatin structure of the highly inducible P-450c isoenzyme in control and carcinogen-treated rat liver nuclei. We find that, in contrast to the P-450m gene, the chromatin structure of the P-450c gene changes upon induction.

Materials and Methods

Preparation of Nuclei. Male Sprague–Dawley rats weighing 160–180 g were obtained from the NIH small animal facility in Frederick, MD. Cytochrome P-450c induction was achieved by injection of 20 mg/kg rat body weight 3-methylcholanthrene (Tridon Chemical Co., Hauppauge, NY) in corn oil into the peritoneal cavity. Following a 24-h induction period, the rats were sacrificed, and the liver and thymus were excised and cooled on ice. All subsequent steps except enzyme digestions were done at 4 °C. Postmitochondrial supernatants (12000 rpm supernatants of liver homogenate, Beckman Microfuge 12, 15 min, 0 °C) were isolated, frozen at −70 °C, and tested for AHH (aryl hydrocarbon hydroxylase) activity (Nebert & Gelboin, 1968). Nuclei were isolated by using modifications of the technique of Chauveau et al. (1956) and Blobel and Potter (1966). Minced liver tissue was homogenized in 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ with a Teflon-glass pestle, mixed with sucrose to 1.6 M, and pelleted through 2.3 M sucrose in the same buffer at 124000*g* in a Beckman SW28 rotor for 45 min. The nuclei were resuspended in the appropriate digestion buffer at 0.5 mg of DNA/mL.

Nuclease Digestion. DNase I (Worthington) digestions were done in 0.34 M sucrose, 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 3 mM $MgCl_2$ with varying amounts of enzyme (μg/L as indicated in figures) for 10 min at 37 °C. Micrococcal nuclease (Worthington) digestions were done in 10 mM Tris-HCl (pH 8.0) and 1.0 mM $CaCl_2$ at 37 °C for 1, 5, 10, and 20 min by using 0.5 enzyme unit (Worthington)/OD_{260}. Released nucleoprotein (S1) was collected by centrifugation of the nuclei, leaving the S1 fraction in the supernatant. The digestion was stopped, and the nuclei were lysed by adding NaEDTA to 5 mM. The lysed nuclei were pelleted away from nucleoprotein released into the supernatant (S2) by centrifugation at 10000 rpm in a Sorvall HB-4 rotor. The DNA was isolated by proteinase K digestion (BRL) overnight at 37 °C, digested with RNase A (Sigma), and subsequently purified by phenol and chloroform extractions. The chromatin DNA was then extensively dialyzed in 10 mM Tris-HCl and 2 mM EDTA, pH 7.5. Restriction enzymes were obtained from BRL and New England BioLabs and were used as recommended by the manufacturer.

Electrophoresis, Transfer, and Hybridization. DNA samples were separated on 3-mm vertical 1% or $1^1/_2$% agarose gels by using a Hoeffer SE600 with one frosted plate/gel. Gels were run (45 V) overnight at 4 °C in Tris–acetate buffer, pH 8.4. Following ethidium bromide staining, the gels were photographed. The DNA was acid-nicked by incubating the gel in 0.25 M HCl for 15 min and was denatured by incubation in 0.5 N NaOH and 1.5 M NaCl for 40 min. Transfer to Gene Screen Plus (New England Nuclear) was done in 12 mM Tris-HCl (pH 7.5), 6 mM sodium acetate, and 0.3 mM EDTA by using a Hoeffer Transfor at 10 V for 45 min followed by 60 min at 40 V at 4 °C. The nylon membrane was air-dried and prehybridized in 1% SDS, 10% dextran sulfate (Pharmacia), 50 mM Tris-HCl (pH 7.5), and 1 M NaCl overnight at 65 °C. The membrane was probed with either 10^7 counts of ^{32}P-labeled plasmid probe (nick translated to $1–2 \times 10^8$ counts/μg of DNA by using the BRL nick translation kit and Amersham ^{32}P-dCTP) with 100 μg/mL denatured salmon sperm DNA (Sigma) as carrier or with 3×10^8 counts of ^{32}P-labeled probe (random primer extension by Lofstrand Corp., Gaithersburg, MD) to 3×10^9 counts/μg of DNA. Plasmid fragments used for hybridization were isolated by transfer to DEAE-cellulose (Schleicher and Schuell) by horizontal electrophoresis. Nick-translated *Hin*dIII-cut λ phage and *Hae*III-cut φX174 phage (BRL) were hybridized as radioactive markers. Hybridization proceeded for 20–48 h at 65 °C with agitation. The membrane was washed with 2 × SSC (SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) at room temperature for 10 min, 2 × SSC with 1% SDS at 65 °C for 60 min, and finally with 0.1 × SSC at room temperature for 60 min. The washed membrane was blotted dry and autoradiographed with Lighting Plus intensifying screens (Du Pont). Rehybridization was performed by allowing the probe to decay and then repeating the prehybridization and hybridization steps or by washing as recommended by the manufacturer.

Plasmids. The 5350 base pair (bp) *Eco*RI fragment of the P-450c gene (see Results and Figure 1) was isolated from a rat genomic library, prepared by T. Sargent, and subcloned in pBR322 by using *Escherichia coli* strain HB101. Various fragments were isolated from pR5 and subcloned in pBR322. Plasmid amplification was done in rich medium containing 240 μg/mL chloramphenicol. Bacteria were lysed by using the Triton X-100 method of Katz et al. (1973), and plasmids were isolated by using the double banding method of Radloff et al. (1967). The NIH guidelines for recombinant DNA research were followed throughout the work.

Results

The genomic map of the rat P-450c gene, derived from published data (Sogawa et al., 1984; Hines et al., 1985), is presented in Figure 1. The bar represents a 6917 base pair

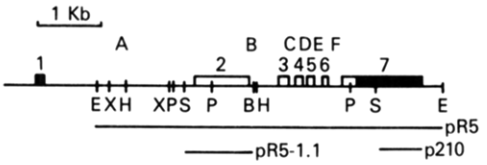

FIGURE 1: Genomic map of the rat P-450c gene (Sogawa et al., 1984) and strategy for mapping hypersensitive sites in the 5′ and 3′ regions of the gene. The 6.9-kb genomic fragment contains the entire coding region of P-450c oriented in a 5′ to 3′, left to right direction. Coding sequences are marked as open boxes and transcribed noncoding sequences are marked as closed boxes. Exons are numbered. Only the restriction sites relevant to discussed data are shown: E, *Eco*RI; B, *Bam*H1; P, *Pst*I; S, *Sst*1; H, *Hin*dIII; X, *Xba*1. pR5, pR5-1.1, and p210 are subclones used in the present studies. Note that probing an *Xba*1 digest of genomic DNA with pR5-1.1 allows mapping of sites in the 3′ direction, while probing a *Bam*H1 digest allows mapping into the 5′ direction. Likewise, probing a *Pst*1 digest with p210 allows mapping of sites in the 3′ region of the gene.

long genomic segment. The residues are numbered from the adenosine of the cap site at the start of exon I, which is not translated. In this diagram (Sogawa et al., 1984), the 5′ respresents residue number −566. The translated region spans 2504 nucleotides, starting from position 2570 located near the 5′ of exon II and ending at position 5074 located within exon VII. We have isolated, from a rat genomic library, a 5350 nucleotide long *Eco*RI fragment and subcloned it into pBR322. Restriction analysis of this construct (plasmid pR5) reveals that it is identical to the major *Eco*RI fragment whose 5′ end is located in the middle of the first intron and whose 3′ end corresponds to that of the sequenced P-450c gene (data not shown). Digestion of pR5 with *Sst*1 and *Bam*H1 yielded a 1.1 kilobase (kb) fragment (subcloned into pBR322 and named pR5-1.1) that was devoid of repetitive sequences and as such was suitable for analysis of DNase I sensitive sites in the 5′ region of the gene. This probe also contains sequences highly homologous to exon 2 of the P-450d gene (Sogawa et al., 1985). Therefore, this probe detects P-450c and P-450d, both of which are highly induced by 3-MC in rat. The 3′ region of the gene was probed with a cDNA construct (p210), which contained a 758-bp fragment, from the 3′ untranslated region of the gene, corresponding to nucleotides 5222–5980 (Fagan et al., 1986). The position of these fragments in the P-450c gene was verified by Southern analysis of various restriction digests of pR5.

Our main goal was to study the chromatin structure of the genes encoding the major 3-methylcholanthrene-induced P-450 genes prior to and following induction of transcription by treatment of rats with 3-methylcholanthrene (3-MC). Induction of transcription by 3-MC was followed by measuring the AHH activity in microsomes isolated from the liver of control and carcinogen-treated rats. Twenty-four hours following intraperitoneal injection of 3-MC, total rat liver microsomal AHH activity increased approximately 3.75-fold, from 152.7 pmol min^{-1} (mg of total microsomal protein)$^{-1}$ to 572.9 pmol min^{-1} (mg of protein)$^{-1}$. This corresponds to an approximate 100-fold increase in expression of the rat liver P-450c gene (Bresnick et al., 1981; Fagan et al., 1986).

In the first set of experiments, we studied the nucleosomal conformation of these genes by digesting, to increasing extents, the nuclei from control and carcinogen-treated rat liver with micrococcal nuclease. Each digest was subjected to a fractionation procedure that separates chromatin into three major fractions (Albright et al., 1980). Fraction S1 contains material that is soluble in divalent metals and that is released from the nuclei during digestion; fraction S2 contains material that is released by EDTA after nuclear disruption, and fraction P

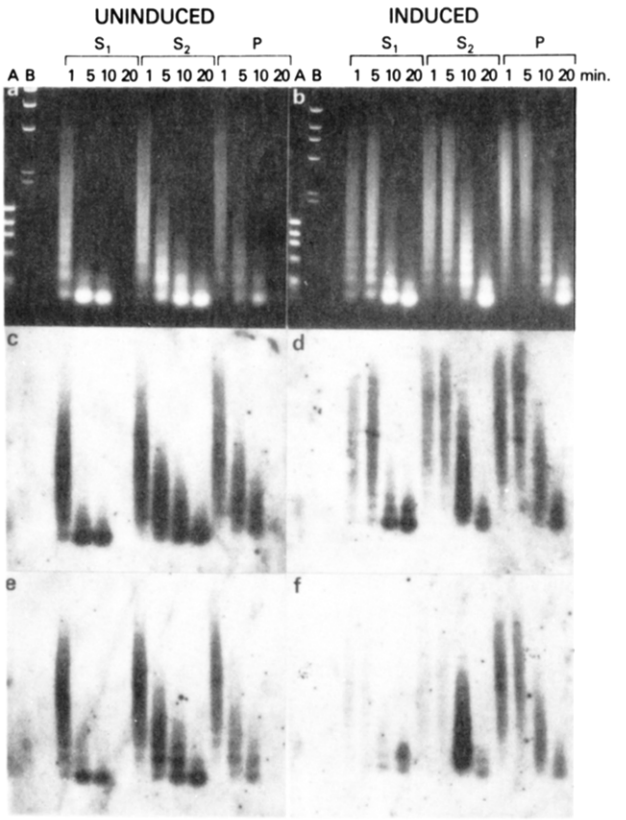

FIGURE 2: Digestions of uninduced and induced rat liver nuclei demonstrate induction-sensitive changes in micrococcal nuclease gel electrophoresis patterns: a, c, and e, uninduced rat liver nuclei; b, d, and f, induced rat liver nuclei. (a and b) Ethidium bromide stained gel showing increased protection of DNA following induction. (c and d) The transferred gel was probed with p210. (e and f) The transferred gel was probed with pR5-1.1. S1, S2, and P refer to various chromatin fractions (see text). The time of digestion of the nuclei with micrococcal nuclease is indicated at the top of each column. Lanes A and B are molecular weight markers.

contains material that is insoluble and presumably associated with the nuclear matrix. Rose and Garrard (1984) presented evidence suggesting that, in some cases, fractions S1 and P are enriched in nucleosomes containing transcriptionally active genes.

The results presented in Figure 2 indicate that bulk DNA of either control or carcinogen-treated rat liver chromatin exhibits typical nucleosomal ladders in all the three chromatin fractions examined. The data presented in Figure 2a,b reveal that the chromatin is digested to nucleosomal monomer more quickly in the uninduced nuclei than in the induced nuclei isolated from the liver of rats treated with 3-MC. Thus, after 5-min digestion, a significant portion of the chromatin in the S1, S2, and P fractions isolated from control nuclei was reduced to mononucleosomal size, while in induced nuclei most of the chromatin was still present as oligonucleosomes.

The DNA from the gels was transferred to Gene Screen Plus and probed either with plasmid pR5-1.1 or with plasmid p210. The results suggest induction-dependent changes in the nuclear organization of the 3-MC-induced genes. Southern blot analysis of DNA obtained from uninduced rats revealed that both the 3′ region of the P-450c gene (Figure 2c) and the 5′ region (Figure 2e) of the P-450c and P-450d genes are present in all the chromatin fractions. The autoradiographic signals seem to mimic faithfully the overall intensity of the ethidium bromide stain. In contrast, the autoradiographic signal of the DNA of the induced rat liver is stronger in the P fraction as compared to the S1 and S2 fractions regardless of whether

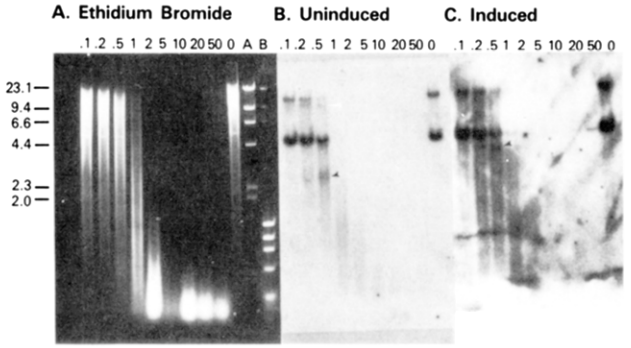

FIGURE 3: Localization of 5′ hypersensitive sites in the rat cytochrome P-450 genes. Panel A represents the ethidium bromide stained agarose gel prior to transfer to Gene Screen Plus. Numbers above the lanes refer to μg/L DNase I. Lanes A and B are molecular weight markers. Panel B represents uninduced rat liver nuclei probed with plasmid pR5-1.1. Panel C represents induced rat liver nuclei probed with plasmid pR5-1.1. Arrows indicate bands generated by the DNase I digestion: black, P-450c; white, P-450d.

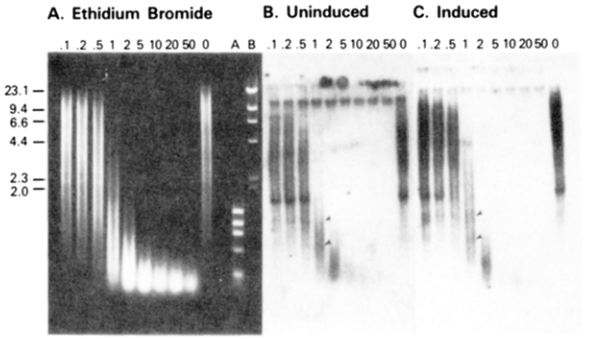

FIGURE 4: Localization of 3′ hypersensitive sites in the rat cytochrome P-450c gene. Panel A represents the ethidium bromide stained agarose gel prior to transfer to Gene Screen Plus. Numbers above the lanes refer to μg/L DNase I. Lanes A and B are molecular weight markers. Panel B represents uninduced rat liver nuclei probed with plasmid p210. Panel C represents induced rat liver nuclei probed with plasmid p210. Arrows indicate bands generated by DNase I.

probed with the 3′ region (Figure 2d) or the 5′ region (Figure 2f) of the gene. More detailed examination of the data indicates that the shift to the P fraction is more pronounced in the 5′ region than in the 3′ region of the gene (compare parts f and d of Figure 2). Thus, transcriptional activation of these genes is correlated with a structural reorganization manifested by an increase in the proportion of the P-450c and P-450d DNA found in fraction P (compare parts c and e of Figure 2 to parts d and f), which is equated with the nuclear matrix.

The autoradiograms also indicate that these genes are not organized in a typical nucleosomal pattern. We have previously demonstrated (Einck et al., 1985) that the autoradiograph generated after probing such transfers with a plasmid containing highly repetitive rat DNA displays a typical nucleosomal pattern similar to that displayed by the ethidium bromide stain. In contrast, the autoradiographic pattern generated by p210 and pR5-1.1 was less well-defined and resembled a smear (Figure 2d,f), suggesting that P-450c and P-450d genomic sequences are not organized in a typical nucleosomal pattern.

Since changes in DNase I hypersensitive sites have been shown to be associated with changes in gene expression, we next analyzed the location of such sites in the chromatin present in rat liver nuclei isolated from both normal and 3-methylcholanthrene-induced rats. In these experiments isolated nuclei were briefly digested with DNase I; the digested DNA was isolated, digested with a restriction enzyme, and fractionated on an agarose gel. The DNA fragments were transferred to a nylon membrane that was probed with ^{32}P-labeled fragments allowing mapping of preferred DNase I digestion sites relative to the known restriction maps of the P-450c and P-450d genes. Probing an *Xba*1 digest of genomic DNA with pR5-1.1 allows mapping of sites in the 3′ direction of the P-450c gene, while probing a *Bam*H1 digest allows mapping in the 5′ direction. Likewise, probing a *Pst*1 digest with p210 allows mapping of sites in the 3′ region of the P-450c gene (see Figure 1). Since the P-450d gene sequences homologous to pR5-1.1 are near the middle of the 15 kb *Bam*H1 fragment that contains the P-450d gene, this probe yields less clear-cut information regarding the location of possible hypersensitive sites within the P-450d gene.

As can be seen from the ethidium bromide stained gel in Figure 3A, increasing DNase I digestion (0–20% acid-soluble nucleotides) greatly reduces the size of the DNA. In the first three lanes of the autoradiogram the intact *Bam*H1/*Bam*H1 gene fragment of 4.5 kb (P-450c gene; Hines et al., 1985) and 15 kb (P-450d gene; Sogawa et al., 1984) can be identified. The probe used is pR5-1.1, an 1100 base pair fragment that abuts the *Bam*H1 restriction enzyme site near the 3′ end of exon 2 of the P-450c. Therefore, it can be used to map preferential DNase I sites by measuring the length of the newly generated DNA fragment upstream from that restriction site. Using this technique, we have identified, in the uninduced nuclei, a DNase-hypersensitive site 2.4 kb 5′ to the *Bam*H1 site of the P-450c gene (small black triangle, Figure 3B). Following induction, the 2.4-kb fragment disappears and a 3.6-kb fragment appears (small black triangle, Figure 3C; arrows Figure 1). The P-450d gene also contains at least one hypersensitive site since under the same digestion condition a new 13 kb long fragment is detectable (white arrow, Figure 3C). The fragment is not observed in DNA extracted from nuclei that were not treated with DNase I (see 0 point in Figure 3C). Since the P-450d gene does not contain the *Bam*H1 site at the 3′ end of intron 2 the hypersensitive site cannot be mapped. In the induced rat we were not able to detect a fragment generated from p-450d by controlled DNase I digestion. Therefore, we conclude that treatment of rats with 3-MC brings about a change in the chromatin structure of the P-450c gene, which is associated with a change in the location of a DNase I hypersensitive site.

Hypersensitive sites can also be measured from the 3′ end of the genomic P-450c clone in an analogous fashion with plasmid p210 as a probe (Figure 4). The ethidium bromide stained gel demonstrating the DNase I digestion is shown in Figure 4A. Hybridization with plasmid p210, which abuts the *Pst*1 site located near the center of exon 7 (Figure 1), allows mapping of hypersensitive sites in the 3′ direction in both uninduced (Figure 4B) and induced (Figure 4C) nuclei. Two DNase I hypersensitive sites, which were not affected by gene induction, were detected 1.1 and 0.7 kb 3′ from the *Pst*1 site. We conclude that gene induction is not associated with detectable changes in the DNase I hypersensitive sites in the 3′ region of the gene.

In an analogous, indirect end-labeling experiment, DNase I digestion of rat thymus nuclei demonstrate that the thymus chromatin lacks DNase I hypersensitive sites in the 5′ region of the P-450c and P-450d genes (Figure 5). The lack of hypersensitive sites near these genes in the thymus indicates that, in the liver, the chromatin structure is altered in some manner so as to allow tissue-specific expression of the P-450 genes.

DNase I digestion is somewhat sequence-specific (Drew, 1984). However, this experiment and analogous experiments

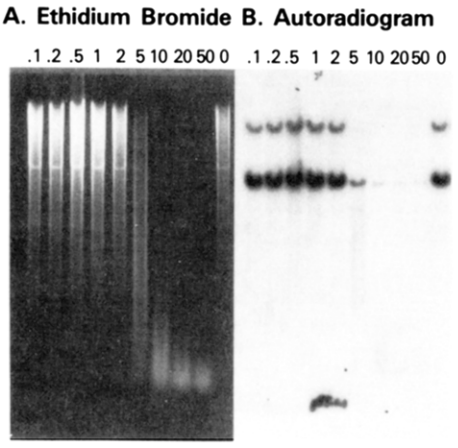

FIGURE 5: Lack of DNase I hypersensitive sites in the rat thymus P-450c and P-450d genes. The experiment is analogous to that presented in Figure 3 except that the nuclei were isolated from rat thymus. Note the absence of small molecular weight DNA fragments in the autoradiogram.

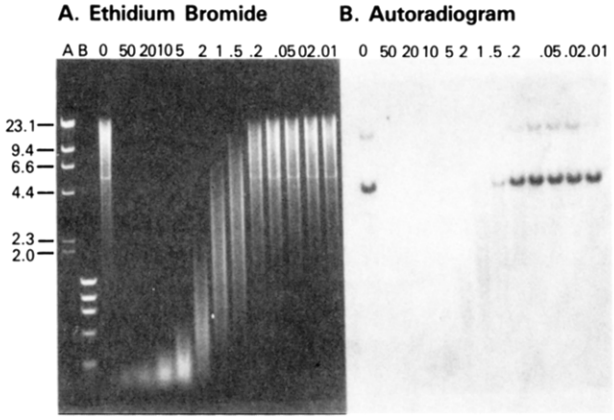

FIGURE 6: Lack of DNase I hypersensitive sites in the isolated rat liver DNA. Experiment done as in Figure 5 except purified DNA was first digested with DNase I and then with *Bam*H1, and the transfer was probed with pR5-1.1.

with purified rat DNA (Figure 6) indicate that the DNase I digestion pattern observed in the liver nuclei reflects the specific nucleoprotein structure of the genes rather than their DNA sequence.

Discussion

The function of the physiologically important cytochrome P-450 family of genes is related to their highly specific induction of gene expression. The question arises as to whether the specific induction of these genes is in any way correlated with observable changes in their chromatin structure. Several genes coding for members of the P-450 isoenzymes have been isolated and characterized (Adesnik & Atchinson, 1985; Nebert & Gonzalez, 1985). Thus far, however, only one study (Einck et al., 1985) involving a P-450 isoenzyme, which is constitutively expressed, addresses the question of gene induction and chromatin structure in this family of genes. The present study addresses this question in the highly inducible cytochrome P-450c and P-450d isoenzymes.

Micrococcal nuclease digestion of nuclei isolated from induced rats reveals that the chromatin structure of these genes are different from that displayed by the genome as a whole. The ethidium bromide stain of the DNA isolated from the supernatant and pellet of the digested nuclei reveals the expected 200 base pair nucleosomal ladder. The digestion was random since, as previously shown, probing the digest with a

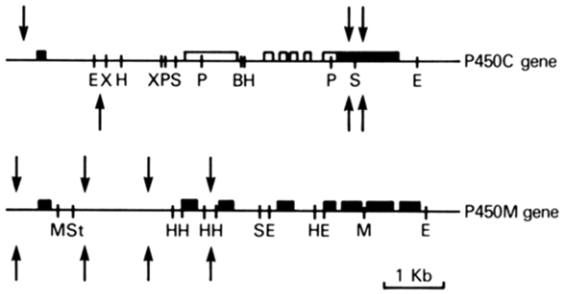

FIGURE 7: DNase I hypersensitive sites in control and 3-MC-induced P-450c and P-450m genes. The bars represent the gene, in a 5′ to 3′ orientation, left to right. Boxes indicate transcribed sequences. Arrows above the bars represent DNase I sites detected in nuclei isolated from rats treated with 3-MC. Arrows under the bars represent DNase I sites present in nuclei isolated from control, untreated rats.

plasmid containing highly repetitive rat DNA resulted in an autoradiograph which faithfully reproduced the ethidium bromide pattern (Einck et al., 1985). In contrast, the transcribed P-450c and P-450d genes, probed with plasmid p5-1.1, or the P-450c gene, probed with plasmid p210, is not organized in the same conformation. The nucleosomal pattern is not as well-defined as that observed by ethidium bromide staining and resembles a smear. In this respect, the highly inducible P-450c and P-450d genes resemble the constitutive P-450m gene (Einck et al., 1985), which also displays a nonnucleosomal conformation.

Nonnucleosomal DNA structure has been observed for other transcribed genes (Wu et al., 1979; Levy & Noll, 1981; Weischet et al., 1983; Bellard et al., 1982; Bloom & Anderson, 1982; Wu & Simpson, 1985). Rose and Garrard (1984) suggested that the irregularity in nucleosome spacing reflects a deficiency in histone H1 and an enrichment in certain nonhistone chromosomal proteins.

Induction of the P-450c and P-450d genes are associated with a reorganization of their nuclear locations. In the uninduced liver nuclei, P-450c and P-450d sequences are equally distributed among the three chromatin fractions, S1, S2, and P. In contrast, in the induced liver the DNA found in the P fraction is highly enriched in P-450c and P-450d sequences. Our previous studies indicate that the P fraction is also enriched in P-450m sequences. These studies suggest, but do not prove, an association between the nuclear matrix and transcriptionally active P-450 sequences.

The results presented here indicate that DNase I hypersensitive sites are present in the 3′ region of the P-450c gene and in the 5′ regions of both the P-450c and P-450d gene. These experiments also demonstrate that the induction of rat P-450c involves chromatin changes that alter the locations of a DNase I sensitive site. The probable positions of these sites, in control and in 3-MC-induced P-450c gene is diagrammed in Figure 7 and compared with the DNase I hypersensitive sites in the P-450m gene (Einck et al., 1985). In the uninduced P-450c gene, a hypersensitive site is detected 2.4 kb 5′ to the *Bam*H1 site, which is located 4010 bp from the 5′ of the genomic clone (see also Figure 1). Thus, this site is located approximately 1050 bp 3′ from the cap site at the start of exon I, i.e., within the first intron. In the induced nuclei this site is not detected and a new site 3.6 kb from the *Bam*H1 site appears. This site is approximately 160 bp 5′ to the cap site, near a sequence of 40 base pairs of alternating purines and pyrimidines.

Two DNase I hypersensitive sites, which are not affected by gene induction, are present in the 3′ region of the P-450c gene. Both sites are located in the DNA sequence coding for the trailer sequence of the mRNA. In the P-450m gene we

have detected (Einck et al., 1985) four hypersensitive sites in the 5′ portion of the gene. These sites are not affected by 3-MC treatment of the rats.

In the present studies, we have used nuclei isolated from liver because in this tissue the P-450 enzymes are active and highly inducible and because we wished to avoid potential artifacts associated with cells grown in culture. Our ability to detect hypersensitive sites in both the major 3-MC-induced P-450 and the P-450m genes in a tissue that contains high level of endogenous nucleases, suggest that these particular features of the chromatin are present in a substantial fraction of the liver cells.

In summary, our results suggest that the chromatin structure of the P-450 genes studied to date is organized into a transcribable conformation, perhaps associated with the nuclear matrix. Their DNA does not display the typical nucleosomal conformation, and hypersensitive sites are present in both the 5′ and 3′ region of the gene. Furthermore, in the thymus the hypersensitive sites are not present, providing additional support for the notion that transcription, as well as the transcriptional potential, of these genes is associated with a specific organization of their chromatin. However, there are some differences between the mechanism of gene regulation at the chromatin level between the P-450c and P-450m genes. The chromatin structure and the location of the hypersensitive sites in the P-450m gene does not change upon gene activation, suggesting that the gene induction is developmentally determined. This gene is expressed constitutively, and upon carcinogen treatment there is only a 4-fold increase in its mRNA synthesis (Fagan et al., 1982). In the highly 3-MC inducible P-450c gene however, induction is correlated with a distinct change in the location of a hypersensitive site, suggesting an induction-sensitive change to an active chromatin configuration similar to that reported in some other genes (Wu, 1980; Peterson, 1985; Burch & Weintraub, 1983; Nahon et al., 1984; Alevy et al., 1984). Because the 100-fold increase in the P-450c mRNA level is temporal, it would be interesting to know whether a decrease in mRNA levels subsequent to induction would also correlate with the pattern of DNase I hypersensitive sites.

The present paper is only the second study on the chromatin structure of a member of the cytochrome P-450 family of enzymes. Analysis of additional genes will be required to clarify the relation between chromatin structure and gene regulation in this family of enzymes.

ACKNOWLEDGMENTS

We thank D. West for performing the AHH assays and Drs. F. J. Gonzalez, D. Landsman, T. Srikantha, and Shawna C. Willey for suggestions and for critical reviews of the manuscript.

Registry No. Cytochrome P-450, 9035-51-2; 3-methylcholanthene, 56-49-5.

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Dithiothreitol Activation of the Insulin Receptor/Kinase Does Not Involve Subunit Dissociation of the Native $\alpha_2\beta_2$ Insulin Receptor Subunit Complex†

Laurel J. Sweet, Peter A. Wilden, and Jeffrey E. Pessin*
Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242
Received March 26, 1986; Revised Manuscript Received July 1, 1986

ABSTRACT: The subunit composition of the dithiothreitol- (DTT) activated insulin receptor/kinase was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and gel filtration chromatography under denaturing (0.1% SDS) or nondenaturing (0.1% Triton X-100) conditions. Pretreatment of ^{32}P-labeled insulin receptors with 50 mM DTT followed by gel filtration chromatography in 0.1% SDS demonstrated the dissociation of the $\alpha_2\beta_2$ insulin receptor complex (M_r 400 000) into the monomeric 95 000 β subunit. In contrast, pretreatment of the insulin receptors with 1-50 mM DTT followed by gel filtration chromatography in 0.1% Triton X-100 resulted in no apparent alteration in mobility compared to the untreated insulin receptors. Resolution of this complex by nonreducing SDS-polyacrylamide gel electrophoresis and autoradiography demonstrated the existence of the $\alpha_2\beta_2$ heterotetrameric complex with essentially no $\alpha\beta$ heterodimeric or free monomeric β subunit species present. This suggests that the insulin receptor can reoxidize into the M_r 400 000 complex after the removal of DTT by gel filtration chromatography. Surprisingly, these apparently reoxidized insulin receptors were also observed to be functional with respect to insulin binding, albeit with a 50% decrease in affinity for insulin and insulin stimulation of the β subunit autophosphorylation. To prevent reoxidation, the insulin receptors were pretreated with 50 mM DTT followed by incubation with excess *N*-ethylmaleimide prior to gel filtration chromatography in 0.1% Triton X-100. Under these conditions the insulin receptors migrated as the M_r 400 000 $\alpha_2\beta_2$ complex. However, when this insulin receptor complex was subjected to nonreducing SDS-polyacrylamide gel electrophoresis, subsequent to gel filtration, only the M_r 95 000 β subunit was detected. These results demonstrate that treatment of the insulin receptors with high concentrations of DTT, followed by removal of DTT by gel filtration, results in reoxidation of the reduced $\alpha_2\beta_2$ insulin receptor complex. Further, these results document that although the DTT stimulation of the insulin receptor/kinase does involve reduction of the insulin receptor subunits, it does not result in dissociation of the native $\alpha_2\beta_2$ insulin receptor subunit complex.

The effects of insulin on cellular metabolism are initiated by the specific binding of insulin to its cell surface receptor on target cells (Czech, 1977; Kahn et al., 1981). The insulin receptor is an integral membrane glycoprotein composed of two M_r 130 000 (α) and two M_r 95 000 (β) subunits covalently linked by disulfide bonds to form the native $\alpha_2\beta_2$ heterotetrameric complex [for reviews see Jacobs and Cuatracasas (1983) and Pessin et al. (1985)]. The complete primary amino acid sequence of the human placental insulin receptor precursor has been deduced from two full-length cDNA clones (Ebina et al., 1985; Ullrich et al., 1985). These data suggest that the insulin binding α subunit is exclusively located on the extracellular face of the plasma membrane and is anchored to the cell surface by disulfide bonds with the transmembrane β subunit. This prediction has been recently confirmed by the release of ^{125}I-insulin cross-linked α subunits from placenta membranes treated with dithiothreitol (DTT)[1] and urea (Grunfeld et al., 1985). The β subunit encodes the tyrosine kinase activity of the insulin receptor (Avruch et al., 1982; Kasuga et al., 1982a,b, 1983a,b; Petruzzelli et al., 1982, 1984; Tamura et al., 1983; Zick et al., 1983), containing both an ATP binding site and tyrosine phosphorylation acceptor sites (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983). Although extensive information has been accumulated regarding the structure of the insulin receptor, the mechanism by which insulin binding to the extracellular α subunit results in an intramolecular transmembrane signal that activates the intracellular β subunit kinase domain has not been elucidated.

Several reports have suggested that the oxidation state of the insulin receptor may play an important role in receptor structure and function. In particular, sulfhydryl reagents have been reported to inhibit insulin biological responsiveness and reducing agents have been observed to act as insulinomimetic

† This work was supported by Research Grants AM33823 and AM25295 from the National Institutes of Health. L.J.S. is supported by Research Fellowship AM07018 from the Diabetes and Endocrinology Research Center, The University of Iowa. J.E.P. is the recipient of a Research Development Award from the American Diabetes Association.
* Author to whom correspondence should be addressed.

[1] Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor.

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