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Isolation and characterization of a novel gene induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rat liver

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The differential display technique was used to identify genes whose expression was regulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Expression of a novel sequence was up-regulated in a dose-dependent fashion in liver of Sprague–Dawley male rats exposed to both chronic and acute treatment with TCDD, as measured by densitometry of Northern blot analyses ($P < 0.01$). A rapid amplification of cDNA ends (RACE) procedure was used to isolate a 1.8 kb cDNA from a rat liver cDNA preparation. This cloned cDNA, called 25-Dx, was sequenced and found to encode a peptide of 223 amino acids. In control rats, the 25-Dx gene was expressed at high levels in lung and liver. A hydrophobic domain of 14 residues followed by a proline-rich domain, both located in the N-terminal region, showed 71% homology with the transmembrane domain of the precursor for the interleukin-6 receptor and a conserved consensus sequence found in the cytokine/growth factor/prolactin receptor superfamily respectively.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD*) evokes a large spectrum of biological effects in a variety of species, including alterations in metabolic pathways, developmental and reproductive changes and neoplasia (1–3). Of special interest is its extremely potent carcinogenicity in rodents, inducing liver tumors at the lowest tested dose of 1 ng/kg/day. In humans, epidemiological studies have recently revealed an association between dioxin exposure and the development of various types of cancer (4–6). Despite extensive research conducted during the past years, the molecular basis of TCDD toxicity and carcinogenicity is poorly understood. Experimental evidence suggests that TCDD triggers a variety of responses by altering the expression of specific TCDD-responsive genes, such as CYP1A1, a member of the P450 family. Up-regulation of CYP1A1 and other target genes involves binding of TCDD to the aromatic hydrocarbon receptor and subsequent interaction of the activated ligand–receptor complex with specific DNA response elements in the promoter region of the target genes (7,8). This transactivation process appears to be similar for other members of the Ah gene battery, such as CYP1A2, aldehyde-3-dehydrogenase and glucuronosyl transferase (9,10). Other changes in gene expression have also been observed following TCDD exposure. Using differential hybridization, Sutter *et al.* (11) reported on the cDNA cloning of two TCDD-responsive genes, plasminogen activator inhibitor 2 and interleukin 1β, from human keratinocytes. The authors demonstrated that, similarly to CYP1A1, enhanced expression of these genes was regulated at the transcriptional level and was associated with altered proliferation and differentiation. TCDD also affects growth factor pathways. For example, TCDD regulates expression of the epidermal growth factor receptor at both the mRNA and protein levels in rat liver and uterus (12,13). Moreover, there have been studies reporting that post-transcriptional events may be responsible for regulation of expression of transforming growth factor-α and -β_2 in human keratinocytes in response to TCDD (14). Although further studies are necessary to establish whether these genes are actively involved in inducing carcinogenesis, these efforts provide evidence that TCDD may alter important proliferative checkpoints in various systems.

For several years, our laboratory has been interested in studying regulatory components that may help to explain the link between TCDD exposure and cancer. In order to identify candidate genes involved in potential TCDD-mediated signal transduction pathways, we used the differential display methodology (DD) (15–18), a PCR-based technique suitable for the rapid isolation of DNA fragments of differentially expressed genes. We focused our attention on expression in rat liver because it provides a well-characterized system to investigate TCDD tumorigenicity. Analysis of rat liver RNA obtained from animals exposed to acute or chronic TCDD treatments allowed the isolation and characterization of a novel cDNA, called 25-Dx. Expression of 25-Dx was enhanced in a dose-dependent fashion over a broad range of doses in response to both acute and chronic TCDD exposure. The sequence of the 25-Dx cDNA suggests the identification of a new member of the cytokine/growth factor/prolactin receptor superfamily.

Materials and methods

Treatments

Livers were isolated from Sprague–Dawley male rats treated with either acute or chronic levels of TCDD as described previously (19,20). Animals were exposed to a single dose (0.5 ml) of 0, 10 or 1000 ng/kg TCDD (acute) and killed 4 days later or to 0, 1 or 125 ng/kg/day TCDD for 30 weeks (chronic). A second high dose acute exposure experiment was carried out in which three animals in each group were exposed to a single dose of 0, 1, 5, 25 or 50 μg TCDD/kg and killed 7 days after treatment. Rat livers were isolated and RNA extracted as described below.

RNA isolation and differential display

Total RNA was isolated using the single-step MRC Tri Reagent procedure (21) according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH). Total RNA preparations were digested with DNase (MessageClean™ Kit; GenHunter Corp., Brookline, MA).

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DD, differential display methodology; MTN, multiple tissue Northern blots; ORF, open reading frame; EST, expressed sequence tag; IL-6R, IL-6 receptor.

DD was performed according to the GenHunter procedure RNAmap (kits A, B, C and D). Briefly, 0.4 μg total RNA from each treatment were reverse transcribed using each of four $T_{12}MN$ oligonucleotides ($T_{12}MA$, $T_{12}MC$, $T_{12}MG$ and $T_{12}MT$, where M pairs with any nucleotide). Samples of 10–20 ng of each cDNA reaction were added to PCRs that contained each of the 20 primers (arbitrary sequence of 10 nt supplied with the RNA map kit) in combination with one of the four $T_{12}MN$ oligonucleotides (80 different PCRs). PCR products were labeled with [α-^{33}P]dATP (2 μCi; Amersham, Arlington Heights, IL). Amplified cDNAs were separated in 6% polyacrylamide sequencing gels and PCR products from differentially expressed mRNAs visualized by autoradiography. Strips of dried gel corresponding to regions containing cDNAs of interest were dissected and cDNAs eluted and re-amplified. PCR products were directly sequenced using the ABI Prism dye terminator kit and an ABI 373A sequencer (Applied Biosystems, Foster City, CA). PCR products obtained from each isolated cDNA were used as probes for Northern hybridization analysis.

Northern analyses

Total RNAs (10 μg) were separated in 1% agarose–formamide gels, stained with ethidium bromide and transferred in 20× SSC to Duralon-UV membranes (Stratagene, La Jolla, CA). Hybridizations were carried out using the QuikHyb procedure (Stratagene) in the presence of [α-^{32}P]dCTP-labeled (Amersham, Arlington Heights, IL) cDNA probe. Probes were labeled using the Random Primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). All the washes were carried out under stringent conditions, according to Sambrook *et al.* (22). Multiple tissue Northern (MTN) blots (rat and human) were obtained from Clontech Laboratories Inc. (Palo Alto, CA) and treated under the same conditions as above. Densitometric analysis of autoradiograms was performed using the Ultrascan XL Enhanced Laser Densitometer (LKB-Pharmacia, Piscataway, NJ). Among several fragments that showed altered expression in response to TCDD, clone 25-Dx was chosen for further characterization as its expression was consistently increased by treatment with TCDD. Regression (dose versus response) analyses were carried out on the values obtained from densitometry of the autoradiograms of the TCDD dose–response Northern blots (SigmaStat; Jandel Scientific, San Rafael,CA)

Cloning and sequencing analysis

For rapid amplification of the 5′-ends of cDNAs we designed specific oligonucleotides for the original 350 bp fragment isolated by DD, according to the RACE procedure (Clontech Laboratories) (23,24). Aliquots of 2 μl (~20 ng) of rat liver 5′-RACE-ready cDNA were used as templates in PCR reactions performed with a 25-Dx-specific primer (5′-CCC ATT ATT GAG TTT TCA NTT TGT TAC TGG-3′) and Anchor Primer (Clontech Laboratories). Dilutions of the primary PCR amplification were used for secondary PCR reactions using a nested 25-Dx-specific primer (5′-CCA GGT GTG TGA GAG TTA C-3′) and Anchor Primer. A PCR product of ~1200 bp was purified from a 3% agarose gel, cloned into pGEM-3Z (Promega, Madison, WI) and sequenced from both directions using the ABI373A sequencer. A 600 bp fragment 3′ of the original 350 bp fragment identified by DD was obtained using 2 μl aliquots of the 5′-RACE-ready cDNA as template and a 25-Dx-specific oligonucleotide (5′-GTC TGA AAA CAA GCC CAT TTT ACC CAA CAG-3′) in combination with an oligo(dT) as primers. The 600 bp fragment was sequenced from both ends. Sequence analysis was performed using the BLAST network service (National Center for Biotechnology Information) (25). Oligonucleotides used for PCR analysis of the 25-Dx gene were synthesized by Bioserve Biotechnologies Inc. (Laurel, MD).

Southern analysis

Genomic DNA was extracted from rat liver of control animals as described previously (22). Liver tissue was homogenized in 0.5% SDS/STE buffer (0.5% SDS, 100 mM NaCl, 20 mM Tris–HCl, pH 7.6, and 10 mM EDTA). Proteinase K (10 mg/ml) was added and the lysate was incubated for 2 h at 37°C, with occasional shaking. After two 1:1 phenol/chloroform and one chloroform extraction, DNA was precipitated in 2 vol. 100% ethanol and the pellet resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA, pH 8.0) at a concentration of 0.5 mg/ml. Aliquots of 5 μg genomic DNA were digested with various restriction enzymes, separated on a 0.7% agarose gel and transferred to a Duralon-UV membrane as described previously. Membranes were hybridized with [α-^{32}P]dCTP-labeled probes containing the full coding region for the 25-Dx gene. Hybridizations were carried out using the QuikHyb solution (Stratagene).

In vitro translation

A cDNA fragment containing the first 1200 bp of the 25-Dx gene and containing the full coding sequence was cloned into the *Eco*RI and *Xma*I sites of the pGEM-3Z vector (Promega) and used for *in vitro* translation (TNT T7 Coupled Reticulocyte Lysate System kit; Promega). [^{35}S]Methionine (1000 Ci/mmol at 10 mCi/ml) was purchased from Amersham. *In vitro* translation products were analyzed by SDS–PAGE.

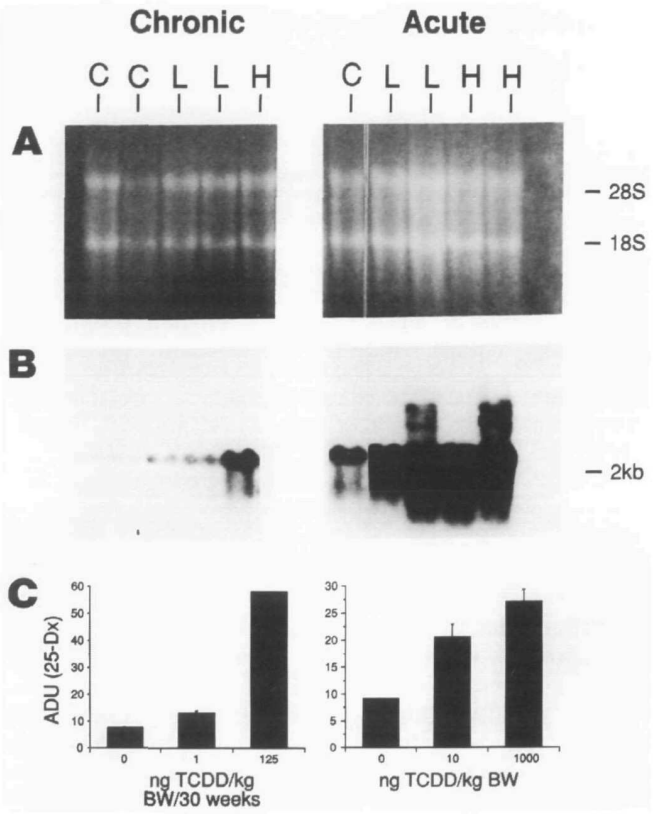

Fig. 1. Differential expression of 25-Dx in rat liver from control and TCDD-treated animals. Total liver RNA (20 μg) was purified from control (C), low (L) or high (H) dose exposed animals from chronic and acute treatments with TCDD. (**A**) Total RNA was run in formamide–1% agarose gels and stained with ethidium bromide. (**B**) Autoradiograms that resulted from hybridization of a [^{32}P]dCTP-labeled 25-Dx cDNA probe to the Northern blot of (A). (**C**) Densitometry analysis of 2 kb bands from (B) was performed and results were expressed in arbitrary densitometric units (ADU).

Results

Differential display

Total RNA samples extracted from livers of control and TCDD-treated (acute and chronic) animals were analyzed using the DD method. Four different pools of cDNAs were generated for each sample by reverse transcription with the $T_{12}MN$ oligonucleotides and then the cDNA pools were subjected to PCR using the same $T_{12}MN$ oligonucleotides in combination with 20 arbitrary sequence primers (80 combinations). Comparison of DD patterns obtained from untreated and treated samples allowed identification of 30 bands corresponding to mRNAs whose expression was potentially altered in response to TCDD (data not shown). These differentially expressed sequences were then re-amplified by PCR using the same combination of primers as in the DD analysis and used as probes in Northern analyses and for direct sequencing.

Expression of 25-Dx

Based on Northern analysis of total RNA using probes obtained from the DD analysis, one of the differentially expressed cDNAs (25-Dx, coding for a 25 kDa peptide, dioxin responsive) was consistently up-regulated in response to dioxin (Figures 1 and 2) and was chosen for further studies. The 350 bp fragment obtained from DD analysis was labeled with [^{32}P]dCTP and hybridized to membranes containing total RNA from control and TCDD-exposed animals (Figure 1B). For

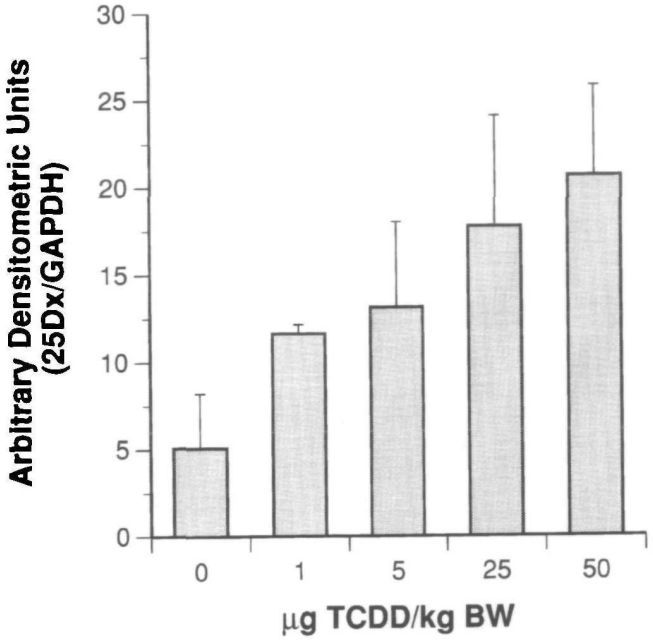

Fig. 2. Densitometric analysis of 25-Dx Northern blots from the high dose acute exposure experiment. Poly(A) RNA from rats treated with 0, 1, 5, 25 or 50 µg TCDD/kg body wt was isolated, separated by electrophoresis, blotted and hybridized with a ^{32}P-labeled 25-Dx cDNA probe. The intensity of each band was expressed in arbitrary densitometric units (ADU) and normalized to the expression of an internal standard (glyceraldehyde-3-phosphate dehydrogenase). Bars are means±SD of three observations for each treatment.

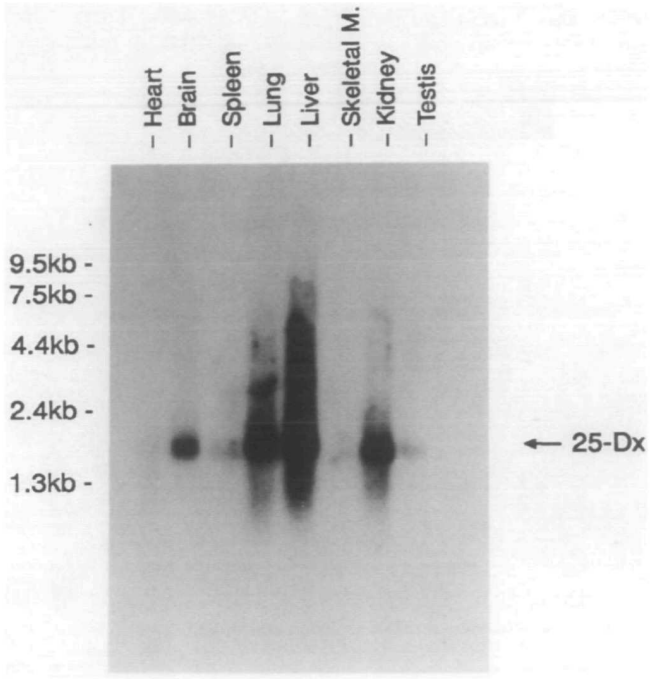

Fig. 3. Northern analysis of 25-Dx expression in untreated rat tissues. Poly(A) RNA blots containing 2 µg poly(A) RNA per lane were purchased from Clontech and hybridized with a ^{32}P-labeled 25-Dx cDNA The autoradiograph was developed after an overnight exposure, as in all other Northern blot experiments, unless otherwise stated.

these analyses, total RNA samples from replicate animals were separated on ethidium bromide stained agarose gels. All the samples were electrophoresed on the same agarose gel and blotted onto the same membrane under identical conditions. Samples showing degradation were omitted from the photograph and from subsequent densitometry analysis (one chronic high dose sample and one acute control sample).

In the chronic exposure (Figure 1C, left), expression of the 25-Dx cDNA was significantly increased (P = 0.0007, adj R^2 = 0.98) to a maximum of 7-fold in samples treated with 125 ng TCDD/kg/30 weeks (lanes H), compared with control samples. The low dose of TCDD exposure (1 ng/kg/30 weeks; lanes L) produced a small increase in expression of 25-Dx but this change was not statistically significant. In the acute

(Figure 1C, right) expression of 25-Dx was
maximum of 2.9-fold with the high dose of
ng/kg; lanes H) and 2-fold with the low dose
s L) relative to the control sample (untreated).
e experiments, the statistical power to detect
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picts the results of an independent high dose
which rats were exposed to a single dose of 0,
g TCDD/kg. These experiments confirmed the
ssion of 25-Dx at the 1 µg/kg dose observed
experiment (Figure 1C) and demonstrated a
nt relationship between dose and response (P =
= 0.48). In this experiment, the maximum
n observed in the high dose experiment was
acute exposures, increases in 25-Dx expression
at doses from 10 ng/kg to 50 µg/kg (a 3.5 log

Figure 3 shows the pattern of 25-Dx gene expression in various rat tissues. In those tissue samples from untreated rats, levels of transcripts for the 25-Dx gene were high in liver, lung, kidney and brain, low in testis and spleen and undetectable in heart and skeletal muscle. Hybridization of the rat 25-Dx probe to a MTN blot of human tissues suggested a similar, but not identical, pattern of expression (data not shown).

Sequence analysis

In Figure 4A, a diagram of the 25-Dx cDNA structure is shown. The cloned nucleotide sequence had a length of 1885 nt and the predicted open reading frame (ORF) consists of 670 bp, encoding for 223 amino acids (Figure 4B). This sequence, which codes for a polypeptide of ~25 kDa, was not previously reported in the GenBank database, when searched using BLAST (25). However, a high degree of homology (between 80 and 97%) was found with expressed sequence tag (EST) cDNAs both from rat (accession no. EST107044) and human libraries (accession nos R59281 and R13334; 26). In addition, we found a 63% homology with a 59 amino acid region of a *Caenorhabditis elegans* protein (U415552) of unknown function, indicating that the 25-Dx protein has a domain that has been conserved over a large evolutionary distance.

The 5′ sequence flanking the first ATG agrees with the Kozak consensus sequence: an A in position –3 and a G in position +4 (27), indicating a putative start codon for the 25-Dx gene. Further attempts to isolate cDNA sequences upstream of nucleotide –96 (Figure 4A; nucleotide 1 in Figure 4B), using a specific primer for the 5′ region of 25-Dx (from –52 to –33) and aliquots of the 5′-RACE-ready cDNA as templates, failed to amplify PCR products. These observations suggested that clone 25-Dx contained most, if not all, of the 5′ untranslated region of 25-Dx. The first stop codon is followed by several

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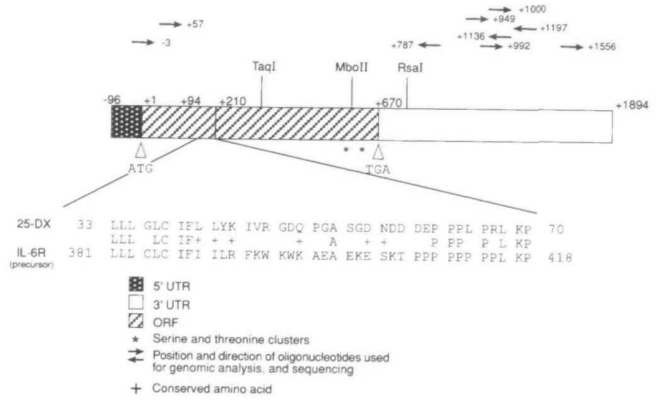


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1
gcaaaaattaatttattggccactgttcgcttagagggcggaggaagccgactgtttcggtctctgcataaacaqqcccaaa 79

cctttgctccagagatcATG GCT GCC GAG GAT GTG GTG GCG ACT GGC GCC GAC CCC AGC GAG CTG 144
                    M  A  A  E  D  V  V  A  T  G  A  D  P  S  E  L  16

GAG GGC GGC GGG CTG CTT CAA GAG ATT TTC ACG TCG CCT CTC AAC CTG CTG CTC CTT GGC  204
E  G  G  G  L  L  Q  E  I  F  T  S  P  L  N  L  L  L  L  G  36

CTC TGC ATC TTC CTG CTC TAC AAG ATC GTT CGC GGG GAC CAG CCC GGT GCC AGT GGG GAC  264
L  C  I  F  L  L  Y  K  I  V  R  G  D  Q  P  G  A  S  G  D  56

AAC GAC GAC GAC GAG CCG CCC CCG CTG CCT CGC CTC AAG CCG CGT GAC TTC ACC CCT GCC  324
N  D  D  D  E  P  P  P  L  P  R  L  K  P  L  D  F  T  P  A  76

GAA CTA AGG CGA TAC GAT GGA GTC CAG GAC CCG CGC ATT CTT ATG GCC ATC AAC GGC AAG  384
E  L  R  R  Y  D  G  V  Q  D  P  R  I  L  M  A  I  N  G  K  96

GTG TTC GAC GTG ACC AAA GGC CGC AAG TTC TAT GGG CCG GAG GGA CCA TAC GGG GTC TTT  444
V  F  D  V  T  K  G  R  K  F  Y  G  P  E  G  P  Y  G  V  F  116

GCT GGA AGA GAT GCA TCC AGG GGC CTT GCC ACA TTT TGC CTG GAC AAA GAA GCA CTG AAG  504
A  G  R  D  A  S  R  G  L  A  T  F  C  L  D  K  E  A  L  K  136

GAT GAG TAT GAT GAC CTT TCT GAC CTC ACT CCT GCC CAG CAG GAG ACC CTG AAT GAC TGG  564
D  E  Y  D  D  L  S  D  L  T  P  A  Q  Q  E  T  L  N  D  W  156

GAC TCT CAG TTC AGT TCA CCT TCA AGT ACC ATC ACG TGG GGA AAA CTG CTT GAA GGA GCG  624
D  S  Q  F  S  S  P  S  S  T  I  T  W  G  K  L  L  E  G  A  176

GAG GAG CCG ATT GTG TAC TCG GAT GAT GAA GAA CAA AAG ATG AGG CTG CTC GGA AGA GTG  684
E  E  P  I  V  Y  S  D  D  E  E  Q  K  M  R  L  L  G  R  V  196

ACT GAA GCA GTC AGT GGA GCA TAT CTA TTT TTG TAT TTT GCA AAA TCA TTT GTA ACA TTC  744
T  E  A  V  S  G  A  Y  L  F  L  Y  F  A  K  S  F  V  T  F  216

CAG TCT GTC TTT ACA ACA TGG TGA tttcaatatttagagaagtttgaacttgtaagttttaagtaac
Q  S  V  F  T  T  W  *                                                    223

atcactagtgacaccgataaaatcaactcgtcttagaatgcatgatgtgttttgtacgtcacaaatccagaaagtgaac
tttagttcttctagatccaaatggtaaagctgtttcctcttctatctgtagtttaaaactgaattacaatttttattttcct
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agtgcttgggtcagttaatacaaacggttttgcccagtaaaaaaaaaaaaaaaaaaa +1885
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Fig. 4. 25-Dx sequence. (**A**) 5′ and 3′ untranslated regions (UTRs) are shown flanking the ORF of the 25-Dx sequence. The hydrophobic segment at the N-terminus is also presented as amino acid sequence. (**B**) DNA sequence and predicted amino acid sequence of the 25-Dx gene. The putative polyadenylation sequence (aataaa) is found in the last line of the figure. The underlined aaagaaaaa sequence in the 3′ UTR may have been primed by the DD primer in our initial DD reactions. This sequence has been deposited in the GenBank database with accession no. U63315.

TGA and TAA triplets and a poly(A) signal was found 15 nt upstream of the poly(A) tail. The deduced amino acid sequence did not contain Asn-X-Ser or Asn-Thr-Ser sequences, which are potential sites for *N*-linked oligosaccharides. However, we identified two clusters of serine and threonine residues at the C-terminus that may represent possible sites for *O*-linked oligosaccharides. In the first cluster there are three threonine and five serine residues spanning the region between amino acids 152 and 168; in the second group three threonine and two serine residues lay between amino acids 212 and 222.

The region between residues 32 and 46 is highly hydrophobic and is suggestive of a transmembrane domain, based on its 71% similarity to the precursor receptor for interleukin-6 (IL-6R, accession no. P08887, Prodom28 release) (Figure 4A). In addition, when comparing the 25-Dx sequence from amino acids 32 to 70, the homology with IL-6R is 55%, with a peak

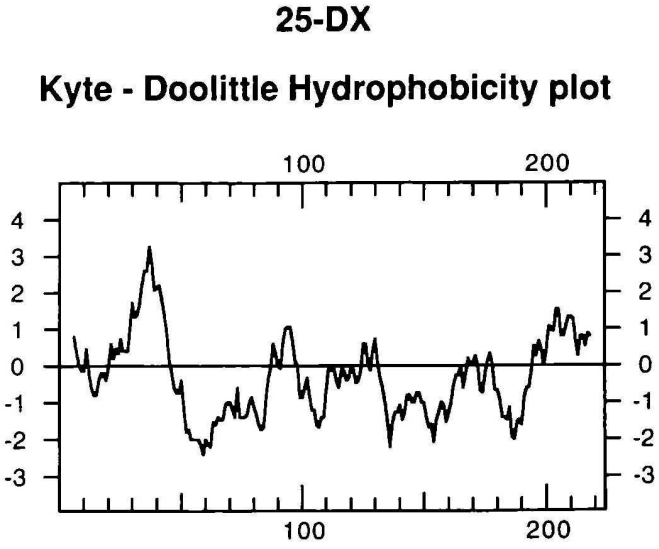

Fig. 5. Kyte–Doolittle hydrophobicity plot of the 25-Dx peptide. The Kyte–Doolittle analysis was used to detect hydrophobic regions in the 25-Dx peptide. The figure shows the hydrophobicity profile based on an 11 amino acid window. Hydrophobic regions are shown on the upper part of the plot, with positive numbers.

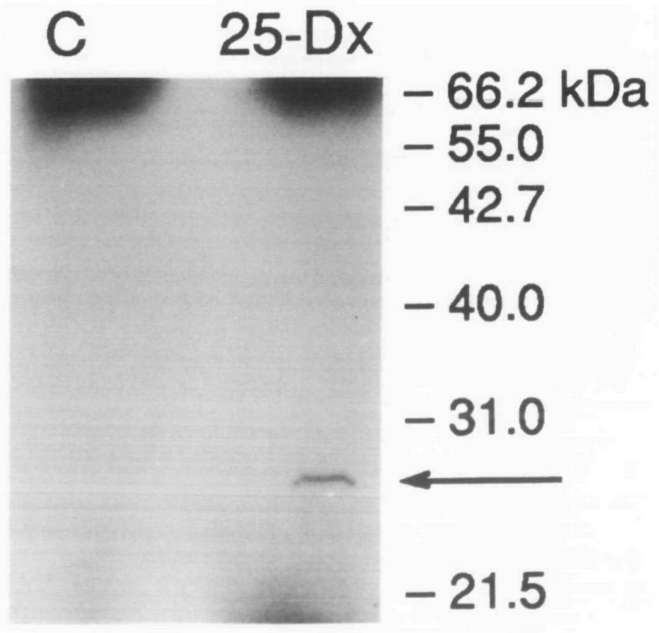

Fig. 6. *In vitro* translation. Aliquots of 1 μg plasmid DNA containing the full coding sequence for the 25-Dx gene (lane 25-Dx) and the same amount of a plasmid DNA missing the 5′ region of 25-Dx (lane C) were *in vitro* transcribed and translated using the Promega TNT T7 kit. The arrow indicates the band corresponding to the *in vitro* translation product, after SDS–PAGE analysis and autoradiography.

of 80% identity corresponding to a region rich in proline (five out of 10 residues) from amino acids 62 to 70. No histidine residues were found in the amino acid sequence of the 25-Dx gene and the protein overall is negatively charged. Figure 5 shows a Kyte–Doolittle hydrophobicity plot for the 25-Dx gene-encoded protein based on the amino acid composition. A second hydrophobic region is present at the C-terminus of 25-Dx.

In vitro translation

To verify whether the newly identified sequence of 25-Dx cDNA encoded for a peptide, we carried out *in vitro* translation using the 1200 bp 25-Dx cDNA containing the full ORF. This region was amplified with RACE and cloned downstream of a T7 promoter sequence in a pGEM-3Z vector. As depicted in Figure 6, the product obtained from the *in vitro* transcription and translation reactions had an apparent mol wt of 24–25 kDa, which is in agreement with the expected size predicted from the amino acid sequence of the 25-Dx cDNA (24.6 kDa).

Genomic analysis

The genomic organization of the 25-Dx gene was investigated by Southern blot hybridization and PCR (Figures 7 and 8). When genomic DNA was digested with *Eco*RI or *Bam*HI, we detected major bands of ~9 and ~3 kb respectively (lanes E and B, Figure 7B) and also bands of less intensity. Two major bands (1.5 and 0.8 kb) were obtained with *Xba*I (lane X), indicating the presence of a *Xba*I restriction site within the 25-Dx cDNA. Digestions carried out with various combinations of the three restriction enzymes (lanes E+B, E+X and B+X) allowed us to determine the position of each restriction site on the 25-Dx cDNA and construct the restriction map shown in Figure 8.

Combinations of primers (Figure 3) spanning the 25-Dx cDNA were used in an exon connection strategy based on PCR amplification to detect the presence of introns. In Figure 7B two primers corresponding to the 5′ region of the 25-Dx cDNA (–3 and +57 bp) were paired with three oligonucleotides covering different stretches of the 25-Dx sequence: +799, +1136 and +1197 respectively in lanes A–C. In lanes D–G three primers overlapping the last 350 bp of 25-Dx (+949, +992 and +1000 bp) were paired in different combinations to two primers corresponding to regions +1136 and +1197 of the 25-Dx gene. Patterns of the amplified cDNA were in agreement with the expected organization of the 25-Dx gene. More importantly, these studies indicated that no introns were present to interrupt the coding sequence of the 25-Dx gene. Figure 8 is a schematic map of the genomic organization of the 25-Dx gene based on the restriction digests and PCR analyses.

Discussion

In this study, we used the DD approach to: (i) identify new genes that may be a target for regulation by TCDD; (ii) dissect novel molecular aspects of TCDD action. For these purposes, we isolated and sequenced 30 differentially expressed fragments of cDNA. Of these, seven were identified as known genes and 13 were not affected by TCDD, as measured by Northern analysis (false positives). The remaining 10 cDNAs encoded for unknown genes, as no homology was found in the GenBank database with any of the published gene sequences, but only one was selected for further study since its expression was consistently up-regulated by dioxin. Densitometric analysis of Northern blots (Figure 1) revealed a 3- to

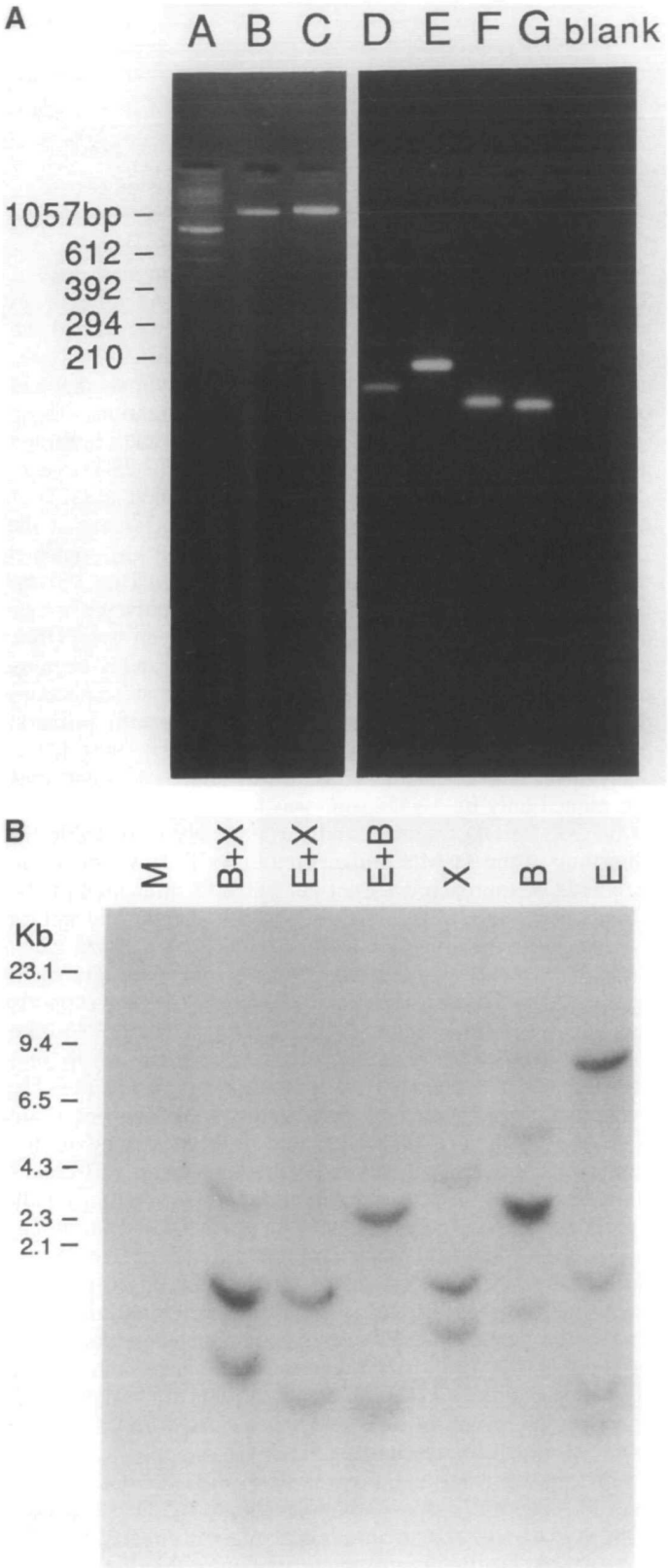

Fig. 7. Genomic analysis of 25-Dx. (**A**) Aliquots of 0.1 μg genomic DNA were used as template for PCR reactions using different pairs of primers. Lane A, the primers used cover the region spanning from –3 to +787; lane B, +57 to +1136; lane C, +57 to +1197; lane D, +949 to +1136; lane E, +992 to +1197; lane F, +992 to +1136; lane G, +1000 to +1136. PCR products were electrophoresed in 3% 3:1 Nusieve agarose–TBE gels and stained with ethidium bromide. (**B**) Aliquots of 5 μg genomic DNA were digested for 6 h with *Eco*RI (E), *Bam*HI (B), *Xba*I (X), *Eco*RI + *Bam*HI (E+B), *Eco*RI + *Xba*I (E+X) and *Bam*HI + *Xba*I (B+X). Digests were electrophoresed overnight in a 1% agarose gel, stained with ethidium bromide and transferred to Duralon-UV membrane in 20× SSC. Samples of 0.5 μg ^{32}P-labeled plasmid DNA containing the entire coding region of 25-Dx were probed with the blot.

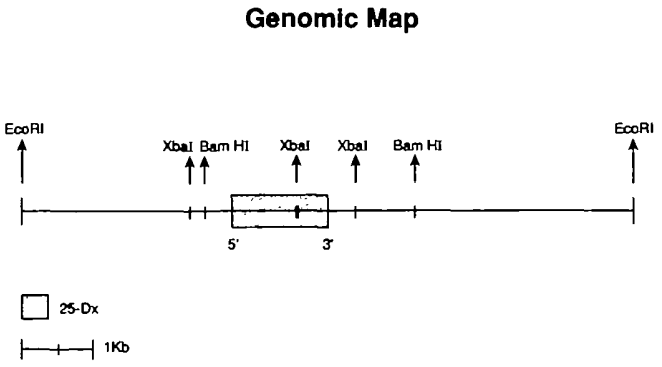

Fig. 8. Genomic map. The genomic map of the 25-Dx gene was predicted based on results from the Southern and genomic (PCR) analyses.

7-fold induction of expression of 25-Dx at the highest dose of TCDD in the acute and chronic groups respectively. In an independent high dose experiment (Figure 2) we studied the expression of 25-Dx by Northern and densitometric analyses, using poly(A) RNA extracted from groups of animals that had been exposed to a wide range of TCDD concentrations. These results strengthened our previous observations and confirmed a stimulatory effect of TCDD on expression of the 25-Dx gene.

Sequence analysis of the 25-Dx cDNA revealed an ORF of 670 bp, encoding for a protein of ~24.6 kDa. The size of the 25-Dx mRNA, based on Northern analysis (~2 kb), matched the size of the sequenced cDNA (1885 bp). The 350 bp fragment isolated by DD did not contain a poly(A) tail or poly(A) signal and was used as a probe to screen two cDNA expression libraries in order to identify both 5′ and 3′ regions of the 25-Dx cDNA. After several attempts that resulted in the isolation of false positives, we designed specific primers, based on the known 25-Dx sequence, to carry out RACE analysis for both 5′- and 3′-ends. A long stretch of adenosines interrupted only by a guanosine was found at the very 3′-end of the 350 bp DD fragment and was probably responsible for annealing of the T_{12}MN primer used in the DD. Although the RACE-PCR approach does not guarantee identification of the 5′-end of a cDNA, several observations support the notion that we have identified the complete ORF of a novel gene. First, the sequence flanking the first methionine is in agreement with the Kozak consensus initiation sequence (27) and supports the fidelity of this stretch of DNA as a start site. Second, analysis of other putative ORFs located upstream of the first methionine and generated by translation of all six possible nucleotide reading frames showed that they did not share homology with any EST sequence or known gene in the database. Third, translation into peptides of several EST clones from human cDNA libraries sharing 85–95% homology with the 25-Dx cDNA indicated that four of them had an amino acid sequence identical or similar (>90% homology) to 25-Dx. Specifically, these sequences covered different or partially overlapping regions of the 25-Dx peptide from amino acid 1 to 183. Two of these EST clones were situated at the 5′-end of their cDNA (see 26), suggesting that there may be no further coding sequence upstream of the first methionine identified in 25-Dx. In addition, one translated EST sequence from rat cDNA (accession no. H35803) was identical to the 25-Dx protein from amino acids –8 to +82. In both human and rat, the EST clones homologous to 25-Dx displayed conservation of the hydrophobic region situated at the N-terminus. Lastly, *in vitro* transcription/translation of a 1200 bp cDNA fragment containing the entire 25-Dx ORF indicated that one single polypeptide is encoded by the 25-Dx cDNA and its molecular weight was in agreement with the size predicted from the amino acid sequence. It is interesting to note that several of the EST clones homologous to the 25-Dx cDNA were sequenced from human infant brain cDNA libraries (26).

Structural analysis of the amino acid sequence of clone 25-Dx revealed interesting similarities with the transmembrane and cytoplasmic regions of the IL-6 receptor (IL-6R). The IL-6R is a protein of 449 amino acids that belongs to the superfamily of cytokine/growth hormone/prolactin receptors. The members of this family share several structural features, including a unique transmembrane domain, an extracellular region containing four conserved cysteine residues and a conserved cytoplasmic domain, called Box 1, which contain a proline-rich sequence known to be involved in protein–protein interaction (28). The IL-6R binds to interleukin-6 through its extracellular domain, which accounts for 75% of the protein. In 25-Dx the hydrophobic region between amino acids 32 and 46 was 71% homologous to the transmembrane domain of the consensus IL-6R (28–29). In addition, a proline-rich region of eight amino acids located in the cytoplasmic domain, near the membrane, is conserved within the superfamily of cytokine receptors (30). This proline-rich domain was found in the 25-Dx sequence from amino acid 62 to 70. It has been documented that this proline-rich region, also known as Box 1, was necessary for the association of the tyrosine kinase Jak2 (31,32) with members of the superfamily of cytokine/growth hormone/prolactin receptors. As with other members of the cytokine superfamily, 25-Dx contains several tyrosine residues in the cytoplasmic region that could serve as potential phosphate acceptor sites during receptor activation. Taken together these observations suggest that the 25-Dx gene encodes a membrane-associated polypeptide and, possibly, a member of the cytokine receptor family. Furthermore, as most of the protein is cytoplasmic, it is possible that 25-Dx may be a mediator of a signal transduction pathway. Finally, several authors have documented the role of TCDD in modulation of the immune system (33–35). In particular, Sutter *et al.* (11) identified plasminogen activator inhibitor factor-2 and interleukin-1β as genes specifically induced by TCDD in human keratinocytes. Both these genes are involved in acute inflammatory responses and growth regulation. Therefore, it is conceivable that 25-Dx may play a role in mediating some aspects of TCDD immunotoxicity.

At the genomic level, Southern analysis suggested the existence of only one gene encoding for 25-Dx. However, hybridization to less intense bands may indicate that one or more related genes may share various degrees of homology with the 25-Dx probe. This finding is in agreement with the hyphothesis that the 25-Dx gene may be a member of a superfamily. Northern analysis confirmed expression of the 25-Dx gene in various rat and human tissues. As high expression levels were detected in brain, lung and liver, but not in skeletal muscle and heart, we surmise that in untreated animals regulation of expression of the 25-Dx gene is tissue specific. Studies to reveal tissue-specific effects of TCDD on 25-Dx expression are in progress.

In summary, we report the isolation and characterization of a novel gene isolated from rat liver. In addition, analysis of the cDNA strongly suggests that 25-Dx may belong to the cytokine/growth factor/prolactin receptor superfamily and thus

may be implicated in mediating TCDD immunotoxicity. Characterization of the polypeptide is in progress to assess the physiological functions of the 25-Dx gene and its tissue and cellular distribution.

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