Phenobarbital Induction of Cytochrome P-450 b,e Genes Is Dependent on Protein Synthesis

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Phenobarbital induces liver cytochrome P-450 b,e proteins mainly by increasing the rate of transcription of these genes. The mechanism responsible for the phenobarbital increment in the rate of transcription of cytochrome P-450 b,e genes is unknown. The objective of this study was to assess whether active protein synthesis was needed for phenobarbital to induce the liver cytochrome P-450 b,e genes. Cycloheximide (2 mg per kg, i.p.) was administered 90 min prior to a single inductive dose of phenobarbital (80 mg per kg, i.p.) and mRNAs measured at 3, 6 and 12 hr by dot-blot hybridization. While phenobarbital increased cytochrome P-450 b,e mRNAs about 12-fold at 3 hr, this induction was abolished by cycloheximide. To define whether the absence of protein synthesis in hepatocytes inhibited the phenobarbital induction of cytochrome P-450 b,e at the transcriptional level, in vitro transcription rates using isolated nuclei were measured. After phenobarbital administration, there was a 20-fold increment in transcriptional rate of cytochrome P-450 b,e genes. This increment was abolished by prior injection of cycloheximide. It is proposed that either preexisting regulatory proteins or transacting factors dependent on active protein synthesis participate in the regulation of liver cytochrome P-450 b,e gene transcription after phenobarbital.

Phenobarbital (PB) exerts several inductive effects on hepatocytes. Among them, the induction of various forms of cytochrome P-450 have elicited considerable interest (1). The molecular mechanism responsible for the induction of cytochrome P-450 b,e genes by PB, the two main components of the PB-inducible cytochrome P-450 family (1), has been defined as an increased rate of transcription of these genes in response to PB (2, 3). However, the induction of cytochrome P-450 proteins in response to PB occurs only in some hepatocytes. These hepatocytes are located in the distal half of the liver acinus (Zones 2 and 3), the microvascular unit of hepatic parenchyma (4–6). This heterogeneous induction of cytochrome P-450 proteins indicated that another intracellular level of regulation in the expression of these P-450 genes must occur. Moreover, recent studies using either isolated hepatocytes (7) or in situ hybridization (8) have shown that the levels of cytochrome P-450 b,e mRNAs were induced predominantly in hepatocytes of Zones 3 and 2 of the liver acinus, respectively. However, the various intracellular events responsible for the increment in transcription rates of cytochrome P-450 b,e genes in response to PB have not been defined.

In this study, the objective was to determine whether active protein synthesis in hepatocytes was necessary for PB to induce cytochrome P-450 b,e genes. Because cycloheximide inhibited the PB-mediated induction of cytochrome P-450 b,e mRNAs in these experiments, as well as the PB increment in transcription rates in nuclear run-off studies, we propose that protein synthesis is required for the PB-mediated induction of these genes.

MATERIALS AND METHODS

Induction of Cytochrome P-450 b,e Genes
Effect of Cycloheximide on the PB Induction of Cytochrome P-450 b,e mRNAs: Nonfasted Sprague-Dawley male rats weighing between 180 and 220 gm were used in all of these experiments. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was freshly prepared by dissolving it in 0.15 M NaCl and injected as a single dose of 2 mg per kg (i.p.). Four groups of three animals each were studied. (a) Controls were injected with 0.15 M NaCl and livers removed after 3, 6 and 12 hr. (c) Cycloheximide-PB-treated rats received cycloheximide (2 mg per kg, i.p.) followed 90 min later by PB. This experimental design assured that PB was injected at the time of maximal inhibition of protein synthesis by this dose of cycloheximide (9). Livers were removed at 3, 6 and 12 hr after PB administration. (d) Cycloheximide-treated rats received 2 mg per kg (i.p.) of cycloheximide only. Livers were removed after 3, 6 and 12 hr.

The NIH Guidelines for the care and treatment of laboratory animals were followed in this study.

Quantitation of Cytochrome P-450 b,e mRNAs
Extraction of RNA: Livers were frozen in liquid nitrogen and stored at −70°C for 1 to 2 weeks prior to RNA extraction. Preliminary studies showed that under these conditions, there is no loss of RNA nor is there significant degradation as assessed by Northern-blot analysis. RNA was extracted from livers of each group of animals according to Chirgwin et al. (10). Only samples with a 260/280 absorbance ratio above 1.8 were used.

Northern Blots: The size of the mRNA hybridizing to cytochrome P-450 b,e cDNA probes was assessed by Northern-
450 mRNA measurements were performed. Thus, albumin mRNA was assayed using the same RNA samples in which cytochrome P-450 genes were measured. Levels of albumin mRNA were determined as if both cytochromes were expressed on the same mRNA molecule. Due to the fact that mRNA hybridizes to the nitrocellulose paper was cut-off in each spot while back-ground radioactivity was assessed by counting a cut-off of the slopes of the lines obtained with each filter. After hybridization, the nitrocellulose paper in which RNA had not been spotted. Non-specific hybridization was assessed simultaneously. Accordingly, measurements have been expressed as cpm bound per microgram of RNA (11). Preparations of Isolated Nuclei: Nuclei were isolated according to Schibler et al. (16) with few modifications. Livers were homogenized in 20 volumes 0.3 M sucrose in buffer A [60 mM KCl, 15 mM NaCl, 0.15 mM spermin, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.5 mM EDTA, 2 mM MgCl₂, 15 mM Hepes (pH 7.5)]. The homogenate was filtered through nylon cloth (mesh width = 100 μm), layered over a 10 ml cushion of 30% sucrose in buffer A and spun for 10 min at 2,500 rpm in a SS 34 rotor (Sorvall). Crude nuclei were resuspended in 42 ml 2 M sucrose in buffer B (prepared as buffer A except that 0.1 mM EDTA and 0.1 mM MgCl₂ were used) and centrifuged at 36,000 rpm for 30 min at 4°C in an SW 41 rotor (Beckman L2-65B digitifuge). The clean nuclei were sedimented in nuclei storage buffer [20 mM Tris-HCl (pH 7.9), 75 mM NaCl, 0.5 mM Na₂EDTA, 0.85 mM dithiothreitol, 50% (v:v) glycerol]. The nuclear pellet was resuspended in nuclei storage buffer at a concentration of 10⁶ nuclei per μl of nuclei storage buffer. Nuclei were stored at -70°C for 24 hr before use. In Vitro Transcription Assay: The transcription reaction was performed according to Love et al. (17). The transcription reaction was initiated by adding 3 × 10⁶ nuclei (300 μl) to 300 μl of the transcription assay solution [ATP, GTP, CTP, 1 mM MgCl₂, 4.4 mM magnesium acetate, 1.7 mM MnCl₂, 7.15 mM Tris (pH 8.0); 1.43 M KCl; 500 μCi [α-³²P]UTP (400 Ci per mmole; Amersham Corp.). The final concentration of [α-³²P] UTP in the reaction was 2.08 μM. The assay was performed at 30°C for 30 min. The reaction was terminated by addition of 4 M guanidinium thiocyanate solution [50 mM NaOH, 5 M guanidinium thiocyanate, 130 mM β-mercaptoethanol, 2% sarcosyl, 10 mM EDTA (pH 8.0)]. The RNA transcripts were purified in a CsCl₂ gradient according to Love et al. (17). Assessment of Transcripts: The elongation of nascent RNA chains was measured by hybridization of the [³²P]RNA transcripts generated in the transcription reaction to nylon membranes (Nytphan®, Schleicher and Schuell) containing the cytochrome P-450 cDNA probe. In these experiments, the cytochrome P-450 cDNA was isolated from the R17 clone as previously described and cloned into the pGem-1 riboprobe. Northern-blot analysis (not shown here) showed that this pGem-1 containing the cytochrome P-450 cDNA hybridized to a mRNA of identical size as that hybridized by the cDNA insert (shown in Figure 1). The pGem-1-cytochrome P-450 b,e cDNA plasmid was linearized (HindIII), denatured in 0.5 N NaOH:1.5 M NaCl and 100 ng (concentration determined by measuring the 260 nm absorbance) blotted onto nylon membranes using a Minifold II apparatus. The method of hybridization used was that of Church and Gilbert (18). Hybridization was performed at 65°C for 18 hr. Each hybridization was performed using 4 ml hybridization buffer, a 6.2 × 2.8 cm nylon membrane and 1 × 10⁶ cpm per ml. At the end of the hybridization period, the blots were washed at high stringency in 0.1× SSC, 0.1% SDS for 1 hr at 65°C in a shaking water bath (65°C × 1 hr).
FIG. 1. Northern-blot analysis of cytochrome P-450 b,e mRNAs. (Lane 1) Control; 10 µg RNA loaded. (Lane 2) PB-induced; RNA extracted 16 hr after a single dose of PB (80 mg per kg, i.p.); 10 µg RNA loaded. (Lane 3) λ-size markers (HindIII digest).

hybridization, the filters were washed (18) and autoradiography performed. Background hybridization was assessed by using pGem-1 plasmid without the cDNA insert.

RESULTS

The effect of blocking protein synthesis at the translational level by cycloheximide on the increments in cytochromes P-450 b,e mRNAs by PB was studied. In these experiments, cycloheximide was injected (2 mg per kg, i.p.) followed 90 min later by the administration of PB (80 mg per kg, i.p.). It should be noted that maximal inhibition of protein synthesis (by at least 80%) has been observed with this dose of cycloheximide (9, 19). Figure

FIG. 2. Cytochrome P-450 b,e mRNAs. Effect of cycloheximide. (a) Dot-blot analysis. Controls = injected with 0.15 M NaCl; PB = PB-treated (80 mg per kg, i.p.); Cx-PB = cycloheximide (2 mg per kg, i.p.) followed 90 min later by PB (80 mg per kg, i.p.); Cx = cycloheximide (2 mg per kg, i.p.). Numbers at the right-hand side of the abbreviation for each group indicate the time interval (in hours) between the injection and the removal of the liver. In Cx-PB, it indicates time after PB administration. At the bottom, the amount of RNA spotted in each slot is indicated. Each sample was analyzed in six different concentrations. The raw data obtained after 6 hr are not shown. (b) The same data calculated as cpm DNA bound per microgram of RNA spotted. Mean ± S.E. of three animals per group. Livers were removed after 3, 6 and 12 hr of the PB injection. Dotted bars = cytochrome P-450 b,e mRNAs after PB administration. Solid bars = cytochrome P-450 b,e mRNAs after PB injection preceded, 90 min earlier, by cycloheximide administration.
at 3 and 12 hr is shown. An entirely similar resolution was obtained with all samples analyzed. Quantitation of dot-bLOTS is presented in Figure 2b. In this figure, results have been expressed as cpm per microgram of RNA spotted. When PB was injected alone, the levels of cytochromes P-450 b,e mRNAs increased about 10-fold at 3 hr and about 30-fold by 12 hr. Cycloheximide injected 90 min before PB administration abolished the induction of the cytochrome P-450 b,e mRNAs. Cycloheximide was injected alone in two experiments, and RNA was extracted at 3, 6, and 12 hr. The levels of cytochrome P-450 b,e mRNAs were low (results not shown) and apparently lower than in controls. However, given the low levels of expression of the cytochrome P-450 b,e genes in controls, it became apparent that many more experiments would be needed to ascertain whether the constitutive expression of these genes was influenced by cycloheximide. This was not the purpose of these studies. To assess the specificity of these results, the effects of cycloheximide on the levels of albumin mRNA were determined using the same RNA in which cytochrome P-450 was measured. Figure 3 shows that albumin mRNA levels were not influenced by cycloheximide administration at the time intervals studied.

To assess whether this inhibitory effect of cycloheximide on cytochrome P-450 b,e induction by PB was exerted at the level of the transcription of these genes, in vitro transcription assays were performed. Figure 4 shows the autoradiography of a representative experiment involving the measurements of in vitro transcription in each of the four groups (control, cycloheximide, cycloheximide-PB and PB alone). The hybridization signals of controls and after cycloheximide alone were faint. In contrast, 3 hr after the administration of PB, there was about a 20-fold increment in the rate of in vitro transcription of the cytochrome P-450 b,e genes. Cycloheximide, injected 90 min before PB, inhibited the PB-induced increment in the rate of in vitro transcription. A total of five experiments measuring in vitro transcription in each of the four groups studied were performed. All experiments showed similar results. Cycloheximide always blocked the induction of cytochrome P-450 b,e genes by PB.

**DISCUSSION**

The aim of this study was to determine whether active protein synthesis in hepatocytes was required for the induction of cytochrome P-450 b,e genes in response to PB. This represents an initial approach to elucidate the molecular mechanisms leading to the transcriptional induction of these genes.

Cycloheximide has been used in several studies in an attempt to answer whether the rate of protein synthesis influences the expression or the induction of genes (19-23). The possibility that cycloheximide may indiscriminately depress gene activity by decreasing RNA polymerase II is unlikely. Following cycloheximide administration, measurements of RNA polymerase II activity have indicated that the activity of this enzyme is increased (20). Moreover, induction of certain genes to levels higher than those observed with the inducer alone or "superinduction" has been reported after cycloheximide administration (21). Such an example is the superinduction of cytochrome P-450 (representative of a different family of P-450 than P-450 b-e) in the presence of cycloheximide and the inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin in mouse hepatoma cells (21). Under these conditions, the induction of the P-450 gene is 10-fold higher than in the presence of the inducer alone. Therefore, the effect of cycloheximide on gene expression appears to be selective and, accordingly, the regulation of genes can be separated into those dependent on, or independent of, protein synthesis (19, 22-23). Moreover, similar responses of genes have been observed when other agents blocking protein synthesis at a translational level, such as emetine, have been used (19). These observations suggest that, in these experiments, cycloheximide was acting in its capacity to inhibit protein synthesis rather than in a nonspecific manner.

In this study, PB was injected in vivo at the time of maximal inhibition of protein synthesis by cycloheximide (9, 19). Cycloheximide blocked the induction of cytochrome P-450 b,e mRNAs by PB. In contrast, the levels of albumin mRNA were not affected within the time interval of these experiments. The mechanism of this apparent difference in the response of cytochrome P-450 b,e genes and of the albumin gene to cycloheximide needs further study.

The nuclear run-off studies indicated that the molecular mechanism responsible for the inhibition of the PB-mediated induction of cytochromes P-450 b,e genes is the inability of PB to increase the transcription rates of these genes in the absence of active protein synthesis in hepatocytes. These observations suggest that a protein(s), either present at the time of PB administration or synthesized in response to PB, modulates the induction of the cytochrome P-450 b,e genes by activating (or derepressing) the rate of transcription of these genes.
we propose that transacting regulatory proteins participate in the modulation of the rate of transcription of the liver cytochrome P-450 b,e genes in response to PB administration.

REFERENCES