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Novel Target Genes of the Ah (Dioxin) Receptor: Transcriptional Induction of *N-Myristoyltransferase 2*[1]

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ABSTRACT

Dioxins are potent mammalian carcinogens and toxins affecting liver, skin, and immune and reproductive systems. The intracellular Ah receptor, a ligand-dependent transcription factor of the basic region/helix-loop-helix/Per-Ahr/Arnt-Sim homology domain (bHLH-PAS) protein family, mediates responses to dioxins. Target genes of the Ah receptor that mediate dioxin toxicity and carcinogenicity are, however, mostly unknown. We used 5L rat hepatoma cells to identify dioxin-inducible genes by suppression subtractive hybridization. Eleven cDNA fragments were identified that represented novel sequences or genes for which induction by dioxins had not been known. *N-myristoyltransferase 2* (*NMT2*) is one of the later dioxin-inducible genes. Induction of *NMT2* was confirmed in livers of mice *in vivo*. *NMT2* induction was a direct consequence of Ah receptor activation in 5L cells. [^{3}H]myristic acid incorporation into 5L cell proteins was inducible by dioxins, indicating that protein myristoylation is a regulated rather than a housekeeping function and that NMT activity is limiting in noninduced 5L cells. Here we show for the first time that expression of *NMT2* and induced protein myristoyltransferase activity are direct responses to carcinogen exposure. Because inappropriate protein NH_2-terminal myristoylation appears to play a role in carcinogenesis, induction of *NMT2* may play a central role in dioxin carcinogenicity.

INTRODUCTION

Dioxins like TCDD[5] evoke a broad spectrum of biochemical and toxic effects in a variety of species including immune suppression, skin alterations, sperm count reduction, body weight loss, and carcinogenicity in the rodent liver as well as in human (1, 2). Dioxin effects are mediated by the AhR, which is a member of the basic region/helix-loop-helix/Per-AhR/Arnt-Sim homology domain (bHLH-PAS) family of transcription factors (3, 4). The AhR functions as a ligand-activated transcription factor. Binding of dioxins to AhR triggers formation of dimeric complexes with the partner protein, Arnt. The AhR/Arnt heterodimer binds to recognition sites in the promoters of target genes and enhances their transcription. Known target genes that have served as model cases for the analysis of AhR function mostly comprise genes involved in xenobiotica metabolism, such as members of the *cytochrome P-450 I* subfamily and enzymes of phase II metabolism (5–7). Genetic evidence in mice (8–10) indicate that most, if not all, activities of dioxins are mediated by the AhR, but there are indications that not all of these activities may require Arnt (11, 12).

Despite the recognition that dioxins exert their biological functions by binding to AhR, mechanisms of dioxin toxicity and carcinogenicity are not well understood because only a few target genes other than those related to xenobiotica metabolism are known. For those target genes that are known, the relevance for dioxin toxicity and carcinogenicity is unclear. Recently, we reported that AhR directly up-regulates the expression of the cell cycle inhibitor *Kip1* and, by that, inhibits cell proliferation both in 5L hepatoma cells and in fetal thymus glands (12). However, AhR target genes that mediate other aspects of toxicity and carcinogenicity in particular are poorly understood. It is likely that the genetic responses to AhR activation are complex and that, in addition to the known dioxin-regulated genes, there are other target genes of AhR that ultimately support tumorigenic transformation of cells in the presence of dioxins. Cell culture models *in vitro* may be of limited value in the study of the process of carcinogenesis as a whole but may provide useful models to identify target genes immediately downstream of the carcinogen-activated AhR.

Several attempts have been made to systematically identify changes in gene expression after TCDD exposure by differential RNA expression screening approaches. Sutter *et al.* cloned several TCDD-inducible cDNAs, including those for tissue plasminogen activator inhibitor 2 and interleukin 1β (13). Gao *et al.* found ecto-ATPase induced by dioxins (14). Selmin *et al.* as well as Donat and Abel reported (15, 16) induction of several genes using the differential display technique. Induction of these genes provides evidence that TCDD alters expression of many genes in different cells/organs, but obvious explanations for most of the toxic activities of dioxins and tumor promotion in particular have not been found.

Thus, there is considerable interest in identifying AhR-regulated genes by technologies that are suitable for detecting all or at least most of the dioxin-induced alterations in mRNA expression, including those concerning low-abundance mRNA species. In this study, we used the dioxin-responsive 5L hepatoma cell line (17–20) to identify AhR target genes by SSH (21, 22). SSH was used to take advantage of a normalization step increasing the relative representation of low-abundance mRNAs; and SSH was preferred over screening of cDNA arrays to allow identification of novel genes. Six known TCDD-inducible genes were found together with 11 novel cDNA fragments. We show that *NMT2* is one of the genes induced by TCDD in 5L cells and in the mouse. At least in 5L cells, the induction was direct and strictly depended on AhR. The increase in *NMT2* mRNA lead to increased protein myristoylation in pulse-labeled 5L cells, providing the first example of protein myristoylation being susceptible to short-term regulation by a chemical carcinogen.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in DMEM supplemented with 10% fetal bovine serum as described previously (12). TCDD was added as 1 μM stock solution in DMSO to a final concentration of 1 nM and control cells were treated with 0.1% DMSO.

Northern and Southern Blot Analyses. Northern blot analysis was done using 5 μg of poly(A)$^+$ RNA. Probes were prepared by PCR or restriction digestion from individual clones as described previously (21, 22). cDNA samples (10 ng each) were labeled in random prime reactions (Amersham) with [^{32}P]dCTP (3000 Ci/mmol), and probes were purified by size exclusion

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[5] Abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Ah, aryl hydrocarbon; AhR, Ah receptor; NMT, *N*-myristoyltransferase; SSH, suppression subtractive hybridization; Arnt, Ahr nuclear translocator; poly(A) RNA, polyadenylated RNA; GAPDH, glyceraldehyde-3-phosphate dyhydrogenase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag.

chromatography (Nick columns, Pharmacia Biotech). Incorporation of radioactivity was determined by liquid scintillation counting. Only probes that had incorporated between 15 and 30 μCi of ^{32}P were used for hybridization reactions. RNA loading was controlled by hybridization with a GAPDH probe. Southern blot analysis was done as described previously (23).

Generation of a Subtracted cDNA Library by SSH. SSH was performed between nontreated 5L cells (driver) and 4-h-TCDD-treated 5L cells (tester), using the PCR-Select cDNA Subtraction kit (Clontech Laboratories, Inc.). All of the PCR and hybridization steps were performed on a Perkin-Elmer 9600 thermal cycler. Double-stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA of the non-treated or the 4-h-TCDD-treated cells as described previously (23). Both double-stranded cDNAs were then *Rsa*I-digested, precipitated, and redissolved in freshly autoclaved water. Tester cDNA was ligated with adaptors (21). The ligation efficiency was verified by comparing PCR products obtained with gene-specific primers for *GAPDH* or one *GAPDH*-specific primer and another adaptor-specific primer. The difference between the intensities of the above PCR reactions was less than 2-fold. For the first hybridization the mixture of driver and tester cDNAs was denatured at 98°C for 90 s and then incubated at 68°C for 11 h. For the second hybridization, fresh driver cDNA was denatured at 98°C for 90 s; then the samples from the first hybridization were mixed together without denaturing in the presence of freshly denatured driver cDNA and were incubated at 68°C for 20 h. At the end of the incubation period, 200 μl of dilution buffer [20 mM HEPES-HCl (pH 8.3), 50 mM NaCl and 0.2 mM EDTA] were added. Differentially expressed cDNAs were selectively amplified in two rounds of PCR as described previously (21).

Colony PCR. To identify TCDD-induced genes, individual clones from the subtractive library were picked, and the inserts were amplified by colony PCR. DNA was denatured with alkali treatment, and duplicate sets of dot blots were prepared in 96-well-format. These dot blots were then hybridized with radioactively labeled double-stranded cDNA derived from either nontreated or 4-h-TCDD-treated 5L cells.

RACE. A commercially available adaptor ligated double-stranded cDNA (Marathon-Ready cDNA from Clontech Laboratories, Inc.) was used as a template. PCR-amplification of 3′ and 5′ regions, extending the novel clone 106, was performed using one adapter and one clone 106-specific primer, each in separate PCR reactions. Primers used were 5′-gcccaagtctgtgaaactacttgatttcc and 5′-gacccagcggtggagtagtgctgtc. Products were sublconed, sequenced, and compared with the database.

RT-PCR Analysis of Mouse Liver Samples. RT-PCR analysis was performed using first-strand cDNA reversely transcribed from 0.1 μg of total liver RNA by oligo(dT) priming and *NMT2*-pecific primers (clone 106 primers, see above) and *GAPDH*-specific primers, 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′ in a single-tube hot-start PCR reaction with the following conditions: 25 cycles for 30 s at 94°C, 45 s at 58°C, and 90 s at 72°C. Pilot experiments had confirmed that the PCR amplification of both *NMT2* and *GAPDH* was in the linear range. Five μl of the PCR products were separated on an agarose gel, and Southern blot analysis was performed with radiolabeled *GAPDH*- and *NMT2*-specific probes. A phosphorimager (FUJIX BAS 2000) was used for quantitative analysis.

***In Vivo* Labeling of Cells.** 5L cells were treated with 1 nM TCDD or 0.1% DMSO solvent for 16 h or 48 h and were then incubated with [^{3}H]myristic acid (50 μCi/ml) for an additional 3, 5, 8, or 12 h. Cell extracts were prepared in SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE, together with radioactively labeled and prestained size markers (Amersham). Autoradiography was performed using the Amplify fluorographic reagent (Amersham Buchler, Braunschweig, Germany). Extracts for immune precipitations were prepared in standard RIPA buffer (PBS, 7% NP40, 0.5% sodium deoxycholate, and 0.1% SDS). Antibodies against Src were Ab-1 (Calbiochem) or sc-18 (Santa Cruz Biotechnology). Against Src-like kinases, we used: Blk (sc-329); Fgr (sc-17); Hck (sc-1428); Lck (sc-433); Lyn (sc-7274; all from Santa Cruz Biotechnology).

RESULTS

Generation of a cDNA Library Enriched for TCDD-induced Genes. Poly(A)$^+$ RNA from nontreated and 4-h-TCDD-treated 5L cells was used for generation of a subtractive cDNA library (TCDD-treated minus nontreated) using the PCR-Select cDNA subtraction method (21, 22). This procedure also normalizes between low and highly abundantly expressed mRNAs allowing for a reasonable probability to also recover low-abundance transcripts from the generated library. Northern hybridizations confirmed the presence and inducibility of known TCDD-induced transcripts such as cytochrome P450 1A1 *(CYP450 1A1)* and glutathione-*S*-transferase Ya *(GST-Ya)* in the mRNA preparations used for library generation (data not shown). Subtraction efficiency was determined by monitoring depletion of cDNAs common to both induced and noninduced populations and enrichment of sequences specific to the induced population. Southern blot analysis of subtracted and unsubtracted cDNA pools showed that one abundant nondifferentially expressed gene, *GAPDH* was almost completely removed. The cDNAs of *CYP450 1A1* and *GST-Ya*, two known TCDD-induced genes, were enriched in the subtracted cDNA population (Fig. 1). This indicated the efficient generation of a pool of cDNA fragments enriched for those fragments derived from TCDD-induced genes. The pool was cloned as plasmid library.

Identification of Individual TCDD-induced Genes in the Subtractive Library. A total of 1500 clones of the library were picked for further analysis by colony PCR amplification of the library inserts and dot blot hybridization. A representative set of hybridizations is shown in Fig. 2. Replica filters of 96 PCR fragments, together with positive (TCDD-inducible *CYP450 1A1*) and negative (non TCDD-inducible *GAPDH*) controls, were hybridized with radiolabeled cDNA derived from RNA of either control cells (upper) or cells that had been treated with TCDD for 4 h (lower). Of the 1500 clones picked, 34 isolates preferentially hybridized with radiolabeled cDNA derived from TCDD-treated cells.

Identity of cDNA clones that hybridized preferentially with radiolabeled cDNA from TCDD-treated cells was determined by plasmid DNA isolation and sequence analysis. The 34 cDNA fragments belonged to 17 apparently independent clusters (Table 1), which included 6 known dioxin-inducible genes, *e.g., CYP450 1A1* (4 isolates), *CYP450 1B1*, *aldehyde dehydrogenase* (13 isolates), *GST-Ya*, *NAD(P)H:quinone oxidoreductase* and *UDP-glucuronosyl transferase* (2 isolates).

Besides the known TCDD-inducible genes, six genes were identified that were hitherto not known to be controlled by dioxins. These were B-cell translocation gene 1 (*BTG-1*; Ref. 24), lost on transformation 1 (*LOT-1*; Ref. 25), *transglutaminase* (26), a cytokine receptor-related protein (*Cyto R4*; Genebank accession no. AF046060), an ABC transporter (*ABC1*; Ref. 27), and a cDNA that is overexpressed

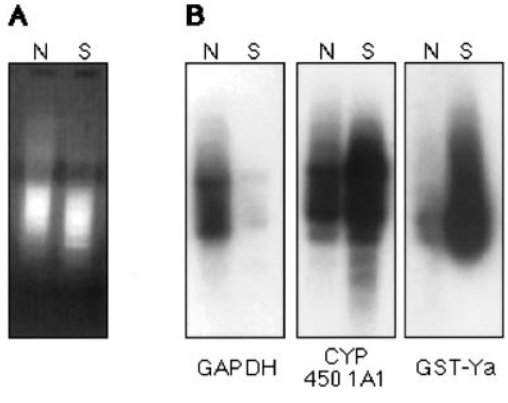

Fig. 1. Efficiency of subtractive suppression hybridization-PCR. 500 ng of PCR-amplified nonsubtracted tester cDNAs before SSH-PCR (*N*) and subtracted tester cDNAs after SSH-PCR (*S*) were separated on agarose gels. In *A*, ethidium bromide staining confirmed loading of comparable amounts of cDNA. In *B*, Sourthern blot analysis showed the relative levels of cDNA fragments derived from the housekeeping gene *GAPDH* and the known dioxin-inducible genes *CYP4501A1* and *GST-Ya*.

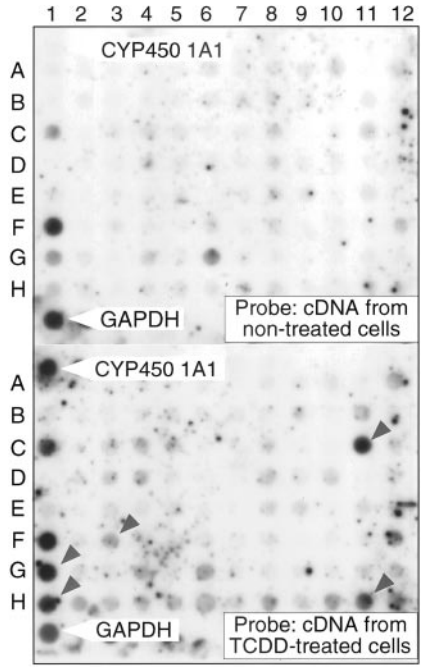

Fig. 2. Differential screening of the subtractive library. Library inserts in bacterial colonies were amplified individually by colony PCRs and duplicate dot blots were prepared. cDNA samples (25 ng) of *GAPDH* and *CYP4501A1* served as controls for comparable efficiency of probe preparation and efficiency of TCDD induction, respectively. Dot blots were hybridized with radiolabeled cDNA from nontreated (*upper blot*) or 4-h-TCDD-treated (*lower blot*) cells. *Arrowheads*, clones corresponding to mRNAs that are differentially expressed in 5L cells after 4 h of TCDD treatment. Equal intensities of signals were obtained with GAPDH, indicating similar hybridization conditions. One representative of 16 similar experiments for all library isolates is shown.

Table 1 *Database queries on TCDD-induced cDNA clones*

Annotation of cDNA[a]	No. of clones[b]	Accession nos.[a]
Group I[c]		
Cytochrome P450 1A1	4	M26129
Cytochrome P450 1B1	1	U09540
Aldehyde dehydrogenase	13	J03637
Glutathione-S-transferase Ya	1	M14986
NAD(P)H:quinone reductase	1	M31805
UDP-glucuronosyl transferase	2	U75903
Group II		
B-cell translocation gene 1	1	L26268
Lost on transformation 1	1	U72620
Transglutaminase	1	M55154
Cytokine receptor-related protein 4	1	AF046060, AF120152
T4 clone	2	U30788
ABC1	1	X75926
Group III		
Clone 106 (= NMT2, see text)	1	Af043327 (by Race, see "Results")
Clone 361	1	
Clone 735	1	
Clone 1396	1	Cluster of ESTs (see "Results")
Clone 1531	1	

[a] The sequence of cDNA clones was compared with the Genbank, EMBL, and EST databases by BLAST analysis (41). If high homologies were found, cDNA annotations and accession numbers are shown.

[b] The number of independent clones corresponding to one cDNA is shown.

[c] Clones of group I correspond to known TCDD-induced genes. Clones of group II represent known genes that had not been reported to be induced by TCDD. Clones of group III are novel sequences of which one could be annotated by RACE analysis (106) and one could be assigned to a cluster of ESTs (1396).

in *N*-methyl-*N*-nitrosourea-induced rat mammary tumors (*T4 clone*; Ref. 28).

One isolate, clone 1396, showed extensive homology to ESTs of the mouse (GenBank accession nos. Ai451155, Be686290, Aw122414, and Be648297). These four ESTs also showed homologies to each other so that a putative contiguous sequence of 1023 bp could be assembled. The sequence is partial, however, because Northern blot hybridization suggested that the endogenous mRNA is ~7.5 kb in size. Four isolates did not match any cDNA database entry (Table 1). These cDNA clones may represent genuine novel genes or may correspond to hitherto not published regions of known genes.

TCDD-inducibility of newly identified genes was confirmed by Northern blot analysis (Fig. 3). In three cases (clone 735, *lot1* and *cyto R4*) the probe identified two bands of which, in the cases of clone 735 and *cyto R4*, only one was inducible by TCDD. These clones may represent two closely related genes out of which only one is induced by TCDD. Alternatively, the double bands on Northern blots may result from an artifact during generation of the subtractive library if fragments from two cDNAs were ligated into one plasmid, although there is currently no evidence from sequence analysis to support this possibility. In all other cases, a single band and bona fide inducibility by TCDD was confirmed.

Furthermore, the Northern blot analysis showed that cDNA fragments had been cloned from both minimally and highly expressed genes. Signal intensity in Northern hybridization analysis as well as the inducibility of the novel genes by TCDD differed considerably (Fig. 3). Some clones were induced more than 10-fold (clones 1531, 735, and *CytoR4*) and others only <4-fold (*BTG1*, *LOT1*, *transglutaminase*, *ABC1*, and clones 106 and 1396). Thus, the chosen experimental approach was suitable to obtain clones corresponding to genes that are not heavily regulated or that are expressed at a low level.

Identification of *NMT2* as a Novel TCDD-induced Gene. We chose to characterize one of the novel sequences, clone 106, in detail. Longer fragments of clone 106 were obtained by RACE. 5′ RACE and 3′ RACE allowed the isolation of 3500 and 2000 bp fragments, respectively. Partial sequence analysis of the 5′ product showed high homology (>95% identity over 400 bp) to the mouse gene coding for *NMT2* (Accession no. Af043327). Northern blot analysis with an *NMT2*-specific probe (220 bp from the 5′ end of the 5′ RACE product) showed TCDD-inducibility in 5L cells, and the pattern of

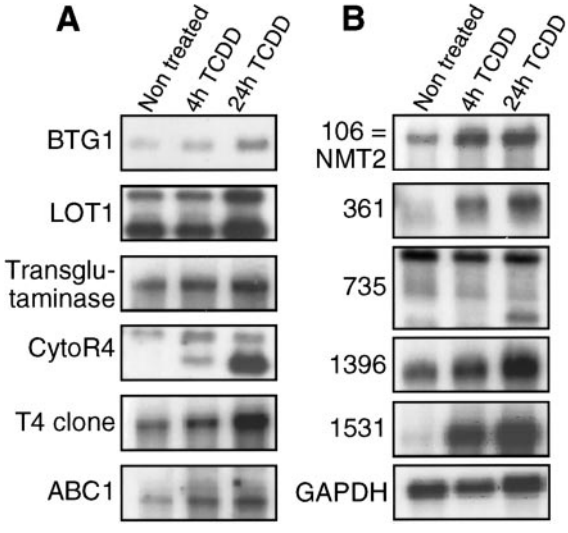

Fig. 3. Northern blot analysis of TCDD-induced clones identified by subtractive hybridization. Five μg of poly(A)$^+$ RNA from nontreated 5L cells or cells treated with TCDD for 4 and 24 h were analyzed by Northern blot hybridization using radiolabeled cDNA from individual clones. Equal loading of RNA was confirmed by rehybridization of the blots with a *GAPDH* probe (*B*). *A*, known genes for which TCDD-inducibility had not been known (*BTG1*, B-cell translocation gene 1; *Lot1*, lost on transformation; *Transglutaminase*; *Cyto R4*, cytokine receptor-related protein 4; *T4 clone*, cDNA that is overexpressed in *N*-methyl-*N*-nitrosourea-induced rat mammary tumors; *ABC1*, novel ABC transporter). *B*, the hybridization with clones that did not match any database entry or could in one case (*1396*) be assigned only to a cluster of ESTs.

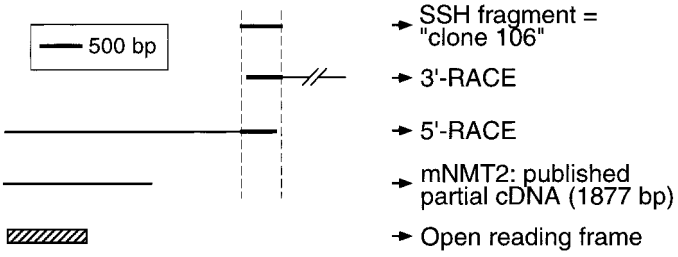

Fig. 4. Schematic representation of clone 106 and RACE products. 3′- and 5′-RACE was performed on clone 106 with double-stranded adapter-ligated cDNA (Clontech Laboratories) as template. The alignment of clone 106 with 5′- and 3′-RACE products is shown. A part of the 5′-RACE product is homologous to the published part of the mouse NMT2 cDNA. ▨, the protein coding open reading frame within the cDNA sequence.

expression is identical to that seen with clone 106 (data not shown). Thus, clone 106 corresponds to a yet unknown fragment of the 3′ untranslated region of rat *NMT2* (Fig. 4; Ref. 29).

AhR-dependent Induction of *NMT2* Transcription by TCDD. To test whether the induction of *NMT2* strictly depends on the AhR, subclones of 5L cells (17, 20) were analyzed. *NMT2* was not inducible by TCDD in the AhR-deficient BP8 subclone, which lacks AhR expression (Fig. 5*A*). The induction was restored by stable expression of AhR in these BP8 cells, which indicated that AhR is required for *NMT2* induction.

NMT2 mRNA was not inducible by TCDD in 5L cells pretreated with the transcriptional inhibitor actinomycin D, which suggests that TCDD-induced *NMT2* mRNA levels are attributable to increased transcription (Fig. 5*B*). Nuclear run-off analysis provided direct evidence for the induction of *NMT2* mRNA synthesis rate by TCDD (Fig. 5*C*). *NMT2* could be a direct target gene of AhR or could require the synthesis of intermediary proteins that induce *NMT2*. To discriminate between the possibilities, TCDD-inducibility of NMT2 mRNA was tested in the presence of the protein synthesis inhibitor cycloheximide (Fig. 5*D*) under conditions that had been shown previously to efficiently block protein synthesis in 5L cells (12). TCDD induced *NMT2* mRNA in both the absence and the presence of cycloheximide. Thus, ongoing or *de novo* protein synthesis are not required for induction of *NMT2* mRNA by TCDD, which indicates that *NMT2* is directly regulated by AhR. This is consistent with the early occurrence of mRNA induction in a time-course analysis (Fig. 5*E*).

***In Vivo* Induction of *NMT2* by TCDD.** A prerequisite for any target gene of AhR to mediate dioxin toxicity or carcinogenicity is expression in the relevant target organ. TCDD is a potent promoter of carcinogenesis in the rodent liver (30). Thus, the inducibility of *NMT2* mRNA in livers of TCDD-treated mice was tested. RT-PCR followed by Southern blotting analysis of the specific product revealed *NMT2* mRNA up-regulation in the liver of mice after 3 (2.5 fold), 5 (2.5 fold), or 7 days (4 fold) of TCDD treatment (Fig. 5*F*). PCR amplification could be expected to be in the linear range because much higher signals, but similar ratios between samples, were obtained when the PCR was continued for five additional cycles (data not shown). Furthermore, at the chosen number of cycles, the product was not yet detectable by ethidium bromide staining of agarose gels.

Dioxin-induced Increase in Protein Myristoylation. 5L cells were labeled *in vivo* with [^{3}H]myristic acid to test whether induction of NMT2 by TCDD is functionally relevant, *i.e.*, would lead to increased myristoylation of cellular proteins. Autoradiography of PAGE-separated whole cell extracts indicated that TCDD pretreatment specifically induced the occurrence of a prominent radiolabeled band at the size of M_r ~55,000 (Fig. 6*A*). Minor inducible bands occurred at M_r ~80,000 and 100,000, whereas the abundance of other radiolabeled bands, *e.g.*, at M_r 40,000, did not depend on TCDD pretreatment. This indicated that in the noninduced cells NMT-2 activity is limiting with respect to several substrate proteins and that TCDD treatment can indeed induce myristoylation of specific target proteins. The c-Src tyrosine kinase is a known myristoylated protein, but the prominent protein is smaller than c-Src (Fig. 6*A*). It comigrates, however, with a protein that is also recognized by one of the antibodies directed against c-Src and, therefore, may correspond to another member of the Src kinase family. In 5L cell extracts, antibodies against known family members detect Blk, Lyn, and low levels of Fgr, in addition to c-Src. Lyn and Blk can be expected to migrate at the position of the inducibly myristoylated protein. In the case of Lyn, a suitable antibody was available for immune precipitation of [^{3}H]myristoylated protein from 5L cell extracts (Fig. 6*B*, *right lanes*). The experiment proves that the chosen approach is suitable for de-

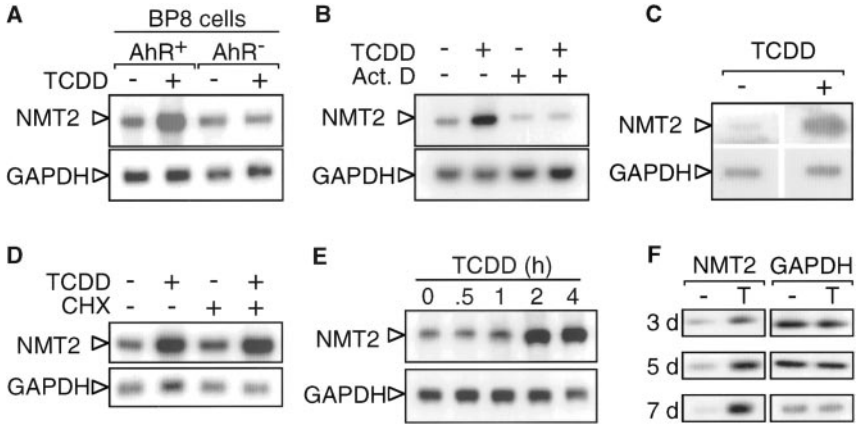

Fig. 5. Induction of *NMT2* gene expression by TCDD both *in vitro* and *in vivo*. *A*, AhR-dependent induction of *NMT2* mRNA by TCDD. Induction of *NMT2* mRNA was tested in AhR-deficient BP8 cells (*BP8, AhR*$^-$) and BP8 cells stably transfected with AhR (*BP8, AhR*+) after 24-h exposure to 1 nM TCDD as described in Fig. 3. In *B*, induction of *NMT2* mRNA was tested in 5L cells after 4-h exposure to 1 nM TCDD or 0.1% DMSO solvent with or without 15-min pretreatment with the transcriptional inhibitor actinomycin D (*Act. D*, 5 μg/ml) by Northern blot analysis. *C*, nuclear run-off analysis. The transcriptional rate of *NMT2* gene was analyzed by a nuclear run-off analysis (12, 23, 42) with nuclei prepared from 5L cells treated for 24 h with 1 nM TCDD or the solvent (DMSO). In *D*, induction of NMT-2 mRNA was tested after 4-h exposure to 1 nM TCDD or 0.1% DMSO solvent with or without 30 min of pretreatment with the translational inhibitor cycloheximide (*CHX*, 20 μg/ml) by Northern blot analysis using 5 μg of poly(A)$^+$ RNA. *E*, time course of *NMT2*-induction by TCDD. Induction of *NMT2* mRNA was tested in 5L cells exposed to 1 nM TCDD for the indicated time. In *F*, *NMT2* mRNA expression was determined in the livers of mice 3, 5, or 7 days after a single injection of solvent or TCDD (1.4 μg/kg body weight). *NMT2* mRNA abundance was measured by RT-PCR/Southern blotting analysis in 0.1 μg of total liver RNA from TCDD-treated or time-matched solvent-treated control mice, using cDNA-specific primers for *NMT2* and *GAPDH* in single-tube reactions. RT-PCR products were collected after 25 cycles of PCR, and the abundance of *NMT2* and *GAPDH* fragments was analyzed by Southern blot hybridization. One representative of three similar experiments is shown.

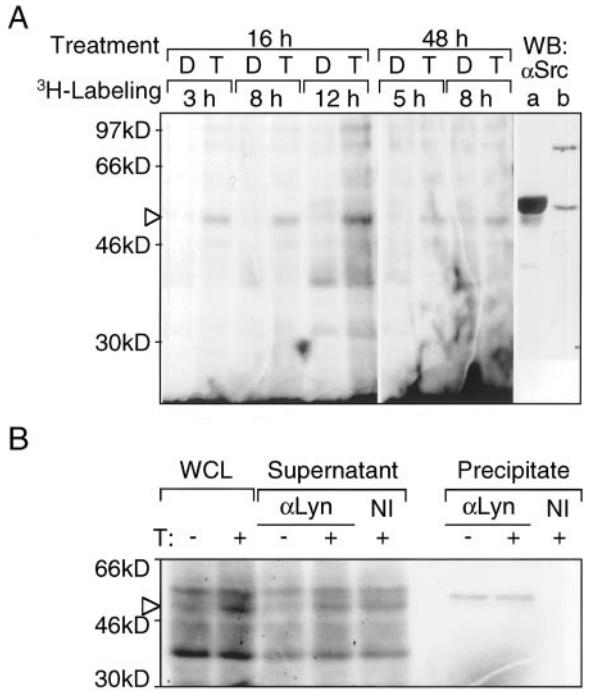

Fig. 6. Increased myristoylation of proteins in TCDD-treated 5L cells. Myristoylation of proteins was tested in control and TCDD-treated 5L cells by *in vivo* labeling with [^{3}H]myristic acid followed by SDS-PAGE analysis and autoradiography. In *A*, 5L cells were treated for 16 h or 48 h with either 1 nM TCDD (*T*) or 0.1% DMSO solvent (*D*). The cells were labeled with [^{3}H]myristic acid for the indicated times. The mobility of the major inducibly myristoylated protein (*arrowhead*) was compared with that of Src by Western blot analysis. Two different antibodies to c-Src directed against either the SH3 domain (*WB: αSrc, right lane*, Ab-1) or the COOH-terminus (sc-18) were used. The latter antibody is known to cross-react with a number of Src-like protein kinases.[6] In *B*, extracts from 5L cells that had been pretreated with TCDD for 16 h, followed by an 8-h labeling period in the presence of [^{3}H]myristic acid, were subjected to immune precipitation using an antibody against Lyn. Immune precipitates (*Precipitate, right lanes*) and 10% aliquots of whole cell lysates (*WCL, left lanes*) or supernatants after immune precipitation (*Supernatant, middle Lanes*) were used for separation and autoradiography. *Arrowhead*, the major inducibly myristoylated protein. *NI*, a nonspecific antibody was used for immune precipitation in these samples. *kD*, molecular weight in thousands.

tecting myristoylation of specific proteins such as Src-like kinases. However, Lyn myristoylation seems not to depend on TCDD treatment of 5L cells, because labeling of precipitated Lyn is not induced, and Lyn migrates slightly more slowly than the predominant inducibly myristoylated protein (Fig. 6*B*). Thus, at present, the identity of the inducibly myristoylated protein remains uncertain, and purification for mass spectroscopic identification will be required.

DISCUSSION

The AhR mediates toxic and carcinogenic effects of dioxins. Known AhR-regulated genes mostly code for xenobiotica-metabolizing enzymes, but AhR-regulated genes responsible for dioxin toxicity were largely unknown. Those genes that do not code for xenobiotica-metabolizing enzymes do not conclusively explain all aspects of dioxin toxicity or carcinogenicity. Thus, we used SSH to identify novel target genes of AhR. The major outcome of this search were the identification of 11 unexpected or novel cDNA fragments and, most importantly, the finding that *NMT2* expression and protein myristoylation underly short-term regulation, *e.g.*, by TCDD.

SSH was chosen instead of a cDNA array-based analysis for several reasons; first, array-hybridization usually does not involve a normalization step, which precludes detection of low-abundance cDNAs. Second, the labeling reaction in common protocols prefers the 3′-end of RNAs and thus limits the analysis to cDNAs and ESTs corresponding to 3′-ends of known cDNAs. Finally, we wanted to allow identification of novel sequences. The analysis of the obtained results indicates that, both, the identification of low-abundantly expressed genes as well as the isolation of novel sequences was successful. Seven cDNA clones including the RACE-extended clone of *NMT2* correspond to known genes for which inducibility by TCDD is novel. Four cDNA fragments represent novel sequences. Some of the known TCDD-induced RNAs were represented by up to 13 different cDNA clones, whereas other RNAs were represented by only one cDNA clone. Thus, a major fraction of the TCDD-induced alterations in the transcriptome appears to be covered, but complete saturation could not be assured despite picking a moderately high number of clones.

This study indicates an estimate of the complexity of AhR-induced early changes in gene expression because several unexpected genes were found. Increased transglutaminase enzyme activity has been reported in the epidermis of hairless mice by topical treatment with TCDD (31) and epidermal cells treated *in vitro* (32). The present study might explain these former findings by showing early induction of *transglutaminase* mRNA by TCDD. Among the other known genes induced by TCDD, *BTG1* is reported to negatively regulate proliferation (24). *LOT1* is a novel gene cloned based on its loss of expression in malignantly transformed cell lines (25). *T4 clone*, a gene containing retroviral sequences, is up-regulated in rat mammary tumors induced by *N*-methyl-*N*-nitrosourea (28). In addition, TCDD also induced *ABC1*, which codes for a member of the large transmembrane ATP-binding cassette protein family. These proteins transport diverse substrates, such as sugars, amino acids, proteins, metals, and xenobiotica across membranes (27). Recently, it is reported that *ABC1* is transcriptionally regulated by sterols through nuclear receptors liver-X-receptor (LXR) and retinoid-X-receptor (RXR; Ref. 33). *ABC1* induction by AhR might, much like xenobiotica-metabolizing enzymes, play a role in the organism's machinery to locate xenobiotica to certain compartments and to eliminate them. The functional and possibly pathophysiololgical roles of other induced genes, such as *BTG1, LOT1, CYTO R4*, and *T4 clone*, are currently not known.

The most prominent result of this study is the finding that *NMT2* expression is inducible by a small diffusible compound, *i.e.*, the xenobiotic TCDD, by a receptor-dependent mechanism. NMT2 is an enzyme-catalyzing protein *N*-myristoylation, *i.e.*, the cotranslational linkage of myristic acid to the NH_2-terminal glycine residues of eukaryotic cellular proteins (34). Examples of NMT substrates include protein tyrosine kinases like $p60^{Src}$, cAMP-dependent protein kinase (PKA), the Gαi2 G-protein subunit and the calcineurin B protein phosphotase. Human and mouse cDNAs for two distinct NMTs called *NMT1* and *NMT2* have been cloned. The specific role of each NMT in protein myristoylation has not yet been studied (29). Furthermore, there are no published data on the transcriptional regulation of *NMT*s. The AhR constitutes the first conditionally regulated pathway that controls expression of either of the *NMT* genes.

NMT2 mRNA inducibility raises the questions as to whether increased *NMT2* expression translates into increased protein myristoylation and whether the induction occurs in the living animal. Increased protein myristoylation could be shown in 5L cells because dioxin pretreatment induced increased appearance of myristoylated proteins. Furthermore, AhR-dependent regulation of *NMT2* appears to be relevant also in the mouse, because *NMT2* mRNA was increased in livers of mice after 3, 5, or 7 days of TCDD treatment. Thus, *NMT2* fulfills essential requirements to be a candidate gene in mediating toxic or carcinogenic properties of dioxins, *e.g.*, its activity is limiting in the absence of the inducing agent, and the induction by TCDD occurs in a target organ of dioxin action.

[6] Santa Cruz Biotechnology product information.

A causal role of NMTs in carcinogenesis is suggested by the finding of elevated levels of NMT protein and NMT activity in carcinomas of the colon (35, 36) or the gallbladder (37). The mechanism(s) of up-regulation are currently not known. One potential myristoylated target protein might be c-src, because $p60^{v\text{-}Src}$ potently induces cell transformation (38), and inhibition of protein myristoylation also prevents transformation (39, 40). Thus, inappropriate protein myristoylation is associated with several forms of cancer and is likely to play a role in pathogenesis, although some of the relevant myristoylated proteins may not yet be known. Because TCDD induces *NMT2 in vivo*, it is tempting to speculate that ectopic *NMT2* expression mediates carcinogenicity of TCDD by induction of inappropriate protein myristoylation.

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