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Nucleotide and Deduced Amino Acid Sequence of a Human cDNA (NQO_2) Corresponding to a Second Member of the NAD(P)H:Quinone Oxidoreductase Gene Family. Extensive Polymorphism at the NQO_2 Gene Locus on Chromosome 6[†,‡]

Anil K. Jaiswal,[*,§] Paula Burnett,[∥] Milton Adesnik,[§] and O. Wesley McBride[∥]

Department of Cell Biology and Kaplan Cancer Center, New York University Medical Center, 550 First Avenue, New York, New York 10016, and Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: NAD(P)H:quinone oxidoreductases (NQOs) are flavoproteins that catalyze the oxidation of NADH or NADPH by various quinones and oxidation–reduction dyes. We have previously described a complementary DNA that encodes a dioxin-inducible cytosolic form of human NAD(P)H:quinone oxidoreductase (NQO_1). In the present report we describe the nucleotide sequence and deduced amino acid sequence for a cDNA clone that is likely to encode a second form of NAD(P)H:quinone oxidoreductase (NQO_2) which was isolated by screening a human liver cDNA library by hybridization with a NQO_1 cDNA probe. The NQO_2 cDNA is 976 nucleotides long and encodes a protein of 231 amino acids (M_r = 25 956). The human NQO_2 cDNA and protein are 54% and 49% similar to human liver cytosolic NQO_1 cDNA and protein, respectively. COS1 cells transfected with NQO_2 cDNA showed a 5–7-fold increase in NAD(P)H:quinone oxidoreductase activity as compared to nontransfected cells when either 2,6-dichlorophenolindophenol or menadione was used as substrate. Western blot analysis of the expressed NQO_1 and NQO_2 cDNA proteins showed cross-reactivity with rat NQO_1 antiserum, indicating that NQO_1 and NQO_2 proteins are immunologically related. Northern blot analysis shows the presence of one NQO_2 mRNA of 1.2 kb in control and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treated human hepatoblastoma Hep-G2 cells and that TCDD treatment does not lead to enhanced levels of NQO_2 mRNA as it does for NQO_1 mRNA. Southern blot analysis of human genomic DNA suggests the presence of a single gene approximately 14–17 kb in length. The NQO_2 gene locus is highly polymorphic as indicated by several restriction fragment length polymorphisms detected with five different restriction enzymes. The NQO_2 gene was localized to human chromosome 6 by Southern analysis of human–rodent somatic cell hybrids. Further analysis of several hybrids containing breaks or translocations involving chromosome 6 allowed regional localization of the NQO_2 gene to chromosome 6pter-q12.

NAD(P)H:quinone oxidoreductases, formerly known as DT-diaphorases (EC 1.6.99.2), are flavoproteins that catalyze the oxidation of NADH or NADPH by various quinones and oxidation–reduction dyes (Lind et al., 1982; Thor et al., 1982; Morrison et al., 1984; Di Monte et al., 1984a; 1984b; Ernster et al., 1960). The physiological functions of these enzymes are not yet understood though they seem to be involved both in the detoxification of nonphysiological quinones (Ernster et al., 1982) and in the bioactivation of vitamin K (Stenflo et al., 1974). In rat liver the oxidoreductase activity is found mainly (approximately 95%) in the cytosolic fraction, but 5–10% of the total cellular activity is recovered in the mi-

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[*] To whom correspondence should be addressed at the Department of Cell Biology, New York University Medical Center, 550 First Ave., New York, NY 10016.
[§] New York University Medical Center.
[∥] National Institutes of Health.

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crosomal (Danielson et al., 1960), mitochondrial (Conover & Ernster, 1963), and Golgi subfractions (Edlund et al., 1982). Several reports concerning the purified proteins and/or antibodies raised to these proteins suggest the presence of at least three different forms of rat liver cytosolic DT-diaphorases (Hojeberg et al., 1981; Raftell & Blomberg, 1980; Prochaska & Talalay, 1986). In human tissues a complex series of oxidoreductase enzymes encoded by four different gene loci (Dia 1 to Dia 4) catalyzing the oxidation of NAD(P)H and using the quinoid redox dye 2,6-dichlorophenolindophenol as electron acceptor have been described (Leroux et al., 1975; Fisher et al., 1977; Edwards et al., 1980). Recently we cloned and sequenced the cDNA encoding human dioxin-inducible cytosolic NAD(P)H:menadione oxidoreductase (NQO_1),[1] which is likely to represent the product of the diaphorase 4 gene locus (Jaiswal et al., 1988). In the present study we report the nucleotide sequence of a cDNA representing a second member of the NQO gene family that is likely to encode another form of NAD(P)H:quinone oxidoreductase. The gene encoding for NQO_2 protein is localized to chromosome 6.

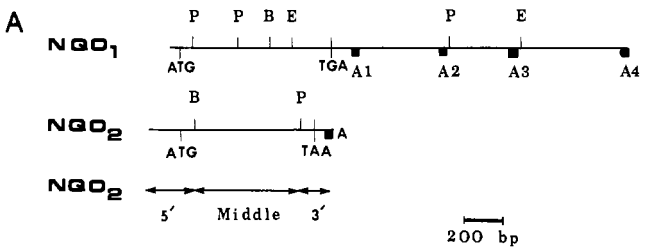
Materials and Methods

Cloning and Sequencing of Human NQO_2 cDNA. Isolation of poly(A)+ RNA from human liver and the construction of cDNA expression library in λgt11 is described (Jaiswal et al., 1988). Approximately 60 000 plaques were screened with the ^{32}P-labeled nick-translated human NQO_1 cDNA ending with first polyadenylation site, as shown in Figure 1A. The filters were hybridized for 16 h at 65 °C in 1 M NaCl, 1% SDS, 50 mM Tris, pH 8.0, 10% Denhardt's solution, and 100 μg/mL denatured salmon sperm DNA. We used 5×10^5 cpm of activity of ^{32}P-labeled probe for each milliliter of hybridization mixture. The filters were washed under conditions of low stringency (three times with 2 × SSC at room temperature, 5 min each; two times with 2 × SSC + 1% SDS at 50 °C, 30 min each; and two times with 0.1 × SSC at room temperature, 15 min each). Several positive clones were plaque-purified, digested with *Eco*RI, and analyzed. Restriction endonuclease/partial sequence analysis showed that six clones corresponded to NQO_1 and two clones to a substantially different sequence. The insert from one of the two latter clones was used to screen 50 000 more plaques to obtain full-length cDNA clones.

The inserts from two clones were isolated by agarose gel electrophoresis and used to prepare a sonication shotgun library (Denninger, 1983) in M13 mp8. Sequencing was carried out by standard M13 cloning protocols and the dideoxy sequencing method (Sanger et al., 1977; Messing et al., 1981) using the Sequenase kit supplied by United Biochemical Corp. Whenever necessary, various restriction enzyme fragments were isolated, cloned into M13, and sequenced. Each stretch of DNA was sequenced on both strands, usually 10–12 times. Nucleotide alignment and analysis of nucleotide and protein data were examined by standard computer programs (Staden, 1980; Brutlag et al., 1982; Orcutt et al., 1982; Wilber & Lipman, 1983; Lipman & Pearson, 1985).

RNA Isolation and Northern Analysis. RNA was isolated from control and TCDD-treated (100 nM, 3 days) human hepatoblastoma cell line Hep-G2 as described (Chirgwin et al., 1979). Poly(A)+-enriched RNA was prepared by two successive passages through oligo(dT)–cellulose. Northern analysis was performed as described (Jaiswal et al., 1985;

[1] Abbreviations: TCDD, tetrachlorodibenzo-*p*-dioxin (dioxin); NQO, NAD(P)H:quinone oxidoreductase; NQO_1, the NQO gene specifically induced by dioxin; NQO_2, the NQO gene not induced by dioxin; bp, base pairs; kb, kilobases; RFLP, restriction fragment length polymorphism.



B

```
                10                  30                  50
NQO2 CCCGGAACCTGGCGCAACTCCTAGAGCGGTCCTTGGGGAGACGCGGGTCCCAGTCCTGCG

                70                  90                 110
NQO2 GCTCCTACTGGGGAGTGCGCTGGTCGGAAGATTGCTGGACTCGCTGAAGAGAGACTACGC
NQO1                                                          C

               130                 150                 170
     AGGAAAGCCCCAGCCACCCATCAAATCAGAGAGAAGGAATCCACCTTCTTACGCTATGGC
     C  C  C A G  ...    G C     C CCCC   C G    ....AGA  C___  T

               190                 210                 230
     AGGTAAGAAAGTACTCATTGTCTATGCACACCAGGAACCCAAGTCTTTCAACGGATCCTT
     C  C GA G  C   G  C  ACTG  T   TCA  GAGG CC  C.      TATG  A

               250                 270                 290
     GAAGAATGTGGCTGTAGATGAACTGAGCAGGCAGGGCTGCACCGTCACAGTGTCTGATTT
        G G CT    C  CG CTT   AG A A A  A  GGAG  GGTG A   G  CC

               310                 330                 350
     GTATGCCATGAACTTTGAGCCGAGGGCCACAGACAAAGATATCACTGGTACTCTTTCTAA
     C              CA T  C TCATTT CAGA  G  C     A    AA  GAAGG

               370                 390                 410
     TCCTGAGGTTTTCAATTATGGAGTGGAAACCCACGAAGCCTACAAGCAAAGGTCTCTGGC
     C    C AAC   TC G   CCT CC  GT TGTTCTG  T  T  AG  G CCA    AG

               430                 450                 470
     TAGCGACATCACTGATGAGCAGAAAAAGGTTCGGGAGGCTGACCTAGTGATATTTCAGTT
     CCCA  T  TGTG C   A  A  G   C GGAA CC  A     T        C

               490                 510                 530
     CCCGCTGTACTGGTTCAGCGTGCCGGCCATCCTGAAGGGCTGGATGGATAGGGTGCTGTG
        C   C G     TG A  C  T     T     A      T T  GC A   T CAT

               550                 570                 590
     CCAGGGCTTTGCCTTTGACATCCCAGGATTCTACGATTCCGGTTTGCTCCAGGGTAAACT
     AGGA AG      T ACACTTA G T CCA G   T   CAAA  ACCCT   G A    GAA

               610                 630                 650
     AGCGCTCCTTTCCGTAACCACGGGAGGCACGGCCGAGATGTACACGAAGACAGGAGTCAA
     G  AG G       A C     T  T     GT G TCC       T TCT CA   GA  C

               670                 690                 710
     TGGAGATTCTCGATACTTCCTGTGGCCACTCCAGCATGGCACATTACACTTCTGTGGATT
     C  G  CATGAATGT A T  C       A T   AG      TTC G  T         C

               730                 750                 770
     TAAAGTCCTTGCCCCTCAGATCAGCTTTGCTCCTGAAATTGCATCCGAAGAAGAAAGAAA
     CC      T A AA      AC G CA A AGCAT   GGCACA TC A C    C CCC   T

               790                 810                 830
     GGGGATGGTGGCTGCGTGGTCCCAGAGGCTGCAGACCATCTGGAAGGAAGAGCCCATCCC
     TCAA   CC    AA GA    AAGA AC C   G    AT  T   G T  GACA  AC GTA

               850                 870                 890
     CTGCACAGCCCACTGGCACTTCGGGCAATAACTCTGTGGCACGTGGGCATCACGTAAGCA
     T TTG TC AAG A C T    T AC T A CT   CAG CAGGA TCTT ATGAAA  AG

               910                 930                 950
     GCACACTAGGAGGCCCAGGCGCAGGCAAAGAGAAGATGGTGCTGTCATGAAATAAAATTA
      GTACAGGATGA GAG AAAA  A A  TTT GCCT TC  TG G CATC C TGGGCA

               970                 990                1010
NQO2 CAACATAGCTACCTGG
NQO1 GTC   CC A  TGACAACCAGATCAAAGCTAGAAAATGAGATTCCTTAGCCTGGATTTC

              1030                1050                1070
NQO1 CTTCTAACATGTTATCAAATCTGGGTATCTTTCCAGGCTTCCCTGACTTGCTTTAGTTTT

              1090                1110                1130
NQO1 TAAGATTTGTGTTTTTCTTTTTCCACAAGGAATAAATGAGAGGGAATCGACCGTATTCGT

NQO1 GCATT
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FIGURE 1: Restriction map and DNA sequence of human NQO_2 cDNA clone. (A) Restriction map of the human NQO_2 and human NQO_1 cDNA clones. P, *Pst*I; E, *Eco*RI; B, *Bam*HI; A, A1–A4; polyadenylation signals (AATAAA). 5′, middle, and 3′ are the three different probes used for restriction fragment length polymorphisms. (B) Comparison of the human NQO_2 and NQO_1 cDNA sequences. The initiation codons, the termination codons, and the putative poly(A)+ addition signal (AATAAA) are underlined. The human NQO_2 cDNA sequence is numbered; the NQO_1 sequence is shown only at those positions where there was no match, and this sequence starts at position 117.

Gonzalez & Kasper, 1982) using the hybridization and washing conditions of Church and Gilbert (1984). The northern blots were prehybridized for 2 h at 65 °C in 15 mL containing 1% BSA, 7% SDS, 0.5 M NaH_2PO_4, pH 7.0, 1 mM EDTA, and 100 μg/mL denatured salmon sperm DNA. The hybridization mixture was the same as the prehybridization mixture. The two probes (NQO_1 and NQO_2 cDNAs) were nick-translated [specific activity = (1–2) × 10^8 cpm/μg of DNA] and added to the hybridization bag to a final concentration of 1 × 10^6 cpm/mL of hybridization mixture. The RNA–DNA hybridization was carried out at 65 °C overnight. Following hybridization, the filters were washed two times (each time 10 min at 55 °C) with 0.5% BSA, 5% SDS, 40 mM NaH_2PO_4, pH 7.0, and 1 mM EDTA and then four times (each time 10 min at 55 °C) with 1% SDS, 40 mM NaH_2PO_4, pH 7.0, and 1 mM EDTA. The filters were then dried and autoradiographed. After autoradiography with intensifying screens, the hybridized probe was removed from the membranes by incubating in 0.005 M Tris-HCl, pH 8.0, 0.0002 M Na_2EDTA, 0.05% sodium pyrophosphate, and 0.1 × Denhardt's solution [1 × Denhardt's solution is 0.02% BSA, 0.02% poly(vinylpyrrolidone), and 0.02% Ficoll] at 65 °C for 2 h. The membranes were rehybridized with human β-actin probe.

Cell Hybrids, DNA Isolation, Southern Hybridization, and Chromosomal Localization. Construction of human x mouse and human x hamster somatic cell hybrids, karyotypic analysis of their banded mitotic chromosomes, and electrophoretic analysis of human biochemical markers in these hybrids have been detailed elsewhere (McBride et al., 1982a–c). Briefly described, the strategy for chromosomal assignment (Roderick et al., 1984; Shows et al., 1982) involves use of a well-characterized cDNA to probe restriction endonuclease digested DNA from a battery of these hybrids. The presence or absence of the human gene in each hybrid cell line is then correlated with the presence or absence of each human chromosome.

The DNA was isolated from 54 human x mouse and 38 human x hamster somatic cell hybrids that have retained varying numbers of human chromosomes after segregation. Ten micrograms of DNA from each hybrid cell line was digested with various restriction enzymes, size-fractionated on agarose gels, and transferred to nylon membranes as described (McBride et al., 1982b,c). Membranes were hybridized for 24–48 h at 42 °C with ^{32}P-labeled probes in 50% formamide containing 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 0.001 M EDTA, pH 7.4), 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% poly(vinylpyrolidone), 0.02% bovine serum albumin, and 0.02% Ficoll], 10% dextran sulfate, 0.2% SDS, and denatured herring sperm DNA at 150 μg/mL. Membranes were washed twice at room temperature in 2 × NaCl/citrate (1 × NaCl/citrate is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.2% SDS and four times with 0.1 × NaCl/citrate plus 0.2% SDS at 55 °C. After autoradiography with intensifying screens, the hybridized probe was removed from the membranes by incubating in 0.4 M NaOH at 42 °C. The membranes were neutralized, prehybridized with carrier DNA, and hybridized with a second probe.

Detection of Restriction Fragment Length Polymorphisms. DNA was isolated from the peripheral leukocytes of ten normal unrelated individuals and size-fractionated restriction digests were transferred to nylon membranes and analyzed for the presence of restriction fragment length polymorphisms (RFLPs) with 5′, middle, and 3′ NQO_2 cDNA probes as depicted in Figure 1A. When RFLPs were detected, DNA isolated from 29 additional individuals were similarly analyzed. After autoradiography, probes were removed from blots with alkali and the same blots were hybridized with additional probes as described above.

Cloning of NQO_2 cDNA into Expression Vector and Transfection of COS Cells. The pMTII vector (Wong et al., 1985) has several features that make it ideal for the transient expression of cloned cDNAs in monkey kidney COS cells. The cDNA inserted into the vector is transcribed under the control of the adenovirus strong major late promoter. The vector contains a portion of the SV40 genome that codes for T-antigen required for viral replication, and it has the SV40 origin of replication. This allows propagation of multiple DNA copies in T-antigen containing COS cells, resulting in overproduction of the cDNA-expressed protein. The pMTII vector also contains the VA RNA gene, the product of which enhances expression of cloned cDNA presumably by counteracting inhibition of translation caused by the presence of double-stranded RNA. Full-length cDNA inserts for NQO_1 and NQO_2 were cloned into expression vector pMTII and transfected into COS cells by the DEAE dextran chloroquine method (Luthman & Magnusson, 1983). Untransfected COS cells served as control. Forty-eight hours after transfection, the cells were washed three times with ice-cold Dulbecco's phosphate-buffered saline without calcium and magnesium, scraped with a rubber policeman, and collected by centrifugation (800*g* for 5 min). The cell pellet from each dish was homogenized briefly in 0.25 M sucrose supplemented with 0.1 mM phenylmethanesulfonyl fluoride (PMSF) corresponding to protein concentrations of 1–5 mg/mL. The homogenate was centrifuged in an Eppendorf centrifuge at 14000 rpm for 15 min at 4 °C, and the supernatant was removed and used for analysis of the expressed NQO_1 and NQO_2 proteins by gel electrophoresis and immunoblotting (Laemmli, 1970; Towbin et al., 1979). The western blots were probed with antiserum against purified rat liver cytosolic NQO_1 protein (Robertson et al., 1986). The 14000 rpm supernatants were also analyzed for their capacity to reduce menadione and 2,6-dichlorophenolindophenol as described earlier (Prochaska & Talalay, 1986; DeLong et al., 1986). The final reaction mixture contained 25 mM Tris-HCl, pH 7.4, 0.18 mg/mL BSA, 5 μM FAD, 0.01% Tween 20, 200 μM NADH or NADPH, and the supernatant protein (0.2 μg in the case of NQO_1 and 20 μg in the case of NQO_2). When menadione was used as substrate, the reaction rate was monitored by measuring the decrease in absorbance at 340 nm due to oxidation of the pyridine nucleotide. When 2,6-dichlorophenolindophenol was used as substrate, the decrease in absorbance due to self-reduction was monitored at 600 nm.

Probes. The full-length NQO_2 cDNA insert from one recombinant bacteriophage was subcloned into pUC13 and isolated from the resultant plasmids by agarose gel electrophoresis and electroelution. The 233 bp *Eco*RI–*Bam*HI (5′ terminus to nucleotide 233), 575 bp *Bam*HI–*Pst*I (positions 234–808), and 168 bp *Pst*I–*Eco*RI (809 to 3′ terminus) segments of NQO_2, designated 5′, middle, and 3′, respectively (Figure 1A), were purified by 5% polyacrylamide gel electrophoresis, electroeluted, and used in addition to the full-length cDNA insert for restriction fragment length polymorphism analysis. The fragments were labeled with [^{32}P]-dCTP by nick translation or random oligonucleotide primed DNA synthesis to a specific activity of at least (1–2) × 10^8 cpm/μg of DNA for use as probes.

Results and Discussion

We reasoned that if human liver contains several immu-

```
                10                  30                  50
NQO2  MAGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGCTVTVSDLYAMNFEPRATDKDITGTL
      :.:...::: :: :..::: ..:..:...:..: .:. :::::::::.:   . :::::.:
NQO1  MVGRRALIVLAHSERTSFNYAMKEAAAAALKKKGWEVVESDLYAMNFNPIISRKDITGKL
                                                  I         II

                70                  90                  110
      SNPEVFNYGVETHEAYKQRSLASDITDEQKKVREADLVIFQFPLYWFSVPAILKGWMDRV
      ..:. :.: .:.  :::.  :..:::.:::::. .:::::::::::: ::.::::::::..::
      KDPANFQYPAESVLAYKEGHLSPDIVAEQKKLEAADLVIFQFPLQWFGVPAILKGWFERV
                                                    III
                130                 150                 170
      LCQGFAFDIPGFYDSGLLQGKLALLSVTTGGTAEMYTKTGVNGDSRYFLWPLQHGTLHFC
      . .:::.. ...::.: ...: :.::.::::...::.  :..:: . .:::.: :.::::
      FIGEFAYTYAAMYDKGPFRSKKAVLSITTGGSGSMYSLQGIHGDMNVILWPIQSGILHFC
                                                                IV
                190                 210                 230
      GFKVLAPQISFAPEIASEEERKGMVAAWSQRLQTIWKEEPIPCTAHWHFGQ
      ::.::.::.... . .....:  ...:.:.::..::.:.:.
      GFQVLEPQLTYSIGHTPADARIQILEGWKKRLENIWDETPLYFAPSSLFDLNFQAGFLMK

                250                 270
NQO1  KEVQDEEKNKKFGLSVGHHLGKSIPTDNQIKARK
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FIGURE 2: Comparison of human NQO_2 and human NQO_1 protein seequences. Identical residues between human NQO_2 and NQO_1 are indicated by (:) and related amino acids by (.). The four highly conserved regions between NQO_2 and NQO_1 are underlined and numbered I–IV. Abbreviations for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; O, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

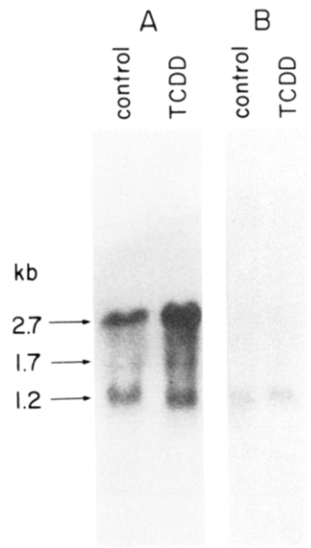

FIGURE 3: Northern blot hybridization analysis of mRNA from TCDD-treated and control cultures. Five micrograms of poly-(A)+-enriched RNA from Hep-G2 cells exposed to control medium or 100 nM TCDD for 3 days was subjected to electrophoresis and, after transfer to nitrocellulose, hybridized with the nick-translated (specific activity 1×10^8 cpm/μg) probes. The full-length cDNA clones used for hybridization were blot A, with NQO_1 cDNA ending with first polyadenylation site, and blot B, with NQO_2 cDNA.

nologically related cytosolic NQOs like rodent liver, then these would be expected to share some structural features not only because of their antigenic similarity but also because they catalyze nearly equivalent reactions, using NADH or NADPH as electron donor albeit with different substrates, and because they all associate with flavin-containing nucleotide (Hojeberg et al., 1981; Raftell & Blomberg, 1980; Prochaska & Talalay, 1986). Recently we have reported (Jaiswal et al., 1988) the cDNA and protein sequence for a dioxin-inducible human liver cytosolic NAD(P)H:quinone oxidoreductase (NQO_1). We attempted to isolate cDNA clones for other NQOs by screening a human liver λgt11 cDNA library with NQO_1 cDNA as a hybridization probe. We isolated eight positive clones, six of which corresponded to NQO_1 cDNAs, and two clones showed restriction maps quite different from that of NQO_1 cDNA. These clones were named NAD(P)H:quinone oxidoreductase (NQO_2) since they were likely to represent cDNA clones for another form of human cytosolic DT-diaphorase.

NQO_2 cDNA Nucleotide Sequence. The human NQO_2 cDNA of 976 bp was completely sequenced (Figure 1B). This cDNA has an open reading frame (positions 176–868) and codes for a protein of 231 amino acids including the initiator methionine. The initiator ATG codon as shown in Figure 1B is believed to be the true initiation codon because (i) it has the adjacent sequences that are characteristic of other eukaryotic initiation codons (Kozak, 1986) and (ii) it aligns perfectly with the initiation codons of human NQO_1 (Figure 1B) and the rat NAD(P)H:menadione oxidoreductase reported by Robertson et al. (1986). Nucleotide sequence comparison between the NQO_1 and NQO_2 cDNA (Figure 1B) shows 54% homology overall. It is noteworthy that the 80% of the 5′ end of NQO_2 cDNA shows greater than 60% sequence similarity to NQO_1 cDNA, while the sequence similarity drops substantially in the remaining 20% of NQO_2 cDNA sequence.

NQO_2 Protein Sequence. The human NQO_2 cDNA codes for a protein of 231 amino acids (M_r = 25 956) that has 49% sequence similarity to the human dioxin-inducible cytosolic NQO_1 protein. The two protein sequences can be aligned without inserting or deleting any residues (Figure 2). The NQO_2 protein is, however, 43 amino acids shorter at its C-terminus compared to NQO_1 protein. Amino acid sequence comparison between NQO_1 and NQO_2 shows four very highly conserved regions that are underlined and designated I–IV in Figure 2. The complete amino acid sequence of NQO_2 protein and the conserved stretches I–IV (Figure 2) were compared to the NBRF data base by using the FASTP program (Lipman & Pearson, 1985), and no highly significant homology with any other protein sequence including the other flavoenzymes was found except the human NAD(P)H:menadione oxidoreductase (NQO_1) reported by us earlier (Jaiswal et al., 1988) and rat NAD(P)H:menadione oxidoreductase (Robertson et al., 1986). The highly conserved stretches of amino acid sequences within the NQO_2 and NQO_1 proteins (I–IV in Figure 2) may play a role in the binding to the cofactors NADH/NADPH or FAD or participate in the enzyme active site and are a subject for future investigation. It is likely that NQO_2 cDNA encodes a cytosolic protein since the hydropathy profile for the human NQO_2 protein (data not shown) does not reveal the presence of a highly hydrophobic sequence that could represent a membrane-anchoring domain or insertion signal for translocation across the ER membrane.

NQO_2 mRNA Is Not Induced by TCDD in Hep-G2 Cells. Northern blot analysis of Hep-G2 mRNA using the hybridization and washing conditions of Church and Gilbert (1984) showed that only one band of 1.2 kb hybridized to NQO_2 cDNA and that the level of this mRNA is not affected by TCDD treatment (Figure 3B). In contrast, as previously reported, the expression of the NQO_1 gene in human Hep-G2 cells in culture (Figure 3A) is increased severalfold in the presence of TCDD. It should be noted that under the Church and Gilbert hybridization conditions the NQO_2 cDNA does not hybridize to NQO_1 mRNA (compare parts A and B of Figure 3). Longer exposures of the northern blot shown in Figure 3B did not reveal the presence of higher molecular weight mRNA species as seen in Figure 3A. The presence of equal amounts of RNA in control and TCDD-treated lanes of blots shown in Figure 3 was confirmed by hybridization of the same filters with human β-actin probe (data not shown). It should be noted that the intensity of the signal seen for NQO_2 in Figure 3B is weaker relative to that obtained with

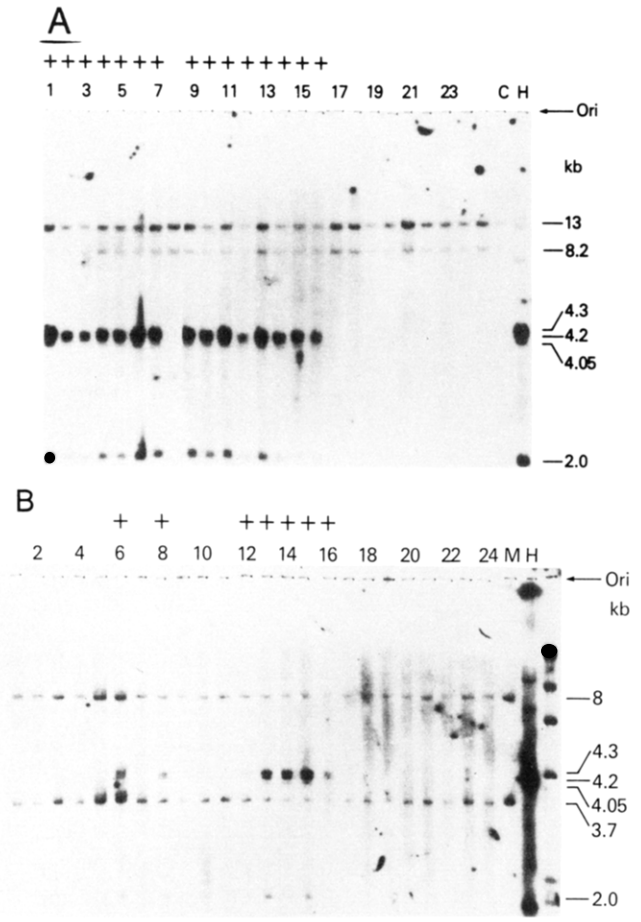

FIGURE 4: Chromosomal localization of the NQO_2 gene. Southern hybridization of representative *Eco*RI-digested human–hamster (A) and human–mouse (B) somatic cell hybrid DNAs with the full-length NQO_2 cDNA probe is shown. Aliquots (10 μg) of DNA were size-fractionated by (0.7%) agarose gel electrophoresis, transferred to nylon membranes, and hybridized as described under Materials and Methods. A different hybrid cell DNA is present in each lane; parental chinese hamster (C), mouse (M), and human placental (H) DNA are also shown. The sizes of hybridizing human (2.0, 4.05, 4.2, and 4.3 kb), hamster (8.2 and 13 kb), and mouse (3.7 and 8 kb) DNA fragments are indicated. The presence (+) of human hybridizing sequences is indicated above the lanes. All hybridizing human DNA fragments segregated concordantly in the hybrid cell DNAs.

NQO_1 in Figure 3A. As both the northern blots were done under similar conditions using equal amount of probes and time of exposure, the difference in the intensity of bands may indicate a low level of expression of NQO_2 gene compared to NQO_1. The one band of NQO_2 mRNA described above for Hep-G2 cells in culture was also detected in RNA isolated from normal human liver samples (data not shown).

Southern Analysis and Assignment of NQO_2 Gene to Human Chromosome 6. As detailed below, a comprehensive Southern blot analysis of human genomic DNA using fragments of the NQO_2 cDNA as probes (Figures 4–6) suggested strongly the presence of a single NQO_2 gene.

To determine the chromosomal location of the human NQO_2 gene, the 1-kb *Eco*RI full-length NQO_2 cDNA probe was used for Southern analysis of *Eco*RI-digested human–rodent somatic cell hybrid DNAs retaining varying numbers of human chromosomes after segregation (Fibure 4). Four hybridizing bands (2.0, 4.05, 4.2, and 4.3 kb) were detected in human DNA, and these bands were well resolved from weakly cross-hybridizing 3.7- and 8-kb or 8.2- and 13-kb bands in mouse and hamster DNAs, respectively. Analysis of 92 human–rodent somatic cell hybrid DNAs (Table I) clearly

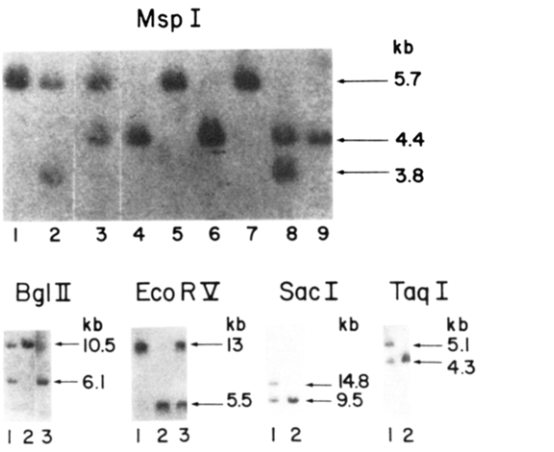

FIGURE 5: Detection of restriction fragment length polymorphisms (RFLPs) with the 3′ NQO_2 cDNA probe. The restriction endonuclease used is indicated above each panel. The sizes of the hybridizing bands are indicated at the right of each photograph. Simple two-allele polymorphisms were found with the 3′ probe in *Eco*RV, *Sac*I, *Bgl*II, and *Taq*I digests. In the case of *Bgl*II, *Sac*I, and *Taq*I digests the heterozygotes (lane 1) and homozygotes (lanes 2 and 3) are shown and lane 3 is absent when homozygotes for only one of the alleles were found. With *Eco*RV digestion, the homozygotes (lanes 1 and 2) and heterozygote (lane 3) are shown. A three-allele polymorphism was observed in *Msp*I digests; homozygotes (lanes 1, 4–7, and 9) and heterozygotes (lanes 2, 3, and 8) are shown.

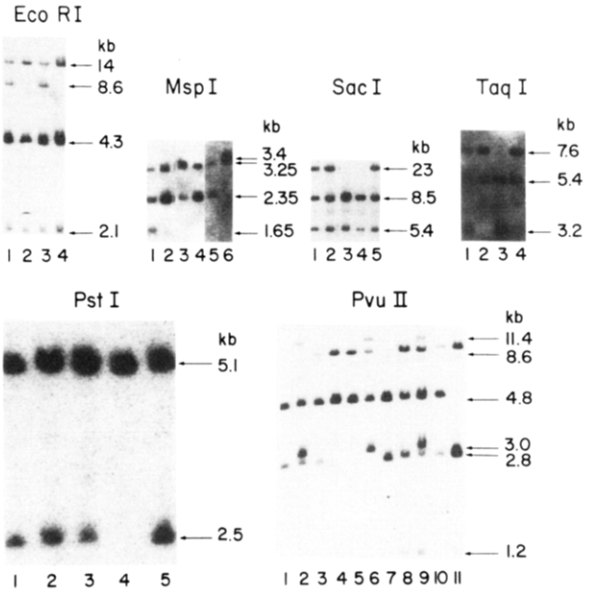

FIGURE 6: Detection of restriction fragment length polymorphisms (RFLPs) with the 5′ NQO_2 cDNA probe. Two-allele polymorphisms were found with the 5′ probe in *Taq*I (3.2- and 7.6-kb alleles), *Eco*RI (8.6- and 14-kb alleles), and *Sac*I (8.5- and 23-kb alleles) digests; constant bands observed were 5.5 kb (*Taq*I), 5.4 kb (*Sac*I), and 2.1 and 4.3 kb (*Eco*RI). A 2.5-kb band was not present in two *Pst*I digests hybridized with the 5′ probe; this band is probably allelic with a 5.1 kb and is not resolved from a constant band of similar size. A 5′ probe three-allele polymoprhism in *Msp*I digests is shown containing a 1.65-kb allele (lane 1), a 2.35-kb allele (lanes 1–5), and a 3.4-kb allele (lanes 3 and 6). A 3.25-kb constant band is present in all lanes. A complex polymorphism was found in *Pvu*II digests with the 5′ probe; a single constant (1.2-kb) band not visible in many cases because of a very weak signal and various combinations of 2.8-, 3.0-, 4.8-, 8.6-, and 11.4-kb variable bands were observed (see Table III and text for discussion).

indicates that this gene is present on chromosome 6 since the gene segregates concordantly with this chromosome and discordantly (>18%) with all other human chromosomes. The assignment of the NQO_2 gene to chromosome 6 was confirmed by analyzing these same *Eco*RI-digested hybrid cell DNA blots

Table I: Segregation of NQO_2 Gene with Human Chromosomes

human chromosome	gene/chromosome +/+	+/−	−/+	−/−	% discordancy
1	31	17	0	44	18
2	24	24	4	40	30
3	22	26	11	33	40
4	33	15	19	25	37
5	18	30	5	39	38
6	48	0	0	44	0
7	22	26	9	35	38
8	19	29	8	36	40
9	15	33	2	42	38
10	17	31	3	41	37
11	22	26	9	35	38
12	24	24	10	34	37
13	22	26	11	33	40
14	33	15	18	26	36
15	33	15	13	31	30
16	16	32	12	32	48
17	12	36	15	29	55
18	21	27	26	18	58
19	30	18	5	39	25
20	34	14	7	37	23
21	29	19	28	16	51
22	23	25	10	34	38
X	30	18	23	21	45

[a] The NQO_2 gene was detected as 2.0-, 4.0-, 4.2-, and 4.3-kb bands in *Eco*RI digests of somatic cell hybrids DNAs after Southern hybridization with the entire NQO_2 cDNA. Detection of the human gene is correlated with the presence or absence of each human chromosome in the group of somatic cell hybrids. Discordancy indicates presence of the gene when the chromosome is absent (+/−) or absence of the gene despite the presence of the chromosome (−/+), and the sum of these numbers divided by total hybrids examined × 100 represents percent disordancy. The human–hamster hybrids consisted of 24 primary clones and 14 subclones (25 positive of 38 total), and the human–mouse hybrids comprised 12 primary hybrids and 42 subclones (23 positive of 54 total). The isolation and characterization of the hybrid cell lines has been described (McBride et al., 1982a–c).

with a 233 bp 5′ cDNA subfragment after removing the previous probe with alkali (data not shown). The 4.2- and 4.3-kb human *Eco*RI bands were not detected with the 5′ cDNA probe, which represents primarily the 5′ untranslated region of the NQO_2 mRNA, but the intensity of hybridization with a 14-kb human band (not clearly visualized with the full-length cDNA probe) was markedly increased and cross-hybridization with rodent sequences was not observed. Hence, it was possible to map all of the hybridizing bands to chromosome 6.

The gene could be regionally localized to the short arm or proximal long arm of chromosome 6 by analysis of several hybrids containing breaks or translocation involving this chromosome. One human–hamster hybrid containing only human chromosome 22, X, and 6pter-q13 also retained the NQO_2 gene. A human–hamster hybrid line resulting from fusion of hamster cells with human fibroblasts containing a reciprocal translocation of chromosomes 2 and 6 (GM2658) retained the 6q12-qter translocation chromosome but not the NQO_2 gene whereas another hybrid retained the reciprocal translocation chromosome and the gene. A human–mouse hybrid and nine subclones retained a chromosome 6 with a deleted long arm and loss of human mitochrondrial superoxide dismutase (SOD-2) activity; the NQO_2 gene was retained in this hybrid. These combined results permit regional localization of the NQO_2 gene (including all hybridizing bands) to chromosome 6pter-q12.

Detection of Restriction Fragment Length Polymorphisms (RFLPs) with the NQO_2 cDNA Probes. RFLPs were found when digested DNAs from 10 individuals were hybridized with the full-length cDNA probe. Multiple bands were usually

Table II: RFLPs Detected with NQO_2 168 bp 3′ cDNA Probe[a]

enzyme	constant bands (kb)	N[b]	alleles (kb)	frequency
*Bgl*II	none	78	A1 = 10.5	0.79
			A2 = 6.1	0.21
*Eco*RV	none	76	B1 = 13	0.17
			B2 = 5.5	0.83
*Sac*I	none	78	C1 = 14.8	0.06
			C2 = 9.5	0.94
*Taq*I	none	86	D1 = 5.1	0.09
			D2 = 4.3	0.91
*Msp*I	none	58	E1 = 5.7	0.48
			E2 = 4.4	0.43
			E3 = 3.8	0.09

[a] All of these polymorphisms were also detected with a 576 bp middle cDNA fragment. [b] N = number of chromosome sets examined.

Table III: RFLPs Detected with NQO_2 223 bp 5′ cDNA Probe

enzyme	constant bands (kb)	N[a]	alleles (kb)	frequency
*Eco*RI	2.1, 4.3	78	A1 = 14	0.88
			A2 = 8.6	0.12
*Sac*I	5.4	78	B1 = 23	0.13
			B2 = 8.5	0.87
*Taq*I	0.4, 5.4	78	C1 = 7.6	0.87
			C2 = 3.2	0.13
*Msp*I	3.2	58	D1 = 3.4	0.22
			D2 = 2.35	0.72
			D3 = 1.65	0.05
*Pst*I	5.1	78	E1 = 2.5	?0.97
			E2 = ?5.1	
*Pvu*II[b]	1.2	58	11.4	
			8.6	
			4.8	
			3.0	
			2.8	

[a] N = number of chromosome sets examined. [b] Complex polymorphism with *Pvu*II with single constant band (1.2 kb). The combinations of variant bands observed (and instances of each) inclued the following: 2.8 + 4.8 + 8.6 (12), 4.8 + 8.6 (5), 2.8 + 4.8 (4), 2.8 + 8.6 (2), 3.0 + 4.8 + 8.6 + 11.4 (3), 2.8 + 3.0 + 4.8 + 11.4 (2), and 2.8 + 3.0 + 4.8 + 8.6 + 11.4 (1).

observed, and the identification of allelic bands was frequently difficult. Hence, these same blots, as well as DNAs from 29 additional individuals, were examined sequentially by using subfragment probes derived from the middle, 5′ end, and 3′ end of the full-length cDNA as depicted in Figure 1. The 3′ cDNA probe detected RFLPs with five different restriction enzymes (Figure 5, Table II), and all appear to result from single base changes causing the gain or loss of a restriction site. These included simple two-allele polymorphisms with *Bgl*II, *Eco*RV, *Sac*I, and *Taq*I all resulting from mutations in flanking sequences distal to the 3′ end of the gene. The three-allele polymorphism with *Msp*I results from alterations at one site in the 3′ flank (i.e., 4.4-kb vs 5.7-kb alleles) and one site in an intron within the 575 bp middle fragment (i.e., 1.9-kb plus 3.8-kb vs 5.8-kb alleles). Additional RFLPs were detected with the 5′ cDNA probe (Table III and Figure 6). The two- and three-allele polymorphisms found with *Sac*I and *Msp*I, respectively, involve restriction sites at the 5′ flank of the gene, and the two-allele polymorphisms with *Eco*RI and *Taq*I probably also involve sites in these flanking sequences. A 2.5-kb band was not found in 2 of the 39 *Pst*I digests; no allelic band was detected, suggesting that this band may not be resolved from a 5.1-kb constant band in these digests. A very complex but highly informative polymorphism was detected in *Pvu*II digests with the 5′ probe. Obviously, this complex band pattern reflects polymorphism at more than a single *Pvu*II restriction site if only one gene is detected. A clear interpretation of this polymorphism awaits the results

Table IV: NQO Activity of Proteins Encoded by NQO_2 and NQO_1 cDNA into Monkey Kidney COS1 Cells[a]

cell/construct	NAD(P)H:quinone oxidoreductase (NQO) activity [μmol of 2,6-dichlorophenolindophenol reduced/(min·mg of protein)]	x-fold increase over untransfected cells
COS1	0.05	1
COS1 + pMTII–NQO_2	0.25–0.35	5–7
COS1 + pMTII–NQO_1	7.50–40.0	150–800

[a] Values obtained from three separate transfections followed by enzyme assays. Similar results were obtained when menadione was used as substrate, in which case the oxidation of NAD(P)H was measured.

of hybridization with smaller subfragments of the 5′ cDNA or oligonucleotide probes.

The above results indicate that the NQO_2 gene constitutes a highly polymorphic locus that is eminently suited for further mapping by genetic linkage studies. Analysis of RFLPs indicates that most individuals can be expected to be informative with at least one of these restriction enzymes combined with the use of both 5′ and 3′ probes.

All of these RFLPs must be considered tentative since Mendelian segregation of the allelic bands has not been demonstrated. It is highly improbable that any of the hybridizing bands represent contamination with extraneous DNA, such as plasmid, since these bands were not detected during reuse of the same blots with at least 20 different probes. In all cases, identical patterns of polymorphic alleles have been identified on at least two different blots prepared at different times using DNAs isolated from unrelated individuals (see Materials and Methods). Hence it is unlikely that the polymorphic bands represent artifacts of incomplete digestion or "star activity" (i.e., reduced restriction site specificity). Finally, a reasonable dosage dependence of hybridization intensity was observed with the polymorphic alleles, and no more than two alleles were detected in any individual. For these reasons, it is likely that the RFLPs described are authentic.

Estimation of NQO_2 Gene Copy Number and Size. Chromosomal mapping studies indicated that all NQO_2 sequences are located on human chromosome 6 but provided no information relating to the gene copy number at that locus. Two lines of evidence strongly suggest that a single NQO_2 gene is detected with the full-length and subfragment cDNA probes. The 3′ cDNA probe clearly detects only one gene since a single band (or allelic bands) was found in 12 different restriction digests of DNAs isolated from 10 or more individuals. It is highly improbable that restriction sites in the flanking sequence of multiple genes would have resulted in bands that were indistinguishable in size in all 12 digests. Moreover, it was anticipated that probes derived from contiguous cDNA sequences would detect a single common hybridizing band in all restriction digests of a single gene, and this was, indeed, observed. In contrast, the number of bands in a restriction digest hybridizing with both contiguous cDNA probes could have provided an estimate of the gene copy number if more than one band had been observed. The gene must contain at least five introns containing restriction sites as determined from the number of hybridizing fragments in restriction digests. From the size of internal restriction fragments, the size of the gene was also estimated to be 14–17 kb.

Expression of NQO_2 and NQO_1 cDNAs into COS1 Cells. In order to characterize and assess the NQO activity of proteins encoded by the NQO_2 and NQO_1 cDNAs, they were cloned into the expression vector pMTII (Wong et al., 1985) and transfected into COS1 cells. The western blot analysis of the homogenates using the antibody against rat liver cytosolic NQO_1 revealed bands of 31 or 25 kDa in the homogenates of COS cells transfected with NQO_1 and NQO_2 cDNAs, respectively (Figure 7), indicating, clearly, that the two proteins are immunologically related. The stronger reaction of the NQO_1-encoded protein with the antibody against rat NQO_1 was most likely due to the higher sequence similarity between human NQO_1 and the orthologous rat NQO_1 protein. The homogenates of the transfected cells were also analyzed for their capacity to reduce 2,6-dichlorophenolindophenol and menadione. It was observed that homogenates of cells transfected with NQO_1 cDNA had a very high capacity to catalyze the reduction of both substrates [7.5 −40 μmol of 2,6-dichlorophenolindophenol reduced/(min·mg of protein)] whereas cells transfected with NQO_2 cDNA showed a substantially lower activity, which was nevertheless 5–7-fold higher than that of homogenates of untransfected cells (Table IV). It should be noted that when 2,6-dichlorophenolindophenol was used as substrate, the activity detected reflected the appearance of its reduced product. When menadione was used as substrate, however, and the reaction was followed by monitoring the decrease in absorbance at 340 nm that reflected the oxidation of NAD(P)H, this activity did not represent the consequence of redox cycling by its interaction with other redox centers as a nonspecific redox partner since the activity of transfected cells was always 4–8 times higher than that of nontransfected cells. The relatively low activity of NQO_2 with 2,6-dichlorophenolindophenol and menadione as substrates would indicate that NQO_2 might have a preference for other, as yet unknown, substrates as electron acceptors. It is noteworthy that diaphorase 5 identified by Edwards et al. (1980) did not utilize 2,6-dichlorophenolindophenol and could be detected only with menadione as electron acceptor.

The human NQO_2 cDNA characterized in the present study will allow us to clone and characterize the structural gene

FIGURE 7: Western blot analysis of homogenates from COS cells transfected with NQO_1 and NQO_2 cDNAs cloned into pMTII vector. Five micrograms of 14 000 rpm supernatant proteins obtained from untransfected COS cells, COS cells transfected with pMTII–NQO_1, and COS cells transfected with pMTII–NQO_2 were electrophoresed on a SDS–polyacrylamide gel and transferred to nitrocellulose membranes, and the transferred proteins were probed with the rabbit antisera against purified rat NAD(P)H:quinone oxidoreductase (rat NQO_1).

encoding the NQO_2 enzyme, to study aspects of its regulation, and to carry out further studies on the structure and function of NQO_2 protein.

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