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Aryl Hydrocarbon Induction of Rat Cytochrome P-450d Results from Increased Precursor RNA Processing

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We have previously demonstrated that cytochrome P-450d mRNA accumulation is induced at a posttranscriptional level by 3-methylcholanthrene (MCA) in primary cultures of rat hepatocytes grown in serum-free hormonally defined medium. Using dactinomycin chase experiments in this culture system, we found that MCA had no effect on the P-450d mRNA half-life. In addition, induction of P-450d occurred both in the presence and in the absence of protein synthesis inhibitors. An analysis of nuclear precursors showed that the accumulation of the primary transcript of the P-450d gene was induced to the same extent as that of the mature mRNA after MCA treatment and that the pattern of accumulation of precursors differed between treated and control liver cells. Since P-450d induction is thought to be a receptor-mediated event, these data are consistent with a model in which a direct interaction occurs between the receptor-ligand complex and the primary transcript.

Exposure of most organisms to environmental pollutants results in the induction of sets of genes whose products act to metabolize these compounds. In rat liver exposed to aryl hydrocarbons such as 3-methylcholanthrene (MCA), β-naphthaflovone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the hydrocarbon-metabolizing enzymes cytochromes P-450c and P-450d are the major induced proteins (12, 13). It has been determined that transcriptional regulation mediated by the cytosolic Ah receptor is primarily responsible for the hydrocarbon-induced accumulation of P-450c and its mouse homolog cytochrome P_1-450 in intact liver (14, 26). However, it has been demonstrated that the Ah receptor-dependent 30-fold induction of P-450d mRNA and its mouse homolog P_3-450 is mediated primarily at a posttranscriptional level (10, 18, 19). We and others (15, 18, 19) have also examined the MCA induction of the P-450c and P-450d genes in primary rat hepatocyte cultures grown in defined medium and have shown that both genes appear to be mainly posttranscriptionally regulated. Thus, the regulation of P-450d in cultured cells is identical with its regulation in liver cells, whereas P-450c loses much of its transcriptional inducibility and becomes regulated at a posttranscriptional step.

To define the mechanism responsible for the hydrocarbon induction of P-450d mRNA, we have analyzed (i) the degradation rate, (ii) the relationship between protein synthesis and MCA induction, and (iii) the accumulation of nuclear mRNA processing intermediates in the presence and absence of MCA. These results show that the Ah receptor-mediated induction of P-450d mRNA is effected at an early step of processing of the nuclear precursor mRNA, perhaps at the process-versus-discard decision point, and not at the level of mature cytoplasmic mRNA turnover. Moreover, the inhibition of protein synthesis has no significant effect on the induction process, suggesting that the Ah receptor, which has been shown to enter the nucleus after hydrocarbon binding (22, 26), may act directly on an early event in the mRNA synthesis of P-450d.

Degradation of P-450d mRNA is unaffected by MCA treatment. We have previously established that the MCA-induced ~30-fold increase in abundance of P-450d mRNA in both intact rat liver and primary hepatocyte culture is due primarily to posttranscriptional regulation (18, 19). In addition, the induction in primary liver cell cultures is enhanced by the presence of dexamethasone in the growth medium (19). To determine whether MCA-induced changes in mRNA turnover rates could account for the induction, we performed dactinomycin chase experiments to directly measure mRNA half-life. Primary rat hepatocytes were prepared from collagenase-perfused Sprague-Dawley liver cells as described previously (19). Hepatocyte cultures were grown overnight in hormonally defined medium (HDM) in the presence or absence of MCA in order to induce high levels of P-450d (18, 19). The medium was then replaced with HDM containing either MCA or its solvent, dimethyl sulfoxide, and also containing a concentration of dactinomycin sufficient to inhibit greater than 98% of RNA synthesis (data not shown). The abundance of P-450d mRNA was then measured as a function of time after addition of dactinomycin. Under these conditions, the degradation rate of the mRNA is a measure of the mRNA half-life.

P-450d mRNA was extremely stable in primary rat hepatocyte cultures, with a $t_{1/2}$ of >18 h in either the presence or the absence of MCA (Fig. 1). Given that the ~30-fold induction of this mRNA occurred within 8 h of MCA treatment and without a transcriptional change, this result shows that induction of accumulation of P-450d must occur at a step prior to mRNA degradation. As controls of the effectiveness of the dactinomycin chase, the levels of abundance of the mRNAs encoding α_1-protease inhibitor (α_1-AT) and tyrosine aminotransferase (TAT) were determined. α_1-AT has been shown to be extremely stable in primary hepatocyte cultures (1), and TAT is known to have a relatively short $t_{1/2}$. Each of these mRNAs behaved as expected, with no apparent effects of MCA (Fig. 1). The fact that the abundance of at least one mRNA species did not change significantly during the 18-h time course implies that wholesale cell death due to dactinomycin treatment did not occur.

Since we have shown previously that dexamethasone has important effects on P-450d accumulation in the presence of MCA, we also measured the effect of the removal of dexamethasone from the HDM upon the $t_{1/2}$ of P-450d mRNA. Although removal of dexamethasone results in a reduction of

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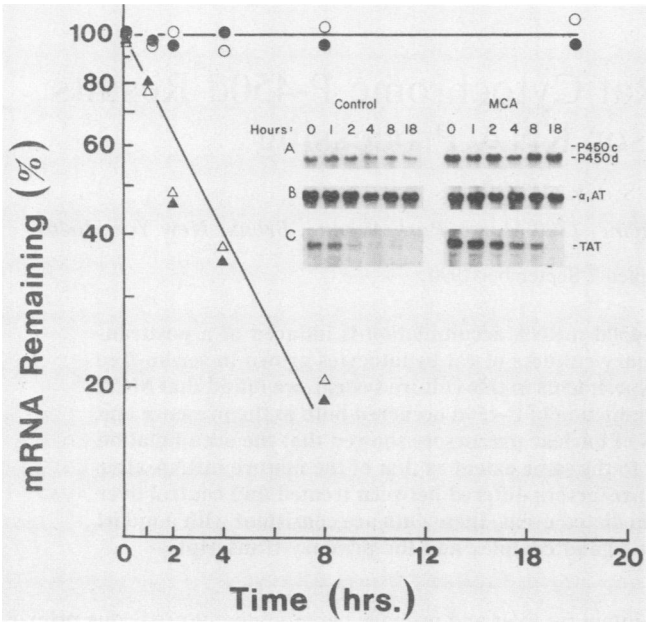

FIG. 1. Effect of MCA on P-450d mRNA half-life. One-day-old primary hepatocyte cultures were grown for 18 h in HDM with or without MCA (1 μg/ml). The medium was then replaced with identical medium supplemented with dactinomycin (10 μg/ml) dissolved in ethanol. The final ethanol concentration was 0.2%. RNA was extracted after 0, 1, 2, 4, 8, and 18 h of incubation and quantitated as described previously (18, 19). Northern blots were hybridized to riboprobes corresponding to a partial cDNA clone of mouse P_3-450 which readily hybridizes to P-450c and P-450d (inset, row A) (18, 19), a mouse α_1-AT cDNA clone (inset, row B) (3, 11), and a mouse TAT cDNA clone (17). The control P-450 panel was exposed for 72 h; the MCA P-450 panel was exposed for 5 h. All other autoradiograms were equally exposed. Quantitation was performed by densitometric scanning of appropriate exposures of the autoradiograms shown in the inset. P-450d expression was normalized to the levels of expression of α_1-AT, which is unaffected by MCA treatment. Symbols: ●, P-450d mRNA, control; ○, P-450d mRNA, MCA treated; ▲, TAT mRNA, control; △, TAT mRNA, MCA treated.

MCA-induced accumulation of P-450d mRNA to about one-fourth the level observed in cells grown in the presence of dexamethasone (19), the $t_{1/2}$ of the mRNA is unaffected by its presence (data not shown). Therefore, the effect of dexamethasone on MCA induction of P-450d must also occur at a step prior to mRNA degradation but after transcriptional initiation.

Induction of P-450d mRNA does not require protein synthesis. Since a posttranscriptional level of control appeared to be the regulated step in P-450d induction, it was important to determine whether it was a primary event associated with MCA treatment or was a secondary effect associated with induction of a primary gene product. Therefore, MCA effects were measured in the presence of inhibitors of protein synthesis. One-day-old cultures were preincubated with a concentration of cycloheximide sufficient to inhibit >98% of protein synthesis and then induced with MCA. Induction of P-450d was apparent within 2 h of MCA treatment, reaching maximum levels of accumulation by 8 h either in the presence or in the absence of cycloheximide (Fig. 2; Table 1). Thus, neither the extent nor the kinetics of induction of P-450d by MCA was affected by the inhibition of protein synthesis.

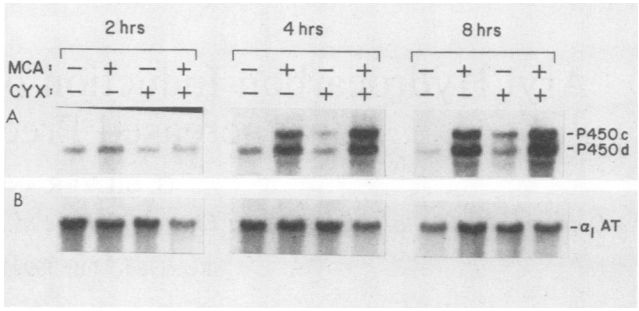

FIG. 2. Effect of protein synthesis inhibition on P-450 expression. One-day-old cultures were incubated with (+) and without (−) cycloheximide (CYX; 10 μg/ml) for 30 min. Cultures were then treated with fresh HDM with or without cycloheximide and either MCA (1 μg/ml in dimethyl sulfoxide) or dimethyl sulfoxide. RNA was extracted after 2, 4, and 8 h, and P-450d was quantitated as described for Fig. 1. Cultures with no cycloheximide and no MCA are identical to zero-time-point cultures. Row A, Hybridization to P-450c and P-450d; row B, hybridization to α_1-AT mRNA.

It is interesting that the level of expression of the P-450c mRNA in the absence of MCA was increased by cycloheximide treatment (Fig. 2; Table 1). It has been shown that inhibition of protein synthesis followed by hydrocarbon treatment results in the superinduction of mouse P_1-450 at the transcriptional level in Hepa I mouse hepatoma cells (8). This effect has been hypothesized to be due to the rapid turnover of a negative regulator of transcription of the P_1-450 gene. We found that cycloheximide, even in the absence of MCA, appeared to induce expression of the normally silent rat P-450c gene and that final levels of induction were comparable to those in MCA alone. This result is consistent with previous work by Teifeld et al. (21), which demonstrated a lower extent of superinduction of P-450 in primary hepatocyte cultures treated with cycloheximide plus hydrocarbon than in hepatoma cell lines. These observations provide further evidence for a significant difference between the mechanisms of regulation of the P-450c and P-450d genes. Although both genes appear to require an interaction with the Ah receptor molecule, clearly P-450d is induced at a posttranscriptional step prior to mRNA turnover, while P-450c is regulated primarily at a transcriptional level.

Hydrocarbon affects an early step in mRNA processing. To better identify which posttranscriptional process in P-450d mRNA synthesis is regulated by MCA treatment, we used intron-specific hybridization probes to determine the levels

TABLE 1. Effect of cycloheximide on relative levels of accumulation of P-450 mRNA

Treatment	Relative level of accumulation[a]					
	2 h		4 h		8 h	
	P-450c	P-450d	P-450c	P-450d	P-450c	P-450d
MCA	4.0	3.2	18.7	5.7	40.6	24.8
Cycloheximide	1.5	0.91	3.9	0.8	20.7	1.3
Cycloheximide + MCA	4.0	2.9	19.1	6.4	43.8	26.3

[a] Data were obtained by densitometric scanning of Northern blots shown in Fig. 2 as previously described (18, 19) and represent averages of two independent experiments. Similar data were obtained in at least three independent experiments. Values are expressed as [level expressed (induced)/level of α_1-antitrypsin (induced)]/[level expressed (uninduced)/level of α_1-antitrypsin (uninduced)].

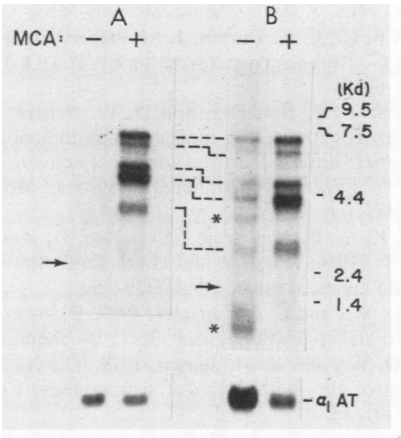

FIG. 3. Northern analysis of P-450d intron 1-containing cellular RNA. Polyadenylated RNA from MCA-treated (+) and corn oil-treated (−) rat liver was hybridized to the P-450d intron 1-specific riboprobe (P_3-450 intron 1 bases 1385 to 1751; 82% identity with P-450d intron 1) and then erased and reprobed with the α_1-AT probe loading control shown at the bottom. (A) A 5-μg sample of each RNA was analyzed on a 1.5% agarose-formaldehyde gel. (B) Either 35 (−) or 5 (+) μg of poly(A)$^+$ RNA was analyzed on a 1.2% agarose-formaldehyde gel. To enhance the signal obtained with the control RNA, the − lane was subjected to 96 h of autoradiography and the + lane was subjected to 18 h of autoradiography. The loading controls (α_1-AT) for both lanes were exposed for equal times. Arrows show the positions of mature P-450d cytoplasmic mRNA; asterisks show the positions of RNAs unique to the control sample. Kd, Kilodaltons.

of accumulation of nuclear pre-mRNA molecules in treated and untreated cells. This analysis would permit us to determine whether nuclear RNA accumulated to the same extent as cytoplasmic mRNA and whether there are qualitative differences in accumulated splicing products between treated and untreated cells. For example, if the MCA induction of P-450d were mediated by a nuclear decision to process rather than discard the majority of gene transcripts, then induction would likely lead to the nuclear accumulation of the major primary transcription product.

The levels of accumulation of P-450d mRNA precursors were measured in MCA-treated and control cells by Northern (RNA) analysis of total cellular polyadenylated RNA isolated from intact rat liver. Although quantitatively and qualitatively similar data were obtained when RNA from primary cell cultures was used (data not shown), adult rat liver was used because of the difficulty of isolating the large amounts of pre-mRNA needed to analyze the nuclear precursors in the untreated animals (i.e., 35 μg per lane; Fig. 3). The blots were probed with both intron 1 and intron 5 probes, which hybridize to precursor molecules and not to fully spliced cytoplasmic mRNA. The intron 1 probe detected RNAs of 6.9, 6.1, 5.0, 4.3, and 3.4 kb in MCA-treated cells (Fig. 3A), while the intron 5 probe detected only the 6.9-kb RNA (data not shown). Since the DNA sequence of the gene predicts the size of the primary transcript to be 6.9 kb (20), the largest detected precursor is likely to be the primary transcript. It is likely that the other, shorter fragments correspond to processing intermediates which retain intron 1 and that intron 5 is usually spliced out of the transcript soon after processing begins. The most significant result seen in a comparison of the accumulation of precursors in MCA-treated and untreated cells is that there appeared to be ~30-fold induction of most of the detected precursors in MCA-treated cells, including the primary transcript, despite the absence of a significant transcriptional induction of the gene. The simplest interpretation of this result is that MCA treatment results in the stabilization of the nuclear precursors of P-450d mRNA and that the increased levels of nuclear RNA can be properly processed to mature mRNA.

MCA induces alterations in the pattern of nuclear precursor accumulation. Careful examination of the accumulation of precursors detected with the intron 1 probe shown in Fig. 3 reveals subtle differences between the MCA-induced and uninduced patterns. To more carefully compare these patterns, higher-resolution Northern analyses comparing the induced and uninduced patterns were performed. Clear differences in the accumulation of intron 1-containing precursors were induced by MCA treatment (Fig. 3B; note the significant differences in gel loading and film exposure). While a 4.3-kb precursor was a major accumulated P-450d product in MCA-treated cells, this band was greatly reduced in untreated cells, which constitutively expressed P-450d. In addition, the untreated cells produced RNAs of 4.0 and 1.2 kb in relatively high abundance (asterisks in Fig. 3). It is worth noting that the 1.2-kb species is significantly smaller than the mature mRNA and may be indicative of degraded RNA or aberrantly spliced mRNA. These data show a clear difference in accumulation patterns between treated and untreated cells and imply either that a difference in the order of intron sequence removal is induced by MCA treatment or that aberrant splicing of the primary transcripts occurs in untreated cells.

Regulation of mRNA accumulation at the level of RNA processing efficiency has been suggested for only a small number of genes, and in no case has this level of control been directly demonstrated. The work described here shows clearly that the ~30-fold induction of cytochrome P-450d mRNA by MCA in primary rat hepatocyte cultures must occur at this level of gene control. Our analysis of the induction process also demonstrated that MCA induction of P-450d occurred both in the presence and in the absence of new protein synthesis. If indeed the Ah receptor is involved in the P-450d induction, this result is consistent with the hypothesis that the receptor acts directly on the P-450d gene or primary transcript rather than through the induction of expression of a second protein which in turn acts to stabilize P-450d nuclear RNA. The DNA-binding activity of the Ah receptor-ligand complex is now clearly documented (2, 4, 6, 9). Despite considerable effort, however, little evidence for an Ah receptor-binding site upstream or within the P-450d gene has been elaborated (7). Thus, our data suggest a novel function for the receptor.

It is interesting that the fivefold potentiation of MCA-induced P-450d accumulation by dexamethasone is also mediated at a step in the posttranscriptional processing of the RNA prior to its final appearance in the cytoplasm. Dexamethasone has been previously shown to affect the stability of some mRNAs (23, 24) or enhance their transcription (5). However, its effect on the posttranscriptional control of P-450d is unusual in that dexamethasone alone has no effect on mRNA accumulation and enhances a nuclear processing step in P-450d mRNA formation. In this regard, it recently has been shown that the dexamethasone enhancement of phenobarbital-induced P-450b and P-450e mRNA levels in rat liver cells also appears to be regulated at a posttranscriptional step (16). In our system, it is possible that dexamethasone enhances the ability of the Ah receptor-ligand complex to interact with its P-450d target or perhaps

simply increases the level of Ah receptor in the cell. Quantitative analysis of the Ah receptor, however, is hampered by a lack of specific reagents for identifying either the protein or the mRNA encoding the receptor (25). Specifically, the lack of an Ah receptor-specific antibody precludes the study of the subcellular localization of the receptor and potential changes induced by the carcinogen.

On the basis of our experiments, we cannot determine the precise step in RNA processing that is affected by MCA. Our working hypothesis is that the Ah receptor-ligand complex interacts directly with either the P-450d gene or the P-450d primary transcript. This interaction stabilizes the primary transcript, perhaps through interaction with the splicing apparatus, and may enhance some aspect of RNA processing such as 3'-end formation or splicing. In the case of a receptor-DNA interaction, this may affect polyadenylation efficiency; in the case of a receptor-RNA interaction, it may induce a more favorable structure for efficient splicing or result in protection of the molecule from rapid nuclear degradation. In either case, the result would be higher levels of accumulation of P-450d precursor RNAs. The role of the alternate patterns of accumulation of mRNA precursors in induced or uninduced cells is obscure but may reflect either high levels of P-450d mRNA precursors or alternate splicing patterns induced by the binding of an Ah receptor-RNA complex. We are currently using reverse genetics approaches to determine the *cis*- and *trans*-acting determinants of the MCA response for the P-450d gene.

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