An Alternatively Spliced Form of NQO₁ (DT-Diaphorase) Messenger RNA Lacking the Putative Quinone Substrate Binding Site Is Present in Human Normal and Tumor Tissues¹

Pamela Y. Gasdaska, Hugh Fisher, and Garth Powis²

Arizona Cancer Center, Arizona Health Sciences Center, University of Arizona, Tucson, Arizona 85724

ABSTRACT

DT-diaphorase is a ubiquitously expressed flavoenzyme responsible for the two-electron reduction of a number of quinone and other anticancer drugs. The majority of DT-diaphorase enzyme activity in human tissues is the product of the NQO₁ gene. We have now identified a novel alternatively spliced form of human NQO₁ mRNA lacking exon 4 at levels equal to or exceeding those of wild-type NQO₁ mRNA. Exon 4 codes for the putative quinone substrate binding site of DT-diaphorase derived from NQO₁ and the recombinant protein from alternatively spliced NQO₁ mRNA lacking exon 4 has minimal enzyme activity with quinoid and other known substrates of DT-diaphorase. The physiological substrate of DT-diaphorase is unknown, and it is possible that the protein derived from the alternatively spliced NQO₁ mRNA could have enzyme activity with an appropriate substrate. We found full-length DT-diaphorase protein but could not detect expression of an appropriately smaller form of DT-diaphorase in human tissues using polyclonal antibody to DT-diaphorase, suggesting that alternatively spliced NQO₁ mRNA lacking exon 4 may not be translated or that the protein product is rapidly degraded. Alternative splicing of NQO₁ RNA could provide an important mechanism for regulating NQO₁ gene expression.

INTRODUCTION

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2), also known by its older name DT-diaphorase, is a homodimeric flavoprotein characterized by its ability to catalyze exclusively the two-electron reduction of quinones (1). It differs from other quinone reductases in the cell in that it uses both NADH and NADPH as cofactors and is inhibited by low concentrations of dicumarol (2). The physiological function of DT-diaphorase remains unclear. In experimental animals, DT-diaphorase activity is increased in response to oxidant stress and carcinogens, which has led to the suggestion that DT-diaphorase may offer protection against the toxic effects of these agents (3–5). Metabolism by DT-diaphorase can lead to the activation of some anticancer drugs, including the quinones mitomycin C (6) and diaziquone (7), the indolequinone EO9 (8), and the dinitrophenylaziridine CB1954 (9). DT-diaphorase activity is elevated in some human primary tumor cell lines (10–12). It has been suggested that DT-diaphorase might be a clinically relevant target for bioreductively activated anticancer drugs (12–16).

The majority of DT-diaphorase activity in human tissues is the product of a dioxin-inducible gene, termed DIA₂ (2) or NQO₂ (17), located on human chromosome 16. The human NQO₁ gene encodes a protein of 273 amino acids (18). Low stringency hybridization using NQO₁ cDNA has identified a second human DT-diaphorase gene, termed NQO₂, that is expressed at only low levels and that is not induced by dioxin (19). The predicted sequence for the human NQO₂ protein is 231 amino acids, which is 43 amino acids shorter than the NQO₁ protein, with 49% overall homology to the NQO₁ protein.

Jaiswal et al. (17) reported three species of NQO₃ mRNA detected by Northern analysis in the human Hep-G2 hepatoma cell line and human liver of approximately 1.2, 1.7, and 2.7 kb. The different NQO₃ mRNAs were suggested to correspond to different polyadenylation sites on the human NQO₁ gene. In our previous studies of NQO₁ mRNA in primary human colon and lung cancer, we observed, in addition to the three larger species of NQO₃ mRNA reported by Jaiswal et al. (17), NQO₁ mRNA species of <1.2 kb (20). During studies to investigate these smaller NQO₃ mRNAs, we discovered an alternatively spliced form of NQO₁ mRNA that lacks exon 4. Alternative splicing offers an important mechanism for the control of gene expression that can give rise to qualitatively different proteins with altered activity (21) or provide quantitative control of gene expression through the production of nonfunctional RNA (22). We have found an alternatively spliced form of NQO₁ mRNA that gives rise to nonfunctional product and could be a regulatory mechanism for NQO₁ gene expression.

MATERIALS AND METHODS

Cell Lines, Tissues, and Chemicals. Human HT-29 colon carcinoma and SW480 colon adenocarcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Other human cell lines were obtained from the Arizona Cancer Center tissue culture service. Cells were grown in DMEM with 10% FCS and were passaged prior to confluence using 0.25% trypsin and 0.02% EDTA. Human primary colon and lung tumors and their paired normal tissue were collected from the surgical pathology laboratory within 1 h of removal and immediately frozen and stored in liquid nitrogen. 2,6-Dichlorophenolindophenol, p-benzoquinone, 2-methyl-1,4-naphthoquinone (menadione), 2-methyl-1,4-benzoquinone, methylene blue, and potassium ferricyanide were obtained from Sigma Chemical Co. (St. Louis, MO). Diaziquone and mitoxantrone were provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. p-Benzoquinone epoxide was synthesized from p-benzoquinone by the method of Brunmark et al. (23).

Analysis of NQO₁ mRNA. Human tissue was homogenized using a Polytron tissue homogenizer (Kinematica AG, Lucerne, Switzerland) in 6 volumes of guanidine isothiocyanate, and total RNA was prepared by isothiocyanate/cesium chloride gradient centrifugation (24). Polyadenylated mRNA was prepared using the FastTrack isolation system (Invitrogen Corp., San Diego, CA). First-strand cDNA was reverse transcribed from 1 μg mRNA using the Superscript pre-amplification system (Gibco BRL, Gaithersburg, MD). The various primers used for PCR reactions in the study are shown in Fig. 1 and Table 1. NQO₁ cDNA was amplified by nested PCR using primer pairs 2691 and 2690, and 2692 and 2693 by standard protocols (25). The PCR amplified cDNA fragments were gel purified using the Geneclean II system (Bio 101, Inc., La Jolla, CA) and digested with NotI (the internal primers 2692 and 2693 were designed with NotI sites) and cloned into NotI-digested pBluescript KS(+) phagemid vector (Stratagene, La Jolla, CA). NQO₁/pBluescript cDNA clones were sequenced using Sequenase T7 DNA polymerase (United States...
was run on 5% acrylamide gel with detection by ethidium bromide fluorescence. Alternatively, NQO1 cDNA fragments prepared from human colon and lung tissues by reverse transcription PCR using primers 3067 and 2869 were electrophoresed on a 3% agarose gel for 18 h and transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) for hybridization with the human NQO1 cDNA probe (Biochemical, Inc., Cleveland, OH; Ref. 26). For analysis, the NQO1 cDNA number in the sequence published by Jaiswal et al. (17).

Autoradiograms of the blot were quantified by densitometric scanning (Video-Densitometer 620; Bio-Rad, Richmond, CA). NQO1 cDNA fragments were corrected for unequal reaction and transfer during blotting using reverse transcription PCR of histone H3 mRNA with specific primers and probe (27). Expression of NQO1. To prepare protein for antibody production, NQO1 cDNA was cloned into the NdeI and BamH1 sites of peT-3a, a T7 expression vector (28). The Escherichia coli strain BL21 was transformed with NQO1/pET-3a and grown in LB broth with 50 μg/ml ampicillin, 25 μg/ml kanamycin, and 2% glucose to an absorbance (600 nm) of 1.0. NQO1 expression was induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for an additional 4.5 h. The cells were harvested, lysed, and recombinant DT-diaphorase protein purified by affinity chromatography using nick-E-NTA resin (Qiagen).

Western Analysis. DT-diaphorase protein in human cancer cell lines was studied using rabbit polyclonal antibody against the full-length recombinant human DT-diaphorase. The antibody also recognized the recombinant alternatively spliced DT-diaphorase protein. Total cell protein (100 μg) from a number of human tumor cell lines was separated by electrophoresis under denaturing conditions on 7.5% acrylamide gel and transferred to a nitrocellulose membrane. DT-diaphorase rabbit polyclonal antiserum was diluted 1:2000 in 20 mM Tris buffer, pH 7.6, 137 mM NaCl, 0.1% Tween 20, and 3% BSA and incubated with the blot for 2 h at room temperature. The blot was extensively washed with 20 mM Tris buffer (pH 7.6)-137 mM NaCl and incubated with goat anti-rabbit alkaline phosphatase-conjugate IgG diluted 1:3000 in 20 mM Tris buffer (pH 7.6), 137 mM NaCl, and 3% BSA for 2 h at room temperature. Color development was completed with nitro blue tetrazolium and monochloroindolyl phosphate.

DT-Diaphorase Assay. DT-diaphorase activity was measured spectrophotometrically by the oxidation of NADPH at 340 nm with quinone and other substrates as previously described (30). Dicumarol (50 μM) was used to confirm the specificity of the assay for DT-diaphorase (1).

RESULTS

NQO1 mRNA in Human Colon Tumor Cells. A typical Northern analysis of NQO1 mRNA in human colon cancer cell lines is shown in Fig. 2. At least four species of mRNA that hybridize with the human NQO1 cDNA probe can be seen of approximately 2.7, 1.8, 1.4, and 1.1 kb. We have previously seen a similar pattern of NQO1 mRNA species in human normal and primary lung tumor samples

| Primer | Sequence
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2690</td>
<td>GGGAGGCTTGAAAGATACCGA 934 915</td>
</tr>
<tr>
<td>2691</td>
<td>CAGGCCCGGGAGCTACGAGAGCC 265 50</td>
</tr>
<tr>
<td>2692</td>
<td>CAGTGGCGCCCGACTGTCGCGAGAGCC 51 74</td>
</tr>
<tr>
<td>2693</td>
<td>AGTGGGCGGCCCGAATCGGCTGAAATCCTCA 896 873</td>
</tr>
<tr>
<td>2689</td>
<td>ATTTGAACTGCGGCTCTGCTG 661 640</td>
</tr>
<tr>
<td>3067</td>
<td>TCGGACCTCTATGCCATGCCATGAACTTTC 168 191</td>
</tr>
</tbody>
</table>

* The underlined sequence is the NsiI restriction site. Numbers correspond to the base number in the sequence published by Jaiswal et al. (17).
ALTERNATIVELY SPliced NQO₁ mRNA

ALTERNATIVELY SPliced NQO₁ mRNA

**Fig. 3.** Analysis of NQO₁ cDNAs obtained by reverse transcription nested PCR of polyadenylated mRNA from HT-29 colon carcinoma cells. A 5% acrylamide gel was used and detection was with ethidium bromide. Band A was sequenced and shown to be full-length NQO₁ cDNA. Band B was sequenced and shown to be NQO₁ cDNA lacking exon 4. The position of molecular weight markers are shown on the right.

Alternatively Spliced NQO₁ mRNA in HT-29 Colon Cancer. In an attempt to further characterize the NQO₁ mRNAs, we used reverse transcription nested PCR of HT-29 colon cancer cell polyadenylated mRNA to obtain the cDNA. We used the primer pairs 2691 and 2690, followed by 2692 and 2693 that, based on the published sequence of NQO₁ cDNA, should give a cDNA of 845 bases (17). Analysis of the cDNA on 5% acrylamide gel revealed a major cDNA species of approximately 0.85 kb and a minor cDNA of approximately 0.74 kb (Fig. 3). Cloning and sequencing (sense and antisense) of the larger cDNA showed it to be the expected sequence of the NQO₁ cDNA between bases 51 and 896 (17). Cloning and sequencing (sense and antisense) of the smaller cDNA showed that it was an alternatively spliced form of NQO₁ lacking bases 354 to 467, i.e., missing exon 4 (Fig. 4). There were no other base differences found between the wild-type cDNA and the alternatively spliced form of NQO₁ cDNA.

Alternatively Spliced NQO₁ mRNA in Human Normal and Primary Tumor Tissue. Reverse transcription PCR using primers 3067 and 2869 was used to obtain NQO₁ cDNA from samples of human primary colon and lung adenocarcinoma and their paired normal tissue. The cDNA was separated by electrophoresis on 3% agarose, blotted, and hybridized with an 862-base human NQO₁ cDNA probe. There were two major species of cDNA found in all the tissues and two minor species in some of the tissues (Fig. 5). The largest major cDNA species (Fig. 5A) corresponded to a 493-base sequence predicted to be obtained from the full-length NQO₁ cDNA with the primers used. The smaller major cDNA species (Fig. 5B) corresponded to a cDNA sequence that was 114 bases shorter, i.e., the alternatively spliced NQO₁ cDNA lacking exon 4. The other two cDNA species of molecular sizes 0.32 kb (Fig. 5C) and 0.25 kb (Fig. 5D) have yet to be characterized but might correspond to alternatively spliced forms lacking exon 2 and a combination of two exons (for example, 1 and 2, 2 and 5, or 3 and 4). Densitometric scanning of the Southern blots of a number of human tissue samples showed that the alternatively spliced NQO₁ mRNA-derived cDNA lacking exon 4 was between 59 and 103% of the full-length NQO₁ mRNA-derived cDNA (Table 2).

**Western Blotting of DT-Diaphorase Protein.** Rabbit polyclonal antibody was raised against recombinant full-length, enzymatically active DT-diaphorase. This antibody also recognized the protein expressed from alternatively spliced NQO₁ cDNA (see below). Western blots using the DT-diaphorase antibody with lysates from HT-29 and a variety of other human cancer cell lines showed a single protein band at approximately Mr 31,000 but no significant smaller protein species that might correspond to an alternatively spliced NQO₁ protein (Fig. 6).
ALTERNATIVELY SPLICED NQO1 mRNA

Expression of Full-length and Alternately Spliced DT-Diaphorase. The protein products from full-length NQO1 cDNA and NQO1 cDNA lacking exon 4 were expressed in E. coli. Both proteins were recognized by a rabbit polyclonal antibody to recombinant full-length human DT-diaphorase. The NADPH oxidizing activity of the expressed proteins was measured using a variety of substrates for DT-diaphorase (Table 3). The full-length NQO1 protein had activity that was both quantitatively and qualitatively similar to that previously reported for human DT-diaphorase (29, 31). The protein from NQO1 cDNA lacking exon 4 had only very low enzyme activity that was barely above the limit of detectability of the assay.

DISCUSSION

The expression of the NQO1 gene is quite variable in some human tissues. Jaiswal et al. (17) identified four polyadenylation sites in the NQO1 gene, three of which were used, giving rise to three species of NQO1 mRNA in human normal liver and a hepatoma cell line. We have observed a fourth NQO1 cDNA species in human lung and colon tissue of <1.2 kb, i.e., less than the full-length reading frame for NQO1. We have now identified a form of NQO1 mRNA present in human normal and tumor tissues that appears to be an alternatively spliced form of the full-length NQO1 mRNA lacking exon 4. This alternatively spliced NQO1 mRNA is 114 bases smaller than the

Fig. 6. Western analysis of DT-diaphorase in human cancer cell lines using rabbit polyclonal antibody against full-length human DT-diaphorase. The antibody also recognizes the alternatively spliced NQO1 protein. Each lane contains 100 μg protein from a total cell lysate. Lane 1, HT-29 colon cancer; Lane 2, 8226 myeloma; Lane 3, V2387 melanoma; Lane 4, UACC 2925 breast cancer; Lane 5, MCF-7 breast cancer; Lane 6, Her-2-transfected MCF-7 breast cancer; Lane 7, UACC 1649 breast cancer; Lane 8, Jurkat T cell leukemia; Lane 9, UACC 812 breast cancer. A and B, the separation of the full-length DT-diaphorase and the alternatively spliced NQO1 protein. The positions of molecular weight markers are shown on the left.
full-length NQO1 mRNA and would not normally be separated from full-length NQO1 mRNA by electrophoresis on a 1% agarose gel analysis. Our work also suggests there may be other alternatively spliced forms of NQO1 mRNA, and it is possible that one of these may give rise to the 0.8-kb NQO1 mRNA species seen on Northern blots. We do not know whether each of the larger NQO1 mRNA species representing the different polyadenylated forms of full-length NQO1 mRNA, i.e., 1.2, 1.7, and 2.7 kb, also contain alternatively spliced forms of NQO1 mRNA.

Our PCR analysis suggests that alternatively spliced NQO1 mRNA lacking exon 4 is present in human normal and tumor tissues in amounts almost equal to that of the full-length NQO1 mRNA. This raises the question of the function, if any, of the alternatively spliced NQO1 mRNA. Alternative splicing is a tightly regulated process and can offer an important mechanism for the control of gene expression. Alternative splicing can occur through the interchange of cassette exons producing changes in protein identity and function without changes in transcriptional activity (21) or through the splicing of an intron that introduces translational termination, giving an apparently nonfunctional RNA (22). The alternatively spliced NQO1 mRNA we identified shows the complete deletion of exon 4 with the reading frame maintained so that a modified protein could be expressed. Although we were able to express protein from the alternatively spliced NQO1 mRNA in E. coli, we were only able to detect a single major species corresponding to full-length DT-diaphorase in human cell lines using a polyclonal antibody that recognized both the full-length DT-diaphorase and the exon 4 deleted form of the protein. Thus, although possessing an open reading frame, the alternatively spliced NQO1 mRNA may, for other reasons, be a nonfunctional mRNA. Another explanation is that the alternatively spliced protein is rapidly degraded, or there may be other reasons for our inability to detect an immunoreactive form of the protein.

Site-directed mutagenesis studies have identified different binding domains of rat NQO1-derived DT-diaphorase important for activity. Residues reported to be important for NAP(P)H binding are Lys$^\text{76}$ (32), Phe$^\text{116}$ and Glu$^\text{117}$, and Asp$^\text{163}$ (33), Gly$^\text{150}$, Ser$^\text{151}$ and Tyr$^\text{155}$ (34). Tyr$^\text{128}$ is important for dicumarol binding (34), Arg$^\text{177}$ for enzyme conformation with mutation leading to altered protein conformation and decreased FAD incorporation (35), and Cys$^\text{179}$ is important for enzyme stability (32). Work by Traver et al. (36) have shown that Pro$^\text{187}$ is absolutely essential for the activity of human NQO1. The alternatively spliced form of human NQO1 mRNA lacking exon 4 is without amino acid residues 102 to 139 of the full-length protein, which includes at least the putative quinone-binding domain and residues important for NAD(P)H binding. Thus, it is not surprising that we found that the protein product of the alternatively spliced NQO1 mRNA lacking exon 4 is enzymatically inactive toward quinoid substrates. Exon 4 of NQO1 may be a cassette exon conferring DT-diaphorase activity to substrates including quinones (1), quinone-imines (37), metals (38), and epoxides (Ref. 23; Fig. 7). Because we do not know the physiological substrate of DT-diaphorase, it is possible that the alternatively spliced form of the protein could be active with other substrates. DT-diaphorase activity is elevated in some human tumor cell lines (10-12) and in some human primary tumors (39). It has been suggested that DT-diaphorase may play a clinically important role in the bioreductive activation of some anticancer quinone and other drugs (12-16). The alternatively spliced NQO1 mRNA would, presumably, have no effect on measurements of DT-diaphorase activity or sensitivity to bioreductively activated anticancer drugs in human tissues but potentially could confound studies of NQO1 gene expression by reverse transcription PCR.

In summary, we have identified a novel alternatively spliced form of NQO1 mRNA lacking exon 4 that is present in human tissues at levels equal to that of wild-type NQO1 mRNA. Exon 4 contains the putative quinone-binding site of NQO1-derived DT-diaphorase. This may explain why we were unable to detect significant enzyme activity with protein expressed from exon 4-deficient NQO1 mRNA, using quinoid or other reported substrates of DT-diaphorase. The physiological substrate(s) of DT-diaphorase is unknown, and the alternatively spliced protein may have enzyme activity with an appropriate substrate. Human tissues were found to contain only one immunoreactive-form DT-diaphorase that corresponded to the full-length NQO1 protein product. Alternative splicing of NQO1 RNA could provide an important mechanism for the regulation of NQO1 gene expression.

ACKNOWLEDGMENTS

We thank Dr. John Riebow (Mayo Clinic, Rochester, Minnesota) for help with the collection of human tissues. The excellent secretarial assistance of Madelon Cook is gratefully acknowledged.

REFERENCES


An Alternatively Spliced Form of NQO₁ (DT-Diaphorase)
Messenger RNA Lacking the Putative Quinone Substrate Binding Site Is Present in Human Normal and Tumor Tissues

Pamela Y. Gasdaska, Hugh Fisher and Garth Powis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/12/2542

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.