

Human NAD(P)H:Quinone Oxidoreductase (NQO₁) Gene Structure and Induction by Dioxin^{†,‡}

Anil K. Jaiswal*

Department of Cell Biology, New York University Medical Center, New York, New York 10016, and Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

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ABSTRACT: The human NAD(P)H:quinone oxidoreductase (NQO₁) gene, 1850 base pairs (bp) of the 5' flanking region, and 67 bp of the 3' flanking region have been sequenced. The human NQO₁ gene is approximately 20 kb in length and has six exons interrupted by five introns. The start site of transcription was determined by primer extension analysis. The first exon is 118 bp in length and codes for two amino acids including the initiating methionine and one G for the first codon of the second exon. The sixth exon is the largest among the exons and is 1833 bp in length. The sequence analysis of the sixth exon revealed the presence of four potential polyadenylation signal sequences (AATAAA) and a single copy of human Alu repetitive sequence. The second intron is the smallest of all the introns (116 bp). Nuclear run-on experiments performed using nuclei isolated from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treated and untreated human hepatoblastoma (Hep-G2) cells demonstrated that TCDD treatment increases the rate of transcription of endogenous NQO₁ gene by 3-fold. The sequence analysis of the 5' flanking region of NQO₁ gene showed the presence of a TATA box in the -37 to -32 bp region, one CCAAT box at nucleotide -649, an AP1 binding site at position -462, an AP2 site at nucleotide position -156, and one copy of the nucleotide sequence GCGTG (at position -740) similar to that found in the xenobiotic/dioxin response elements (XREs and DREs) present in the cytochrome P-450_{1A1} (CYP1A1) gene and known for binding to the Ah receptor-inducer complex and increasing the expression of CYP1A1 gene in response to polycyclic aromatic hydrocarbons (PAHs) and TCDD. The sequence analysis also showed the presence of one copy of an antioxidant response element (ARE) between residues -467 and -447 of the human NQO₁ gene. The ARE sequence has recently been identified in the regulatory region of *ya* subunit of glutathione *S*-transferase gene and rat NAD(P)H:quinone reductase gene and shown to mediate the basal expression and its induction in response to planar aromatic compounds and phenolic antioxidants. It is noteworthy that XRE in human NQO₁ gene is located 5' to the ARE compared to its 3' location in the rat quinone reductase gene. The nucleotide sequence of the ARE characterized in the rat quinone reductase gene is highly conserved in the human NQO₁ gene. Interestingly, the consensus sequence for binding to AP1 protein (TGACTCA) is contained within the ARE sequence (TCACAGTGACTCAGCAGAATC) of the human NQO₁ gene. The 1850 bp of 5' flanking region of the NQO₁ gene containing one copy each of the XRE and the ARE, when attached to the CAT gene and transfected into human hepatoblastoma (Hep-G2) and mouse hepatoma (Hepa-1) cells, increased the expression of CAT gene upon treatment with TCDD. By deletion mutagenesis and transfection studies, we have identified a segment of DNA in the upstream region of the human NQO₁ gene (between positions -780 and -365) required for a high level of expression in hepatoma cells and its induction by TCDD.

NAD(P)H:quinone oxidoreductase (NQO₁) is a flavo-protein that catalyzes the two-electron reduction of different quinones, quinone imines, and azo dyes for further metabolism and excretion from the body (Talalay & Benson, 1982; Iyanagi, 1987). Evidence has been presented that NQO₁ may have a protective effect against carcinogenicity, mutagenicity, and other toxicities caused by quinones and their metabolic precursors (Lind et al., 1982; Di Monte et al., 1984a,b).

The NQO₁ activity is known to be inducible by 3-methylcholanthrene (Robertson et al., 1986; Williams et al., 1986), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) (Beatty & Neal, 1976; Jaiswal et al., 1988), and phenolic antioxidants (Prochaska et al., 1985). Our laboratory is interested in investigating the molecular mechanism of NQO₁ induction by

PAHs and TCDD and in particular in determining if the aromatic hydrocarbon (Ah) receptor that mediates PAH- and TCDD-induced transcriptional activation of cytochrome P-450_{1A1} (CYP1A1) gene expression [reviewed in Nebert and Gonzalez (1987), Whitlock (1987) Gonzalez (1989), and Nebert and Jones (1989)] also plays a role in NQO₁ gene induction by the similar compounds. In the present report we have cloned the human NQO₁ gene, determined its exon-intron organization, and shown that the increase in the NQO₁ gene expression by TCDD in Hep-G2 cells is due to an increase in the transcription of the gene. We also show that the 1850 bp of 5' flanking region of NQO₁ gene when attached to chloramphenicol acetyltransferase (CAT) gene and transfected into human Hep-G2 and mouse Hepa-1 cells increased the expression of CAT gene by severalfold upon treatment with TCDD. The sequence analysis of this 1850 bp region of NQO₁ gene revealed the presence of potential binding sites for the aromatic hydrocarbon (Ah) receptor (xenobiotic response element), as were also present in the regulatory region of the CYP1A1 gene in response to TCDD (Nebert & Gonzalez,

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[‡]The nucleic acid sequence of the human NQO₁ gene reported in this paper has been submitted to GenBank under Accession Number J05348.

*Address correspondence to this author at the Fox Chase Cancer Center.

1987; Whitlock, 1987; Gonzalez, 1989; Nebert & Jones, 1989), as well as binding sites for the AP1 and AP2 proteins, known for their involvement in 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induction of gene expression (Mitchell & Tjian, 1989). In addition, sequence analysis also showed the presence of consensus sequence of the antioxidant response element (ARE) identified recently in the upstream region of glutathione *S*-transferase γ subunit gene and rat NAD(P)H:quinone reductase gene (Rushmore et al., 1990; Rushmore & Pickett, 1990; Favreau & Pickett, 1991). Deletion mutagenesis studies on the 5' flanking region of human NQO₁ gene have been performed to identify the DNA segments required for a basal level of expression in hepatoma cells and its induction by TCDD.

MATERIALS AND METHODS

Cloning and Sequencing of the Human NQO₁ Gene. Human liver genomic DNA was isolated (Maniatis et al., 1982), partially digested with *Sau*3A, and fractionated on a 10–40% sucrose density gradient. Fragments of 15–25 kb were collected (Maniatis et al., 1982). The DNA fragments were inserted into the *Bam*HI site of λ EMBL3 (Frishauf et al., 1983). The ligated DNA was packaged in vitro with extracts derived from *Escherichia coli* BHB2688 and BHB2690, as described (Maniatis et al., 1982; Hohn, 1979). Recombinant phage were then plated onto *E. coli* LE392, and approximately 1.5×10^6 plaque-forming units were screened (Jaiswal et al., 1985) by hybridization with human NQO₁ cDNA inserts that had been ³²P-labeled by nick translation (Rigby et al., 1977). Five positive plaques were purified by a minimum of three rounds of plaque purification. DNA was isolated by CsCl gradient centrifugation (Maniatis et al., 1982). Overlapping clones were aligned by restriction endonuclease mapping and Southern blot hybridization analysis using NQO₁-5' (nucleotides 1–657) and NQO₁-3' (nucleotides 658 to the first polyadenylation site) cDNA as the probes. All the positive clones showed a similar restriction enzyme overlapping pattern consistent with a single NQO₁ gene. One of the clones named λ hNQO₁-g-18 was found to contain the entire gene with flanking regions and was used for further studies. Various restriction fragments carrying the entire human NQO₁ gene and approximately 2 kb each of 5' and 3' flanking regions were excised out from the λ hNQO₁-g-18 clone and subcloned in pUC18 for further refined mapping and nucleotide sequencing.

Determination of Exon–Intron Junctions. To find the nucleotide sequences encompassing the exon–intron junctions of NQO₁ gene without sequencing the whole gene, we synthesized several oligonucleotides 15–17 bp in length selected at random from the corresponding NQO₁ cDNA sequence and used these as primers for sequencing the NQO₁ gene fragments subcloned into pUC18 plasmid as published (Sal et al., 1989). This process of sequencing continued until we recovered all of the cDNA sequences from the corresponding gene.

Determination of 5' and 3' Flanking Sequence of the NQO₁ Gene. A 3-kb *Pvu*II fragment from NQO₁ gene containing 5' flanking region, the first exon and a portion of the first intron was excised out by digestion of the λ hNQO₁-g-18 clone with *Pvu*II restriction enzyme and subcloned into pUC18 at the *Sma*I site. The insert was removed by digestion with *Hind*III and *Eco*RI, separated by agarose gel electrophoresis, and used to make a sonication shotgun library in M13mp10 (Deninger, 1983). Sequencing was carried out by standard M13 cloning protocols and the dideoxy sequencing method (Sanger et al., 1977; Messing et al., 1981). Each strand of DNA was sequenced at least five times. Wherever necessary, we also used oligonucleotides synthesized from known sequence as primers

to sequence the pUC18 plasmid containing the 5' flanking DNA of NQO₁ gene. Nucleotide sequence alignments and analysis of nucleotide and protein sequence data were carried out by standard computer programs. By the use of a synthesized oligonucleotide from the 3' end of NQO₁ cDNA, we were able to obtain 67 bp of sequence in the 3' flanking region of the NQO₁ gene.

Determination of Start Site of Transcription for the NQO₁ Gene. We used the primer extension method to determine the start site of transcription in the NQO₁ gene. Twenty-three base pair long synthetic oligonucleotide selected from the 5' leader sequence of coding as well as noncoding strands of NQO₁ cDNA was used as primers. The oligonucleotide selected from coding strand worked as control. The primers after end labeling with ³²P were hybridized to total RNA isolated from TCDD-treated (100 nM for 48 h) and untreated Hep-G2 cells and then extended using AMV reverse transcriptase (Bhat et al., 1990). The primer-extended fragments of 61 bp were detected when oligonucleotide from noncoding strand was used. Nothing was detected with oligonucleotide primer selected from the coding strand because of the similar sequence to that of the NQO₁ mRNA.

Nuclear Run-On Transcription. Nuclear run-on experiments were performed using nuclei isolated from TCDD-treated (100 nM for 48 h) and untreated Hep-G2 cells (McKnight & Palmiter, 1979). The human B-actin DNA and human CYP1A1 cDNA were used as controls.

Construction of NQO₁–CAT Recombinant Plasmids. The 1960 bp DNA segment was excised out by digesting the pUC18 plasmid carrying the 3-kb *Pvu*II fragment of the NQO₁ gene with *Nco*I restriction enzyme. This fragment contained 1850 bp of the 5' flanking region and 110 bp of first exon. The *Nco*I fragment was first treated with mung bean nuclease to remove the initiation of translation codon for NQO₁ protein, followed by treatment with Klenow polymerase, and then cloned into the pBLCAT3 vector (Luckow & Schutz, 1987) after adding *Bgl*II linkers. The resulting recombinant plasmid was called pNQO₁CAT1.85. The deletion mutants pNQO₁CAT1.55 and -0.13 were made by digestion of pNQO₁CAT1.85 plasmid with *Hind*III and *Pst*I restriction enzymes in separate experiments. The *Hind*III enzyme created a deletion of 307 bp from the 5' end of the NQO₁ gene promoter and was named pNQO₁CAT1.55. Similarly, the *Pst*I enzyme created a deletion of 1720 bp from the 5' end of the NQO₁ promoter and was called pNQO₁CAT0.13. The 475 bp (*Sau*3A to *Bgl*II) fragment of DNA containing 365 bp of 5' flanking region and 110 bp of the first exon of the human NQO₁ gene was isolated by digestion of the 1960 bp *Bgl*II fragment with *Sau*3A and subcloned into the *Bgl*II site of the pBLCAT3 vector to give rise to plasmid pNQO₁CAT0.365. The recombinant plasmids pNQO₁CAT1.34, -0.837, and -0.786 were created by controlled digestion of linearized pNQO₁CAT1.55 plasmid with nuclease *Bal*31 and subsequent cloning of different lengths of DNA fragments at the *Bgl*II site of vector pBLCAT3 after adding *Bgl*II linkers (Davis et al., 1986). The 1013 bp *Bgl*II–*Rsa*I fragment from the 5' end of the NQO₁ gene promoter (1960 bp *Bgl*II fragment) was isolated and blunt ended and after adding *Sal*I linkers was inserted into the *Sal*I site of the plasmid pNQO₁CAT0.365 to create the recombinant plasmid pNQO₁CAT Δ 0.837–0.365 with an internal deletion of the DNA segment in the region –837 to –365. The *Sau*3A segment of DNA (in the region –780 to –365) of the NQO₁ gene promoter was cloned at the *Bam*HI site of the vector pBLCAT2 (a derivative of pBLCAT3, containing the basal tk promoter, designed to study

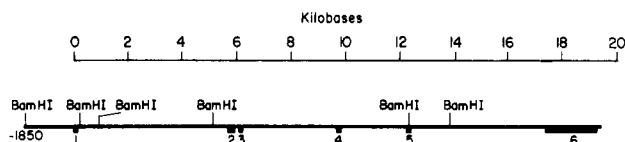


FIGURE 1: Human NAD(P)H:quinone oxidoreductase gene structure. Exons are denoted by black boxes and introns by the thick dark lines. The flanking regions, all the exons, and the second introns were completely sequenced. The sizes of all other introns were determined by restriction digestion and Southern hybridization.

the effect of a regulatory sequence on heterologous promoter, Luckow & Schutz, 1987) and was named pNQO₁tkCAT0.780–0.365.

Transfection and Expression of NQO₁–CAT Recombinant Plasmids into Hep-G2 and Hepa-1 Cells. The NQO₁–CAT chimeric DNAs were transiently transfected into the human Hep-G2 and mouse Hepa-1 cells by the calcium phosphate procedure (Parker & Stark, 1979). The CAT gene expression was monitored by measuring the CAT activity (Davies et al., 1980). The pBLCAT3 is a promoterless plasmid and was used as control. The RSV–βGal plasmid was cotransfected in each case to normalize the transfection efficiency by measuring the β-galactosidase activity (Guarente, 1983).

RESULTS AND DISCUSSION

Exon–Intron Organization and Sequence Analysis of the 5' Flanking Region of the Human NQO₁ Gene. The human NQO₁ gene structure is shown in Figure 1. The 5' flanking sequences, exons, introns, and 3' flanking sequences of NQO₁ gene have been determined (Figure 2). The human NQO₁ gene is approximately 20 kb in length and has six exons of 118, 165, 131, 114, 102, and 1883 bp interrupted by five introns of approximately 5500, 116, 3500, 2500, and 5000 bp in length. All the introns began with GT and ended with AG. Exons 2–5 are remarkably conserved in both nucleotide similarity and actual number of nucleotides between human NQO₁ gene cloned by us and recently reported rat quinone reductase gene (Bayney et al., 1989). The initiation of transcription for human NQO₁ gene was determined by primer extension analysis (data not shown) and is indicated at position +1 (Figure 2). From the primer-extended fragment of 61 bp we determined the size of the first exon as 118 bp. The first exon codes for two amino acids including the initiating methionine and provides one G for the first codon of the second exon. The sixth exon in human NQO₁ gene is 1883 bp in length compared to 907 bp in the corresponding rat gene (Bayney et al., 1989). Sequence analysis of the sixth exon in human reveals the presence of four potential polyadenylation signal sequence (AATAAA) and a single copy of human Alu repetitive sequence between the second and third polyadenylation signal site. In contrast, the sixth exon of the rat gene has only one polyadenylation signal sequence and no Alu repetitive sequence (Bayney et al., 1989). The sizes of introns in human and rat genes could not be compared as the same was not reported for the rat gene except the size of the second intron. The second intron in human NQO₁ gene is the smallest of all the introns (116 bp) and is 46 bp larger than the second intron of rat quinone reductase gene. The TATA box present in many eukaryotic genes and known for its role in correct initiation of transcription (Maniatis et al., 1987) was found located in the –37 to –32 bp region upstream from the cap site of the human NQO₁ gene. The position (distance measured from the initiation of translation codon and not from the cap sites) and nucleotide sequence of the TATA box region are highly conserved between human and rat genes. However, the first exon in human is 118 bp compared to 126 bp in the rat

gene. Sequence analysis also showed the presence of one CAT box sequence (Maniatis et al., 1987) at nucleotide –649. The CAT box is found in many eukaryotic promoters and has been shown to increase the rate of transcription of the gene in which it is present (Maniatis et al., 1987; Mitchell & Tjian, 1989).

Nucleotide sequence analysis (Figure 2) of the 1850 bp of the NQO₁ gene promoter showed the presence of one copy of the sequence GCGTG, the five invariant bases of the xenobiotic response element (XRE) at position –740. The XRE sequences present in the regulatory region of the CYP1A1 gene are known for binding to the Ah receptor–inducer complex and increasing the expression of the CYP1A1 gene (Whitlock, 1987). The sequence analysis also showed the presence of a single copy of the antioxidant response element (ARE) at position –467 of the NQO₁ gene promoter. The ARE motif has recently been identified in the regulatory region of the ya subunit of the glutathione S-transferase gene (Rushmore et al., 1990; Rushmore & Pickett, 1990) and rat NAD(P)H:quinone reductase gene (Favreau & Pickett, 1991) and shown to mediate the basal expression and its induction in response to planar aromatic compounds (e.g., β-naphthoflavone) and phenolic antioxidants. The position and sequence of the rat ARE are highly conserved in the human NQO₁ gene (Figure 2).

We also found the consensus binding sites for the AP1 (at position –462) and AP2 (at position –156) proteins in the 5' flanking region of the NQO₁ gene promoter. The AP1 and AP2 proteins have been shown to affect the transcription of many eukaryotic genes in the presence of TPA (12-*O*-tetradecanoylphorbol 13-acetate) (Mitchell & Tjian, 1989).

NQO₁ Gene Is Transcriptionally Activated in Response to TCDD. Nuclear run-on experiments performed using nuclei isolated from TCDD-treated and untreated Hep-G2 cells demonstrated that TCDD treatment increases the rate of transcription of NQO₁ gene by 3-fold (Figure 3). In the same experiment the human CYP1A1 gene transcription was found elevated 10-fold due to TCDD treatment as expected from previous studies (Cresteil et al., 1987). The low magnitude of the increase in transcription of the NQO₁ gene may be due to the high basal level of transcription of the NQO₁ gene in Hep-G2 cells (Figure 3).

Basal Expression and TCDD Induction of Human NQO₁ Gene Are Expected To Be Localized within a 415 bp Fragment of the DNA in the Upstream Region of the NQO₁ Gene. The NQO₁–CAT recombinant plasmids (pNQO₁CAT1.85, –1.55, –1.34, –0.837, –0.786, and –0.13) were transiently transfected into the human Hep-G2 and mouse Hepa-1 cells, and the CAT gene expression under the control of different lengths of promoter (as represented by numbers in kilobases) was monitored in the presence and absence of TCDD. As shown in Figure 4, the mouse Hepa-1 cells transfected with plasmid pNQO₁CAT1.85 expressed the CAT gene at high level, which increased in a dose-dependent fashion upon treatment with different concentrations of TCDD. In a related experiment our measurements showed that when we used plasmid pNQO₁CAT1.85 for transfection into Hep-G2 and Hepa-1 cells, the basal CAT activity was 31- and 111-fold higher compared to the promoterless plasmid pBLCAT3 (Figure 5). Treatment of the transfected Hep-G2 and Hepa-1 cells with 100 nM TCDD showed an increase of approximately 4- and 3-fold, respectively, in the expression of the CAT gene (Figure 5). An increase in the concentration of TCDD of more than 100 nM for treatment did not result in a further increase in fold induction of CAT activity in either cell type (Data not shown). Similar levels of basal and induced expression of CAT

-1850

*****GGGTGACCTGCAGGTCACCGATCCTGCCACTGCATCCAGCCTGGGCGACACA
GTGAACCCCGCCTCAAAAAATAATTTTTAAAAATAATTTAAATACAATAAATCAACT
TCTTCTCAGTCCACTGGCCACTTTTTTTTAAATTTCCATTTTTTTTCTTTTATG
AGTCCACCACTGCGGCCTTGAATTCCTGGGCTCAAGCGATCTTTTGGCTCAGATCCTGA
GTAGCTGGGACACAGGCGCTGCCACACGCGCTGGCTGCTGGCCACATTTCCAG*GCTCA
ATAGCTACTGAAGCTTGTGGCTACTTCTGGGCAGCAGATAGGGAAGTTCTATT
GGACAGGCTGCTACCTAGCCCTTTTTTAAACGCTCTTTTATACATTTTGTGCAGATG
GGGTCTCACTTTGTGCTCAGGCTTTTCTCAAACTCTTGGGCTCAAGTGATCCTCCTGCC
TCAGCTCCCAAAGTAATTTGTAATACGTTGTAATAATATAGTCTCTCTTGTGTGCATC
AAAAATAAGTCAAAATGTACAGTGGGCGCGGCGCGGTGGCTCATGCTGTAATCCCA
GCATCTCGGAGGCGGAGGCGGTAGGATCACCTGAGGTGCGGAGTGGAAACAGTTTTC
TGGTGCTCTTAACCTTTTCTGGTGCCTCTTAACCTTTGCACTGGAGGGAACACGGAAG
GGCCCTGTGTACCTCCTACCTCCCCCATCTGTAATTTCTTCTGATGGTTAATTACCACTTA
AAGCTCTAGTTCTTTTCTTCACTACTGAGAGTATATCTCCTCTTTTACAGTAACCTT
TACTTTATAGAGTTTTTTTGAGACGGAGTCTCACTTCGTCAACCGGCGGAGTGCACT
GGCAGATCTCGACCACTCAACCTCCGCTTCCGGGTTCAAGCGATCTCTCTGCTCA
GCTCCGAGTAGCTGGAGCTACAGGCGCGGCGGCTACGCGCACTAATTTTTCAGTA
CAGACGGGCTTACCAGGTTGGCCAGGCTGCTCGAAGCTCCTGACCTCAGGTGATCCA

-740 XRE -736

CCCACCTCGGCTCCCATATTGCTGGGATTACAG**CGGTG**AGCACCGCGCTGGTCCC
GTTTTGGCTATTCTTTAATATGCTTCCCTATAACTGCTATCTCCACACCGCTTGCCTT

-649 CAT BOX -645

AGCTCATGGGAACAAA**CCAAT**TAGTTTCCCCATTACCTGCCTTGAGGAGCAGGGGT
GGTGCAAGTGGCATGCACCGGGAAGTGTGTGTATGGGCCCCAAGTGACAGAACTGAA
TCTTGCAGGAAGAAACAAATTCGTCTCCACGGAGCATGTCTCCCGAGGACTCTCAGCC

-467 ARE -447 & -461 AP1 -455

TTCCAAATCGCAG**TCACAGCTGACTCAGCAGAATC**TGAGCCTAGGGCA
CCACAGTAATGCGATCGGCTCTTTTTTGTGCTCAATTCGTGCCAAGGCTAAAGGGCATG
CCCCTTGATCTGAGCTCTCTTGGGAGGACTTCCACCTGCATCCTCTTGCACCTCAGG
GCACAGTGGCAGATGGGCTTGCCTTAGCAGCCCCAGCCAGATTTTGGAGGCTCTGTCA
CACACACCCCTACAACTCCCTCCCGGAGGCTTTCTTGAGCTTCCACAGCTT

-157 AP2 -149

GCTCCGGCGGGTGAGAGTGGAGAGGCGCCTCTTCTATC**CCCCAGGCT**CCCTCCCT
TCCTGGAGCTGCAGCTCAGCATCTCCGCCAGCACCCAGGATTACGGCTTGGGTCC

-37 TATA BOX -33

CGCCCTTGAGGCTCTCCACCTCAAAACGGGCGGACAGGATA**TATAA**GAGAGAAATGC

+1

ACCGTGCACTACACACGCGACT CCCACAAGGTTGCAGCGGAGCGGCCAGCTCA
CCGAGAGCCTAGTTCCGGCCAGGTCGCCCCGCAACACGAGCCAGCCAATCAGCGCC

M V

CCGAGCTGCACAGAGCC ATG GTC GTGAGTGCTGCAAAAGGCGGGGTG
CTTGGCGGTGCTCTCGAGACCTGGTGCCTGTGGAGGAGGTTGTAGGGGCTTGGCGCTA
ATTTTGTCTTGTACTCAAAACCCACAAAGGGAAGAGATTAGGGACCTGGGATGAGCCTT

GATCGGACTCCGGGAGGAGGAGGCTTCTGTTTGTATCCGGTGTGCGGGATCCTTCTCA
CTCCACCCCGAAGCTCTAGTGGGCAATTTAGGAGAACTTGACCTTGGAGCGAATAATC
CTCCAAAAGCTGTGGTCAGAACTGGCCGTGACAGGCGCAAACTCAGGCTTACGGTCAAGC
CCATACCTCTTTTGGTCTATAGTATCTAAACACTTTGAAATAAGTTGCTCAACATTAATA
TGGGTGATTCTTCACTTAAATGTGGATTCCGAGCTTCTGAAGAAATGAGAATATCTGGC
GATTGGTGGACAGGAGTGGAGGAACTGATGCTCTTGGTTTACGAGGAGACGAGAACCGGC
TCCAGGCTCGGAGCAGAGGTTTGTGTTTGTGTTTGTGTTTGTGTTGAGACGAGGTT
TGCTCTTGTGACCCAGGTTGTAGTCAATGGCGCTATCTCAGCTCAGCGAACTTCCGCTT
CCCGGGTTCAAGCGATTCTCCCATCTCAGCCTCCGAGTAGCTGGGATTACAGGAGTGGC
CCATCAGCGCGGTAATTTTATATTTTATAGTAGAGCGGGTTTCGCCAAGTTGGCCAG
CGCGGTCTTGAACCTCCTCAGCTCAGGTATCCACCCACCTCAGCTCCCAAGTGGTGTGAG
ATTACTGACGTGAGCCAGCGCTGCTGCGCTTGGGAGGCAATTTCCATTGAGGCGCTTGA
TTAGGCAAACTGATCAGATCGTACTGGATCCGTTTCTAGTATTGGGTCCACTTCACTGTA
TAGAGCTCTCCCACTCGGCCCTGACCCCTAGGGGAACAGAGGTCACCCCGCTCCACC
CAAGCATATAATAACCAAGCTGAGACAACTGCAG-----**INTRON 1**-----

-----TGCGAGGTGCTCAAACTCCTGACCTCAGGTGATCTGCCCGCTTGGCCTCCCA
AAGTAGCTGGGATTACAGTTATGAGCCAGCCAGTCTGCAACATTTATTTAAAT
CAGCCATGATTTAAATGCTTCTTTAATCTTCACTGCTTTTAAAGGGAAGCAAGTCTTCT
GGCTACAGAGATGGAATGCAATAACCTAATGTCAACAAAGCGCCCTCCTTTACAG
ACTGCAACTCCCCGTGAGGTTTGTGCTGGTGGTATGGGTTTCCCTGTGTGCTT

G R R A L I V L A H S
TGTGTGTCTCTGTAG GC AGA AGA GCA CTG ATC GTA CTG GCT CAC TCA

E R T S F N Y A M K E A A A A
GAG AGG ACG TCC TTC AAC TAT GCC ATG AAG GAG GCT GCT GCA GCG

A L K K K G W E V V E S D L Y
GCT TTG AAG AAG AAA GGA TGG GAG GTG GAG TCG GAC CTC TAT

A M N F N P I I S R K D I T
GCC ATG AAC TTC AAT CCC ATC ATT TCC AGA AAG GAC ATC ACA G GT

AGGAGGAGTTCTCTCTCCCTCTTTAATAGTTCTTTTGGGATCTCCTTGCTGTGGGTG

INTRON 2

CTCTGGCCAGCTTTGGCCCTCTGGCCCGAGCCTCTTTTTTCTTCTCTGCG GT AA

L K D P A N F Q Y P A E S V L
A CTG AAG GAC CCT GCG AAC TTT CAG TAT CCT GCC GAG TCT GTT CT

A Y K E G H L S P D I V A E Q
G GCT TAT AAA GAA GGC CAT CTG AGC CCA GAT ATT GTG GCT GAA CA

K K L E A A D L V I F Q
A AAG AAG CTG GAA GCC GCA GAC CTT GTG ATA TTC CAG GTATGGGGGG

ACATCGGAAGGGGTGTCAGGACATTTGCGTGCTTATTGTCTAGACATGACCTAATTA
GCTATGAGATCTTAAACAGTTTACCACTTCTGCTGCTGCAAAATAGGGTGATTAC
TTTAAAGGTGCGATTTACTGGATTTCACGATAT---**INTRON 3**---CCAACAGTCA
TATTGTTGATACCACTTACTAAGCAAACTCTTGGAAATACCTGATTGAAAGCAACAGC
ATGAAGCAAACTTGTGCAATCCTTGTCTATTAGACTTTGCTGCCAAATGTGATTCA

TACAAACAAGTTTCATATCAACAAGTCTTCTGTGTATCTAGCTTTACTCGGACCCACTCA
ATATTTGCATTTTCAGGGAAGAAAGACTGTCAAGTTGGCTGACCAAGGACAATAATGA
TCTCTTCCCTTAAAGTGCTAATCCCCAGGAGGAATGGGAAGGTGTGAAGAGGGGGTTC
CCACACAGTGCCATCATGGGAGCGGCTCAGCACTCCGAGCCACCTTCTGGGCTTGGGA

GCCCTTGGTCTTACCTCAATGATGTCTTCTGTCCACAG TTC CCC CTG CAG TGG

F G V P A I L K G W F E R V F
TTT GGA GTC CCT GCC ATT CTG AAA GGC TGG TTT GAG CGA GTG TTC

I G E F A Y T Y A A M Y D K G
ATA GGA GAG TTT GCT TAC ACT TAC GCT GCC ATG TAT GAC AAA GGA

P F R

CCC TTC CGG GTAGGTGGATGTTTCTGAATGCTCTGACAGCCAGCTTCTGGGTGGTCT

GTACTGATGCAGGGGTGTTTGTGTTTGTGTTTGTGTTGAGATGGAGCTTCCCTCTT----
INTRON 4----AGTCCAGCCCTGGGAGGATGGATAAGAACAACCAATAACTCCACCT
AGGAGCCAGCAGCCAGCACTGATTTTGGATATGGTGACAGCCTCGCTTAAAGCCACATG
TGGGGGTTTGGAACTCCTTTCATTTCGCCAGGCCAGCGTCTGTAGACCATCTAGGCTGGC
ACTCCTGCAGAGTCATATTATGTAGCTCAGGGGAGCCAAAGATGTAAGTAATATAACAGC
CAAAATAGGACAGACTTGTGTGTTCTTTTCCCTGGAGCTGTTGATTAACTTCTTGTGCAAC

AAGGGGATGTGGAGGGAAGTGAAGTTTGTATTGCTTTTCTTTTTCGAG AGT AAG AA

A V L S I T T G G S G S M Y S
G GCA GTG CTT TCC ATC ACC ACT GGT GGC AGT GGC TCC ATG TAC TC

L Q G I H G D M N V I L W P I
T CTG CAA GGG ATC CAC GGG GAC ATG AAT GTC ATT CTC TGG CCA AT

Q

T AG GTACCTCATTTTCTTTTCTTTTCTTTTCTTTTCTTTTGTGAGACGGAGTTACTCTGTG

ATCTAGGCTGGAGTGCAGTGACACCATCTTAGTTCACTACAAGCTCCGCCGCTGGGTTC
GAGCAATTTCTCCTGCTCAGCCTACTGACAGGCACCCGCCACCACTCGGCTAATTTT
TGTATTTTTTAGTAGAGACGGGCTTGGCCATGTTAGCCAGGCTGGTTTCAACACTTGA

CTCAAAATGATTCTCCTGCCTCAGCCTCCCAAGGTGCTGATTACAGGCGTGAGCAGT
CACCTGGCCAGCCCTCATTTTTCACGAACTTTCAGGGGAATTTATCTATTGAG-----
INTRON 5----GAGAGAGACGCTAGCTCTGAAGTATTCTCTAGTGTGCTCAGGC
CTCCTTATCAGAGTGCTTACTGAGAAGCCCAACTCTCTGTTGTATAGCAACT

GCATGGAATTGGTTGACTTACCTCTCTGTGCTTTCTGTATCTCTCAG AGT GGC ATT C

L H F T C G F Q V L E P Q L T Y
TG CAT TTC TGT GGC TTC CAA GTC TTA GAA CCT CAA CTG ACA TAT A

S I G H T P A D A R I Q I L E
GC ATT GGG CAC ACT CCA GCA GAC GCC CGA ATT CAA ATC CTG GAA G

G W K K R L E N I W D E T P L
GA TGG AAG AAA CGC CTG GAG AAT ATT TGG GAT GAG ACA CCA CTG T

Y F A P S S L F D L N F Q A G
AT TTT GCT CCA AGC AGC CTC TTT GAC CTA AAC TTC CAG GCA GGA T

F L M K K E V Q G D E E K N K K
TC TTA ATG AAA AAA GAG GTA CAG GAT GAG GAG AAA AAC AAG AAA T

TT GGC CTT TCT GTG GGC CAT CAC TTG GGC AAG TCC ATC CCA CT G

D N Q I K A R K *
AC AAC CAG ATC AAA GCT AGA AAA TGA GATTCTTAGCCTGATTTCCTTCT

AACATGTTATCAAACTGGGTATCTTTCCAGGCTTCCCTGACTTGTCTTATGTTTAAAGA
TTTGTGTTTTTCTTTTCCACAAGGAATAAATGAGAGGGAATCGACCGTATTCGTGCAAT
TTTGGATGCAATTTTAACTGATTCTTATGATTACTATGCAATATCAACCAAAATCCGA
CTGGGCTCAAGAGGCCACTTAGGGAAGATGTAGAAGATGCTAGAAAATGTTCTTTAA
AGGCATCTACCAATTTAATCTCTTTTATGGGCTAAAGTTTATGGGTACAGTTTGGCT
AGGTATCATCTCAACTCTCCAATGTCTATTATACCTCTCTGTGATTTATGGCAGAAAG
GAATTGCTCAGAGAAAGTAAAAGACTGAATCTACCTGCCCTAAGGGACTTAACTTGTGTT
GTAGTTAGCCATCTAATGCTTGTGTTATGATATTTCTTGTCTTCAATTACAAAGCAGTTAC
TAATATGCCTAGCAAGTACCACCTTGGTGCAGCTTTGTTGTTTATATACAGTACACA
GATACCTTGAAGGAAGAGCTAATAAATCTCTTCTGCTGCAGTCATCTACTTTTCTTT
TAATTAATAAAAAATTTTTTTTGAAGCAGTCTTGTCTGTTACCCAGGCTGGAGTGCAGT
GGTGTGATCTCGGCTCACTGCAACCTCTGCCCTCCAGGTTCCAGCAATCTCTCTGCTCA
GCCTCCCTAGTAGCTGGGATGACAGGCGCTGCCATAGCTGCAATTAATTTTGTATTT
TAGTAGAGAGCGCGTTTACCATTGTGGCCAGGCTGGTCTCAAACTCCTGACCTCAGGT
GATCCGCTACCTCAGCCTCCCAAGTGTCTGGGATTCAGCGGCTGACTCCACACACCTG
GCCCTTGAATCTTCTACTTTAAGGTTTGCAGAGATAAACCAATTAATCCACACCTGTACA
TCTGCAATATGAATTTCAAGAAAGGAATAGTACCTCTCAATACTTAAATCTTCTTCCA
CAAAAAATCTTTATTTCTGATCTATACAAATTTTCAAGGTTATTTTCTTTATCATTG

CTAAACTGATGACTTACCATGGGATGGGTCAGTCCCATGACCTTGGGGTACAAATTTGTA
AACCTAGAGTTTATCAACTTTTGGTGAACAGTTTGGCATTAATAGTCAATTTCTACTTCT
GGAAGTCATCTCATCTCACTGTTGGTATTATATAATCAAGGAGAATATGATAAAACACT
GCCCTCTTGTGGTGCATTTGAAAGAGAGATGAGAAATGATGAAAGGTTGCTGAAATAAT
GGGAGACAGCCTCTTACTTGGCAAGAAAATGAAGGATTTGGACCGAGCTGGAAACCTCT
TTACCAAGTGCTGACTGGCAGTGGTGGTGTGTTGCTCTGCAGCATGTCCCAATAGCTGTA
CGGCTGGGTGTTCAGTTTGTGAAATATTTTGTGCTCTGATCTTCACTGCAATTTGTGT
AAATTTCTCAAGATCTGAATTAATAAATAAATTCATTCTTACAGACCA -----C
ACTCAATGGTATGCTGAAGCTAATGATAGTGAATCTAGTGGAGGCGAGATCCGTGAC
CTGAG

FIGURE 2: Nucleotide and amino acid sequence of the human NQO₁ gene and flanking regions. A total of 1850 bp of the 5' flanking region, exon-intron junctions, and 67 bp of the 3' flanking region were sequenced. The start site of transcription is designated as +1. The position and sequences of TATA and CCAAT boxes, AP-1 and AP-2 binding sites, xenobiotic response elements (XREs), and antioxidant response elements (AREs) are denoted in bold letters.

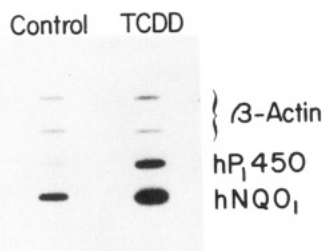


FIGURE 3: Nuclear transcription run-on experiment of the NQO₁ and hP₁₄₅₀ (human CYP1A1) genes in TCDD-treated and untreated human hepatoblastoma (Hep-G2) cells. RNA transcripts from TCDD-treated and untreated Hep-G2 nuclei hybridizing to B-actin, hP₁₄₅₀ (CYP1A1), and human NQO₁ cDNA inserts are shown. The hP₁₄₅₀ and hNQO₁ cDNA inserts determine the rate of transcription of human CYP1A1 and NQO₁ genes, respectively. B-Actin gene transcription in Hep-G2 cells is unaffected by TCDD treatment.

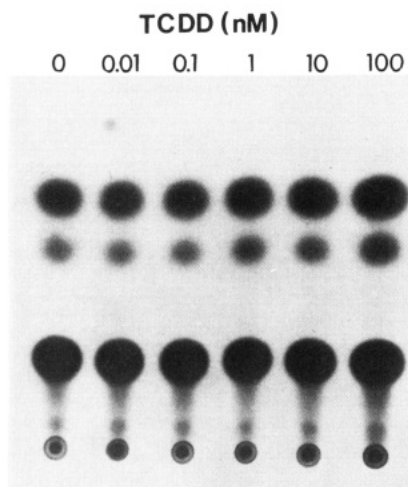


FIGURE 4: Transient expression and TCDD induction of pNQO₁CAT1.85 recombinant plasmid into mouse hepatoma (Hepa-1) cells. Ten micrograms of the pNQO₁CAT1.85 recombinant plasmid containing 1.85 kb of the 5' flanking region of the human NQO₁ gene promoter was transiently transfected into mouse Hepa-1 cells by the calcium phosphate procedure. The CAT gene expression was monitored by measuring the CAT activity. RSV-βGal plasmid was used in each case to normalize the transfection efficiency. The transfected cells were treated with different concentrations of TCDD as shown on the top of each lane for 24 h.

gene were also observed when plasmids pNQO₁CAT1.55, -1.34, -0.837, and -0.786 were used for transfection (Figure 5). However, highly significant decrease in the basal levels of CAT gene expression and no induction upon treatment with TCDD were observed with plasmids pNQO₁CAT0.0365 and -0.13 (Figure 5). These results might indicate that the DNA segment between -0.837 and -0.365 kb of the NQO₁ gene promoter is essential for high basal expression of CAT gene and its induction by TCDD. To confirm this, we used the plasmid pNQO₁CATΔ0.837-0.365 (containing an internal deletion of the region between -0.837 and -0.365 from the initial 1.85-kb plasmid of the NQO₁ gene promoter) for transfection into Hep-G2 and Hepa-1 cells. As expected, this plasmid expressed very low levels of CAT activity which was not inducible by TCDD (Figure 5), indicating the need for the deleted fragment (-0.780 to -0.365) for high basal expression and its induction in response to TCDD.

The *Sau*3A fragment of DNA (region between -0.780 and -0.365 kb containing the cis-acting DNA elements responsible for high levels of expression of the NQO₁ gene in hepatoma cells and its induction by TCDD) was attached to the basal thymidine kinase promoter (no enhancer) hooked to the CAT gene by cloning in the vector pBLCAT2, a derivative of

pBLCAT3, to test if this segment of DNA can confer its effect on the heterologous promoter. This recombinant plasmid pNQO₁tkCAT0.780-0.365 upon transfection into Hep-G2 and Hepa-1 cells showed 10- and 57-fold higher levels of CAT activity, respectively, compared to the control plasmid pBLCAT2 which increased approximately 5-fold in case of both cell lines upon treatment with 100 nM TCDD (Figure 5), clearly establishing that the 415 bp *Sau*3A fragment indeed contains cis-acting DNA elements responsible for high levels of expression of NQO₁ gene in hepatoma cells and its induction by TCDD. The nucleotide sequence analysis of this 415 bp fragment of DNA indicated presence of one copy each of ARE and XRE. Because the ARE sequence is highly conserved between human and rat quinone oxidoreductase gene (Figure 6), it is possible that the human ARE, as in the case of rat, mediates the high level of expression of the NQO₁ gene in hepatoma cells, but this remains to be proven by further experiments. Interestingly, the sequence analysis of the human NQO₁ gene showed presence of a consensus binding site for AP1 protein contained within the ARE sequence (Figure 2). Extensive studies have clearly established that the AP1 protein forms homo- and heterodimers with itself and other members of the AP1 family of proteins (e.g., junB, JunD, fos, fra1, etc.) through leucine zipper motifs and plays an important role in transcriptional regulation by creating a variety of transcription factors with different functional properties [reviewed in Mitchell and Tjian (1989)]. Moreover, recently, an AP1 binding site located between nucleotides -58 and -65 in the regulatory region of the glutathione *S*-transferase π gene has been shown to be required for a basal level of expression into several different human cell lines including Hep-G2 (Xia et al., 1991). The presence of a perfect binding site for AP1 protein in human ARE might indicate its role in basal expression of the NQO₁ gene and remains to be investigated by further experiments. It is noteworthy that in the case of the rat ARE the first five bases of the consensus binding site for AP1 protein are conversed.

Involvement of Ah Receptor in the Induction of NQO₁ Gene Expression by TCDD. It is well-known that PAHs and TCDD induce the expression of a group of genes involved in the xenobiotic metabolism [reviewed in Nebert and Gonzalez (1987)]. Among these genes the best studied is the CYP1A1 gene induction by PAHs and TCDD (Nebert & Gonzalez, 1987; Whitlock, 1987; Gonzalez, 1989; Nebert & Jones, 1989). In brief, the TCDD and PAHs bind to the protein product of the Ah locus, the so-called Ah receptor. Following translocation of the inducer-receptor complex into the nucleus it recognizes and binds to xenobiotic/dioxin response elements (XREs) with five invariant bases, GCGTG, in the 5' flanking region of the CYP1A1 gene, resulting in the transcriptional activation of the gene. Increased mRNA concentrations lead to increased levels of the CYP1A1 protein and consequently to an increased capacity to metabolize benzo[*a*]pyrene and other aromatic hydrocarbons. It should be noted that the induction of expression of CYP1A2 gene by TCDD is presumably not mediated by the Ah receptor because the induction of this gene in the rat is regulated primarily at the posttranscriptional level (Silver & Krauser, 1988). Also, no XREs have been found in the mouse, rat, and human CYP1A2 genes (Ikeya et al., 1989), further supporting the notion that induction of the CYP1A2 gene is due to post-transcriptional modifications. Because both CYP1A1 and NQO₁ genes are induced by TCDD, it will be interesting to find out if these two genes are coordinately regulated by the same Ah receptor. The small quantity of data available in-

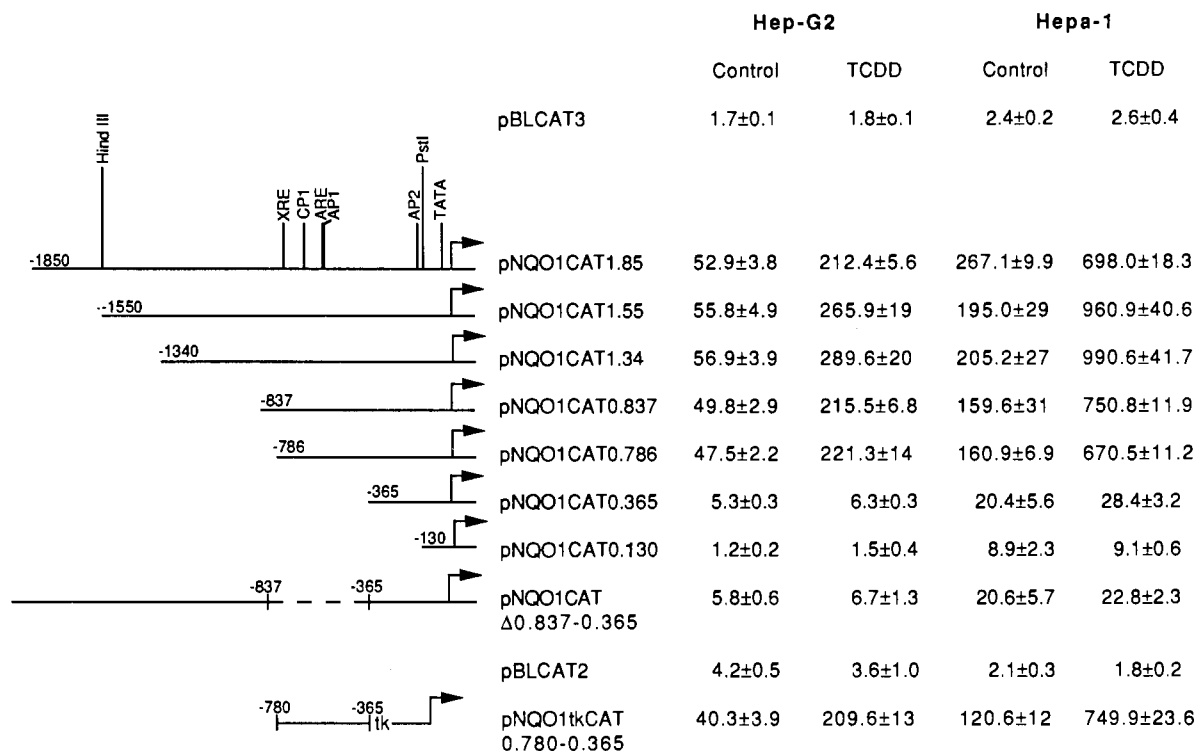


FIGURE 5: Transient expression and TCDD induction of NQO₁-CAT recombinant plasmids in human hepatoblastoma (Hep-G2) and mouse hepatoma (Hepa-1) cells. The NQO₁-CAT chimeric plasmid DNAs from 1.85 to 0.13 contain different lengths of the NQO₁ gene promoter as represented by numbers in kilobase pairs in each construct. The plasmid pNQO₁CATΔ0.837-0.365 contains an internal deletion of -0.837 to -0.365 kb in the 1.85 kb of initial NQO₁ gene promoter. The *Sau*3A fragment (region between -0.780 and -0.365 kb) of the NQO₁ gene promoter was attached to the tk promoter hooked to the CAT gene to construct the plasmid pNQO₁tkCAT0.780-0.365. The pBLCAT3 is a promoterless plasmid, and pBLCAT2 is a derivative of pBLCAT3 containing basal tk promoter. The pBLCAT3 and pBLCAT2 plasmids were used as control plasmids. The various CAT plasmids were transiently transfected into the Hep-G2 and Hepa-1 cells, and their expression in the absence and presence of TCDD (100 nM for 24 h) was monitored by measuring the CAT activity. RSV-βGal plasmid was cotransfected in each case to normalize the transfection efficiency.

Rat ARE TCACAGTGAAGTTGGCAAAATC
 Human ARE TCACAGTGAAGTCAGCAGAATC

FIGURE 6: Comparison of the nucleotide sequence of human and rat NAD(P)H:quinone oxidoreductase AREs. The AREs found in human and rat NAD(P)H:quinone oxidoreductase gene have been aligned to show the sequence homology. The sequence for rat ARE has been taken from recently published work of Favreau and Pickett (1991). The human ARE was identified by nucleotide sequence analysis of the human NQO₁ gene promoter. (:) denotes that the bases are identical.

indicates conflicting views on the involvement of Ah receptor in the induction of NQO₁ gene by PAHs and TCDD. It has been suggested (Kumaki et al., 1977) primarily on genetic grounds that the same Ah receptor mediated process involved in the CYP1A1 gene activation might be responsible for enhancing NQO₁ activity. However, an obligatory association of the induction of NQO₁ and CYP1A1 gene has not been established. More recently, an alternate mechanism has been suggested that first involves the metabolism of PAHs by increased CYP1A1 activity to electrophilic products which in turn provide a redox signal for increased expression of the NQO₁ gene (De Long et al., 1987). In this case the Ah receptor would be indirectly involved in the induction of NQO₁ gene expression by PAHs by interacting with sequences in the 5' flanking region of CYP1A1 gene rather than the NQO₁ gene. Recently, a similar type of mechanism mediating through an antioxidant response element (ARE)/electrophile response element (EpRE) has been reported for the induction of expression of the ya subunit gene of glutathione S-transferase and the rat quinone reductase gene in response to polycyclic aromatic hydrocarbons and phenolic antioxidants

(Rushmore et al., 1990; Rushmore & Pickett, 1990; Friling et al., 1990). However, Favreau and Pickett (1991) suggested an XRE-mediated mechanism of induction for the rat quinone reductase gene by TCDD. We, working on the regulation of human NQO₁ gene, have identified a 415 bp fragment of DNA (the region between -0.780 and -0.365) in the 5' flanking region as responsible for induction of the NQO₁ gene expression in response to TCDD. The nucleotide sequence analysis of this fragment of DNA indicated presence of an XRE sequence at position -740 which is 5' to the human ARE (Figure 2) compared to its 3' location with respect to ARE in the rat quinone reductase gene (Favreau & Pickett, 1991). It remains to be investigated if this XRE sequence binds to Ah receptor and mediates induction of the NQO₁ gene expression by TCDD.

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