Presence of a heterozygous substitution and its relationship to DT-diaphorase activity

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Summary A point mutation in the mRNA of NDPH:quinone oxidoreductase 1 (NQO1, DT-diaphorase) is believed to be responsible for reduced enzyme activity in the adenocarcinoma BE cell line. The present study examined nine cultured human non-cancerous fibroblast cell strains, five of which were from members of a single cancer-prone family, which demonstrated widely varying activity levels of DT-diaphorase (41–346 nmol min⁻¹ mg⁻¹ protein), to determine if genetic alteration of the NQO1 or NQO2 gene was involved in determining enzyme activity. All cell strains expressed NQO1, and NQO1 mRNA as measured by a quantitative polymerase chain reaction amplification technique. No relationship was found between the level of mRNA expressed and the enzyme activity in the cells. Sequencing of the entire complementary DNA from the cell strains revealed only a single base substitution at nucleotide 609 in one allele encoding NQO1, in every cell strain from members of the cancer-prone family, except for one cell strain which expressed only the T at nucleotide 609 in both alleles. Subsequent examination of genomic DNA from 44 individuals revealed that this base substitution is present in approximately 50% of the population. The presence of the T at nucleotide 609 in the NQO1 locus does not appear to be directly causal for altered DT-diaphorase activity.

Keywords: DT-diaphorase: base alteration; fibroblast

DT-diaphorase is a two-electron-reducing flavoenzyme which catalyses the oxidation of NADH or NADPH (Ernster, 1987). It belongs to the family of phase II detoxification enzymes, which includes glutathione S-transferase and glutathione peroxidase along with other transferases and reductases (Nebert, 1994). This enzyme family is responsible for diverting potentially reactive electrophiles from damaging interactions with the nucleophilic groups of DNA and ultimately functions to protect tissue against carcinogenic and mutagenic compounds (Talalay and Benson, 1982). Two-electron reduction bypasses the formation of the semiquinone, which in the presence of oxygen can be efficiently back-oxidised, leading to the production of active oxygen species (Lind et al., 1982; Thor et al., 1982; Fisher et al., 1992, 1993). Once quinone-containing compounds form the semiquinone or hydroquinone a rearrangement may occur, producing an active alkylating species (Tomasz et al., 1982a, b).

Two NAD(P)H:quinone oxidoreductase isozymes were first identified in human liver. NQO1 and NQO2 (Jaiswal et al., 1988, 1990). NQO1 is expressed in all tissues while NQO2 is only expressed in heart, lung, liver, brain and skeletal muscle (Jaiswal, 1994). NQO2 is an inducible homodimeric enzyme in the active state. NQO1 is believed to mediate most cellular quinone reduction since quinone-containing compounds are good substrates for purified human NQO1 (Gibson et al., 1992; Siegel et al., 1992; Ross et al., 1993). NQO2 also appears to be an inducible enzyme which is 54% similar to NQO1 at the cDNA level (Jaiswal et al., 1990; Jaiswal, 1994). The function of NQO1 has not yet been determined, although it is known that NQO1 is less effective at reducing certain quinone-containing compounds than NQO2 (Jaiswal et al., 1990; Jaiswal, 1994). The physiological role of each form still remains uncertain (Belinsky and Jaiswal, 1993). Diaphorase activity coded by independent gene loci and with distinct biochemical characteristics has been identified in most cell types, including red blood cells and sperm. Only NQO1 (also known as DT-diaphorase and diaphorase-4) and NQO2 are able to utilise either NADPH or NADH as co-factor (Fisher et al., 1977; Jaiswal, 1990; Belinsky and Jaiswal, 1993).

NQO1, is known to be induced by several procarcinogens, and a perturbation in the expression of this enzyme might occur during carcinogenesis. Increased levels of NQO1 gene expression have been observed in liver, lung and colon tumours, as well as in premalignant growths, indicating that this enzyme may have a role to play either in the carcinogenic process or in cellular defence mechanisms during tumour initiation (Cresteil and Jaiswal, 1991; Riley and Workman, 1992). It is possible that a deficiency in NQO1 may decrease the ability of the cell to detoxify carcinogens, thereby affecting cellular metabolic pathways and increasing the carcinogenic burden, and perhaps predisposing the affected individual to malignant disease.

Studies by Marshall et al. (1991) demonstrated a relationship between reduced DT-diaphorase activity and enhanced resistance of some cultured human fibroblast cell strains to mitomycin C (MMC), a quinone-containing, bioreductive DNA-alkylating chemotherapeutic agent (Lin et al., 1976; Sartorelli, 1988). These DT-diaphorase-deficient cell strains were derived from members of a cancer-prone family, some of whom had developed malignancies (Fraumeni et al., 1968). Further studies by Marshall et al. (1991) and Traver et al. (1992) revealed that NQO1 protein could not be detected by Western analysis in cell strains lines which have a very low level of DT-diaphorase activity using a polyclonal antibody against rat DT-diaphorase, even though mRNA was expressed and detected by Northern blot analysis or quantitative PCR. Traver's group, using two human adenocarcinoma cell lines, HT-29 and BE, with high and low DT-diaphorase activities respectively, found a missense mutation in the mRNA of the BE cell line at nucleotide 609, which is a predicted proline to serine change at this residue. They concluded that this missense mutation may alter the secondary structure of the enzyme and thus decrease enzyme activity without affecting mRNA synthesis (Traver et al., 1992).

The present work has extended the earlier studies of Marshall et al. (1991) and examined nine cultured human non-cancerous fibroblast cell strains with varying levels of DT-diaphorase activity, five of which were derived from a single cancer-prone family. Studying human fibroblast cell strains

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of cancer-prone families may allow genetic and biochemical changes to be detected which predispose individuals to cancer (Paterson et al., 1986). The cell strains were assessed for expression of both NQO1 and NQO2 and the possible presence of a genetic alteration which might decrease the enzymatic activity of NQO1, and perhaps be a link to the increased susceptibility to cancer noted in this family. The presence of an alteration in those cell strains with low enzyme activity would provide further evidence for the importance of the fidelity of the gene in maintaining enzymatic activity. The results reveal that all cell strains examined expressed both NQO1 and NQO2 mRNA. Furthermore, the base substitution at nucleotide 609 is present in approximately 50% of the normal population. While this base substitution is present in all of the cell strains from members of the cancer-prone family, it does not appear to have a direct effect on DT-diaphorase activity.

### Materials and methods

#### Chemicals and enzymes

MLMV-RT, random hexanucleotides, guadininium isothiocyanate, phenol, chloroform and caesium chloride were obtained from Life Technologies, Burlington, Ontario, Canada. ([35S]dATP (1000 Ci mmol⁻¹, 10 mCi ml⁻¹) was obtained from Dupont NEN, Boston, MA, USA; recombinant Taq DNA polymerase (AmpliTaq) was obtained from Perkin Elmer Cetus Corporation, Norwalk, CT, USA; Sequenase Recombinant T7 DNA polymerase (Version 2.0) was obtained from US Biochemicals, Cleveland, OH, USA.

#### Cell strains lines

The GM00038B (GM38) cell strain was obtained from NIGMS Human Genetic Mutant Cell Repository, Cornell Institute for Medical Research, Camden, NJ, USA. The BE and HT-29 cell lines were obtained from Dr T Mulkahy, Wisconsin Clinical Cancer Centre, Madison, WI, USA. Previous publications have described the origin and general variations of the cell strains from the cancer-prone family (3437T, 3701T, 3702T, 3703T, 3704T) (Paterson et al., 1986; Marshall et al., 1989). In one cell strain from a Li-Fraumeni family (2800T) and two unrelated donors (GM730A, 3424T) (Mizzayans et al., 1995). A brief description of the donors and their relationship within the cancer prone pedigree is presented in Table I. The human fibroblast cell strains were used between passages 15 and 23. All cell strains lines were maintained as exponentially growing monolayer cultures in growth medium consisting of alpha-minimal essential medium (Life Technologies) supplemented with 15% (cell strains) or 10% (cell lines) fetal bovine serum (whitaker Bioproducts, Walkersville, MD, USA, and Sigma, St Louis, MO, USA) and antibiotics (penicillin and streptomycin); cells were kept at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air in 175 cm² polystyrene tissue culture flasks (Nunc, Life Technologies).

### Isolation of total RNA

Cells were grown to confluence in 175 cm² flasks, the culture medium was removed, and the cells were washed once with cold phosphate-buffered saline (PBS) and lysed directly in the tissue culture flasks with 2.5 ml of guadininium isothiocyanate (4 M) mixture (Sambrook et al., 1989). The resulting lysate was then transferred to a polypropylene tube on ice and the lysate further disrupted by drawing it slowly through an 18 gauge needle five times. This lysate was layered over a 5.7 M caesium chloride cushion in Beckman ultracentrifuge tubes (13 × 51 mm) and centrifuged at 122 000 g at 20°C for 18 h in a Beckman SW55 rotor. The pellet was resuspended in 400 μl of Tris EDTA SDS solution and extracted once with phenol–chloroform–isoamyl alcohol (25:24:1) and once with chloroform–isoamyl alcohol (24:1). The RNA was then precipitated overnight at −20°C following the addition of 0.1 volumes of 2.5 M potassium acetate (pH 5.0) and two volumes of 100% isopropanol alcohol. The precipitates were centrifuged at 10 000 g at 4°C for 30 min. The resulting pellets were washed with 75% ethanol, reconstituted briefly, dried at 65°C and resuspended in 100 μl of diethylpyrocarbonate (DEPC)-treated water. Concentration was determined by measuring absorbance at 260 nm. RNA was stored at −70°C at a concentration of 1 μg μl⁻¹.

### Reverse transcription of RNA

NQO1 and NQO2 mRNA was transcribed into cDNA for quantitation and sequencing purposes. Briefly. 7.5 μg of RNA was added to a 13 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM potassium chloride, 3 mM magnesium chloride, 10 mM diethyNlpyrocarbonate (DTT), 2 mM deoxynucleotide triphosphates (Boehringer Mannheim, Montréal, Quebec, Canada), 10 ng μl⁻¹ random hexanucleotides, 40 units μl⁻¹ RNasin (Promega, Madison, WI, USA) and 10 units μl⁻¹ MMLV-RT. This mix was incubated at 37°C for 1 h, terminated by heating to 94°C for 5 min and the reaction quenched on ice. The resulting cDNA was then immediately amplified using the PCR reaction.

### PCR amplification

The cDNA first-strand reactions of NQO1 and NQO2 were used as templates for amplification by PCR in separate reactions. The reaction mixture consisted of 10 μl of cDNA first strand, 50 mM potassium chloride, 10 mM Tris–HCl (pH 8.4), 1.5 mM magnesium chloride, 0.001% gelatin, 0.2 mM deoxy nucleotide triphosphates, 10 units of AmpliTaq polymerase. 1 μg of each primer (synthesised using an ABI 392 DNA synthesiser) in a final volume of 100 μl. Mineral oil was layered on top of the aqueous layer. Tubes were loaded into a thermal cycler model TC1 or model 480 (Perkin-Elmer Cetus) at 94°C and 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1.5 min) and elongation (72°C, 3 min) were completed. The NQO cDNA was amplified using a 5′-NQO, specific sense oligonucleotide and a 3′-NQO, specific antisense oligonucleotide (NQO1–S1 and NQO1–AS1 respectively). Likewise, NQO2 cDNA was amplified using NQO2–S1 5′-specific sense oligonucleotide and NQO2–AS1 3′-specific antisense oligonucleotide. The expected full-length polymerase chain reaction (PCR) products of 876 (NQO1) and 766 (NQO2) nucleotides were obtained. Following amplification of cDNA the resulting fragments were purified on 1% Tris–acetic acid-EDTA agarose gel and extracted from the agarose using Qiaex beads (Qiagen, Chatsworth, CA, USA).

### Table I Clinical status and patient relationship information

| Cell strain | Clinical status of donor | Age | Sex | Relation to 3437T |
|-------------|-------------------------|-----|-----|------------------|
| 3437T       | Glioblastoma ANL        | 26  | Female | Donor            |
| 3701T       | Endometrial carcinoma   | 75  | Female | Paternal aunt    |
| 3702T       | Normal                  | 66  | Male  | Paternal uncle   |
| 3703T       | Normal                  | 60  | Female | None             |
| 3704T       | Leiomyoma               | 52  | Male  | None             |
| GM38        | Normal                  | 9   | Female | None             |
| GM730A      | Normal                  | 45  | Male  | None             |
| 3424T       | Normal                  | 55  | Female | None             |
| 2800T       | Polycythemia Nervosa    | 71  | Male  | None             |
DNA sequencing

The entire coding sequence was analysed directly from the PCR-amplified product using a series of NQO1- (S1, S2, S3, AS1, AS2) and NQO2- (S1, S2, AS1) specific oligonucleotides as sequencing primers by the method of Winship (1989).

Quantitation of mRNA

NQO1 and NQO2 gene expression was measured in all 11 cell strains lines by the method of Noonan et al. (1990) with further modifications. β-m and hydroxymethylbilane synthase (BDG) mRNA served as endogenous standards to normalise for amplification quantitation. Product sizes of NQO2, NQO1. BDG and β-m were 313, 253, 120 and 113 nucleotides respectively. RNA samples (0.8 µg) were reverse transcribed in a reaction mixture as described above with a total volume of 16 µl. The resulting cDNA (16 µl) was combined in a reaction mixture containing 50 mM potassium chloride, 10 mM Tris–HCl (pH 8.4), 1.5 mM magnesium chloride, 0.001% gelatin, 0.2 mM deoxyribonucleotide triphosphates, 4 units of AmpliTaq polymerase and 0.61 µg of NQO1-REV, 0.59 µg of NQO1-REV, 0.44 µg of NQO2-REV, 0.87 µg of NQO2-REV, 0.59 µg of BDG1 and 0.59 µg of BDG2A or 0.18 µg of β-mA3 and 0.23 µg of β-mB3 primers in a final volume of 48 µl. Aliquots of 12 µl were added to each 0.5 ml tube. Overlaid with oil and the tubes were loaded into the thermocycler, which was preheated to 94°C. Tubes were removed after 20, 22, 24 and 26 cycles of amplification with denaturation (94°C, 30 s), annealing (60°C, 30 s) and elongation (72°C, 60 s) and placed on ice. To analyse each transcription product, 3 µl of gel loading solution (0.25% bromophenol blue, 0.25% xylene cyanol and 15% Ficoll) was added to each reaction mixture and the mixture was electrophoresed on a 12% native polyacrylamide gel. Gels were then stained with ethidium bromide, destained with water and photographed using Kodak 665 positive–negative film. The negatives were developed according to the manufacturer's instructions. The negatives were then examined using a laser scanning densitometer (Molecular Dynamics model 373A) with ImageQuant v3.3 software. In order to ensure that the NQO1, NQO2, and endogenous standards were amplified together in the linear amplification range, both β-m and BDG were used as high and low cDNA expression standards, respectively, in all reactions. By using both endogenous standards, cell lines with both high and low expression of product were comparable. To normalise the expression of NQO2 and NQO1 to that of the BDG or β-m, the ratio between the amount of product within the linear amplification range (previously determined, data not shown) of the target genes and the endogenous standard was calculated as follows:

\[
\text{Ratio of PCR products} = \frac{\text{volume target gene}_{\text{sample}}}{} \times \frac{\text{volume internal control gene}_{\text{standard}}}{\text{volume internal control gene}_{\text{sample}}}
\]

GM38 was chosen as a standard against which to compare the remaining cells for NQO1 and NQO2 expression while BE was the standard for the 345 nucleotide product.

Primers

NQO1-5' ACTGCAAGCTTTACGCGGCCGGACTG [bases 23–40 of NQO1, with a HindIII restriction site (underlined) 5' adjacent]

NQO1-5' GGAAACGCGACAGCTTTGTGATATT [bases 326–349 of NQO1]

NQO1-3' GAAGCCGATGCCTTCCATCA [bases 473–492 of NQO1].

Restriction digestion

Genomic DNA from the lymphocytes of 44 normal individuals was obtained from Dr Mark Minden, Ontario Cancer Institute, Toronto, Canada. One microgram of this DNA was PCR amplified, as previously described, using the following touchdown PCR amplification cycles (Don et al., 1994): 2 min at 94°C, two cycles of 94°C for 15 s, 69°C for 15 s and 74°C for 30 s; two cycles of 94°C for 15 s, 67°C for 15 s and 74°C for 30 s; two cycles of 94°C for 15 s, 65°C for 30 s and 74°C for 60 s using primers specific for NQO1. exon 6 (exon 6A and exon 6B). The amplified product was approximately 211 nucleotides in length. Following amplification 10 µl of the DNA was digested with HindIII for 1 h at 37°C. The HindIII-digested product yielded 165 and 46 nucleotide products. The product was then analysed on a 12% native polyacrylamide gel. Gels were stained with ethidium bromide, destained with water and photographed.

Enzymatic activity

Cells were grown to subconfluence before being washed with PBS and harvested with an 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) solution in citrate saline. Cells were pelleted by centrifugation at 2400 g for 6 min and washed twice with PBS and resuspended at 106 cells ml-1 in sterile deionised water. Cells were disrupted by three 10-min cycles of freeze thawing in dry ice–methanol. Protein concentration was determined using a Total Protein Diagnostic Kit (Sigma). Reduction of the substrate 2,6-dichlorophenolindophenol (DCPIP) was measured to determine DT-diaphorase activity by the method of Benson et al. (1980) with modifications as previously described (Kuehl et al., 1993). The activity of P450R was measured using cytochrome c as the electron acceptor according to the procedure of Strobel.
and Dignam (1978) with modifications as noted previously (Kuehl et al., 1993).

Results

Table I demonstrates the clinical status and pedigree relationship of the cell strains obtained from members of a single cancer-prone family (3437T, 3701T, 3702T, 3703T, 3704T). Three control cell strains (GM38, GM730A and 3424T) obtained from three clinically normal volunteers, one non-related cell strain from a Li–Fraumeni family member (2800T) as well as two human adenocarcinoma cell lines HT-29 and BE (Table II) were also examined. Table III shows the range of DT-diaphorase [reported as dicoumarol (DIC) inhibited] enzyme activity for these cells. The DT-diaphorase activity levels of these cell strains differed over a 200-fold range (18–3462 nmol min⁻¹ mg⁻¹ protein). Table III, with the BE cell line expressing the lowest activity and the 3424T cell strain expressing the highest. Cellular DT-diaphorase activity is measured as a functional assay (materials and methods), such that the activity reported is that portion of the activity inhibited by DIC, a reversible and competitive inhibitor of DT-diaphorase (Halliwell and Gutteridge, 1984). It is believed that NQO and DT-diaphorase are the same enzyme, although it remains in question if the assay also measures some low level of NQO activity; even though it is much less active towards the substrate DCPIP than NQO (Jaiswal et al., 1988; Jaiswal, 1994). Enzyme activity is therefore presented as DT-diaphorase activity. The P450R activity levels only differed over a range of 3-fold (1–3 nmol min⁻¹ mg⁻¹ protein, data not shown).

To determine if the present cell strains lines expressed both NQO, and NQO; mRNAs, and the level of expression of the transcripts, quantitative reverse transcription RT–PCR was performed to obtain predefined product sizes. Table III shows the levels of NQO, and NQO mRNA expression normalised to the endogenous standards β-m and BDG, and relative to GM38 or BE levels in the cell strains lines studied. All of the cell strains lines express both NQO, and NQO, mRNA, and results for three cell strains lines are shown in Figure 1. This technique also revealed the presence of a larger transcript (approximately 345 nucleotides) in the cells (Figure 1). This transcript is most prevalent in the BE cell line which expresses only trace amounts of the NQO product (Figure 1 and Table III), but can be observed in moderate levels in the 3437T and 3703T cell strains, and in trace amounts (<0.2 of BE level) in the remaining cells (Table III). There does not appear to be any obvious correlation between mRNA expression for NQO or NQO, and DT-diaphorase activity in these cell strains lines.

Sequencing of the entire NQO cDNA from all cell strains lines revealed the presence of a single base change (as reported by Jaiswal et al., 1988), from C to G, at base 98 in the coding region of the NQO cDNA, analogous to that found by Traver et al. (1992). This base change does not affect the encoded amino acid. Neither the GM38, GM730A, 3424T, 2800T cell strains nor the HT-29 cell line demonstrated any additional deviations from the reported sequence (Jaiswal et al., 1988). The 3437T, 3702T, 3703T and 3704T cell strains and the BE cell line contained both a C and a T at nucleotide 609 in the coding region, suggesting that these cells express both the wild-type and an altered form of NQO, mRNA. The 3701T cell strain expresses only the T at nucleotide 609. Figure 2 shows a representative sequencing gel of nucleotides 600–615 from each cell type (wild-type C, heterozygous C,T, homozygous T). This nucleotide substitution, which is predicted to change a proline to a serine residue, was found even after multiple samples (2–4) were sequenced indicative that they were not incorporated PCR errors. The NQO, cDNA from both the 3437T and the GM38 cell strains were sequenced and matched the NQO, coding sequence (Jaiswal et al., 1990).

The presence of the substituted T at position 609 creates a Hin1I restriction site (G ANTNC). Figure 3 demonstrates the results of the digestion for three of the human fibroblast cell strains (GM38, 3437T and 3701T). The figure shows that in the amplified product no digestion occurs in the absence of the T (GM38, 211 nucleotide fragment), while partial digestion occurs in the presence of both the C and T (3437T, 211 and 165 nucleotide fragments) and complete digestion occurs in the presence of the T only (3701T, 165 nucleotide fragment). The 45 nucleotide fragment could not be detected due to poor resolution of the gel. This restriction site was exploited to determine if this base substitution at 609 was a polymorphism present in the population or a mutation carried in this cancer-prone family. Restriction digestion of the 211 nucleotide fragment of NQO, exon 6 (Jaiswal, 1991) from 44 normal individuals with Hin1I revealed that approximately 40% (18/44) of this population express both the C and T and 9% (4/44) express only the T at this position.

Table II Cell origin and patient relationship information

| Cell line | Cell origin | Age | Sex | Relation to 3437T |
|-----------|-------------|-----|-----|------------------|
| HT-29     | Adenocarcinoma | 44  | Female | None |
| BE        | Adenocarcinoma | 59  | Male  | None |

Table III Analysis of the biochemical and molecular characteristics of the experimental cell strain lines

| Cell strain line | DT-diaphorase activity | NQO1 | NQO2 | Nucleotide |
|------------------|------------------------|------|------|------------|
|                  | / nmol min⁻¹ mg⁻¹ protein | RNA1 | RNA2 |            |
| 3437T            | 72 ± 12                | 2.0 ± 0.13 | 1.3 ± 0.33 | C T |
| 3701T            | 41 ± 8                 | 1.3 ± 0.1 | 0.95 ± 0.11 | T |
| 3702T            | 863 ± 184              | 0.82 ± 0.05 | 1.1 ± 0.16 | C T |
| 3703T            | 1823 ± 104             | 1.7 ± 0.13 | 1.1 ± 0.06 | C T |
| 3704T            | 657 ± 139              | 1.2 ± 0.08 | 1.1 ± 0.07 | C T |
| GM38             | 1242 ± 414             | 1.1 ± 0.02 | 1.1 ± 0.19 | C |
| GM730A           | 2112 ± 738             | 0.62 ± 0.02 | 1.2 ± 0.15 | C |
| 3424T            | 3462 ± 201             | 1.1 ± 0.01 | 0.81 ± 0.15 | C |
| 2800T            | 1162 ± 406             | 1.1 ± 0.03 | 1.2 ± 0.31 | C |
| HT-29            | 2037 ± 1326            | 1.4 ± 0.07 | 0.45 ± 0.04 | C |
| BE               | 18 ± 10                | 0.89 ± 0.07 | 0.13 ± 0.03 | C T |

*Values shown are mean ± s.d. of at least three independent cell extracts. Values shown are mean ± s.e. of three determinations from three independent RT–PCR reactions. †Values shown are mean ± s.d. of at least three independent RT–PCR reactions. ‡Values relative to BE NQO, to 345 nucleotide product: 3437T, 0.49 ± 0.1; 3703T, 0.50 ± 0.08. All others < 0.2. ‡Ratio of GM38 NQO, NQO, expression is 3:1.
Altered activity levels of DT-diaphorase have been observed in many different cell strains lines. Recent reports have suggested that the presence of a missense mutation at position 609 (Traver et al., 1992) and the subsequent loss of heterozygosity at the NQO1 locus (Eickelmann et al., 1994a) are responsible for reduced DT-diaphorase activity in an adenocarcinoma cell line (Traver et al., 1992) and a bladder carcinoma cell line (Eickelmann et al., 1994a). To determine if genetic alterations are involved in DT-diaphorase activity levels, the current study has further characterised cell strains from members of a cancer-prone family, as well as three non-related donors, which have varying DT-diaphorase activities. The present work, as well as previous work by others (Marshall et al., 1991; Traver et al., 1992), shows that, although the DT-diaphorase activity levels differ markedly, the cell strains lines examined express similar levels of NQO1 mRNA. These other groups have attempted to detect NQO1 protein by immunoprecipitation and Western blot analysis using a rat polyclonal antibody against DT-diaphorase and were able to detect protein in the cells which had high levels of NQO1 activity but not in those cells with extremely low NQO1 activity (343T, 3701T and BE) (Marshall et al., 1991; Traver et al., 1992). Traver et al. (1992) sequenced the NQO1 cDNA in both the BE and HT-29 human colon carcinoma cell lines and found a C to T base change at nucleotide 609 in the BE cell line and a second base change at nucleotide 98 in both cell lines. The first nucleotide change at position 609 would probably result in the conversion of proline 187 to a serine while the second base change at nucleotide 98 would not affect the encoded amino acid. They suggested that the loss of the proline residue may alter the secondary structure of the protein, possibly affecting the pyridine binding site of the enzyme or cause conformational changes around the cysteine residue. Reduced enzyme activity in this case might be explained by an altered co-factor binding or other necessary tertiary interactions.

When the cell strains lines were examined for NQO1 and NQO2 gene expression, it was found that all expressed NQO1 and NQO2 mRNA, and that there was no relationship between the expression of the messages and enzyme activity. This differs from the work reported by Traver et al. (1992), which does conclude that a correlation exists between NQO1 mRNA and DT-diaphorase activity in carcinoma cell lines. The lack of correlation in cell strains may reflect a more natural system which undergoes ageing-related changes. The BE cells appear to express only trace amounts of the NQO2 product but express a large amount of a larger 345 nucleotide product (Figure 1 and Table III). The remaining cells appear to also express this larger product, but in varying amounts. The 345 nucleotide product appears to be more prevalent in those cells from the members of the cancer-prone family as well as in the BE cells. The significance of this larger product and the low level of the NQO2 product in BE cells is unknown and further studies are under way in an attempt to resolve this question. Similar to the results of Traver et al. (1992), this work also found a single base change from a C to a G at base 98 in the coding region of the NQO1 cDNA in all of the cell strains lines examined. This change may represent a population
polymorphism or, more likely, the originally reported sequence (Jaiswal et al., 1988) at base 98 is incorrect and the G is the wild-type nucleotide. A greater number of individuals will need to be examined to verify this observation.

When the cDNA from the cell strains/lines were sequenced for genetic alterations in the NQO1 gene, it was found that four of the cell strains from the related members of the cancer prone family (3437T, 3702T, 3703T, 3704T) as well as the BE cell line contained both C and T nucleotides at position 609 of the NQO1 locus (Table III). The 3701T cell strain appears to be homozygous for the T at nucleotide 609. In the previous report by Traver et al. (1992) the BE cell line was also reported to only express a T at nucleotide 609, in contrast to the present report, in which the BE cell line is heterozygous at nucleotide 609. One possible explanation for this discrepancy is that the BE cells previously reported have either gained a second alteration at this nucleotide or lost their wild-type allele, although the more likely explanation is the different PCR amplification and sequencing techniques employed in the present work and the previous work. Traver et al. (1992) amplified and sequenced single-stranded as opposed to double-stranded DNA. This technique may have selectively amplified the T-containing mRNA over the C-containing mRNA, and hence the C would not be observed.

When exon 6 was examined in the genomic DNA of 44 normal individuals it was found that approximately 40% expressed both the C and the T while 9% expressed only the T nucleotide. Previously, it has been reported that 4–10% of the population lack DT-diaphorase activity (Edwards et al., 1980; Eckelmann et al., 1994a) and that approximately 11% of the population have an intermediate level (Edwards et al., 1980). Several groups have speculated that the T substitution at nucleotide 609 is responsible for reduced or undetectable DT-diaphorase expression in human tissue samples (Rovold et al., 1993; Eckelmann et al., 1994a,b). Eckelmann et al. (1994a) also demonstrated a lack of measurable DT-diaphorase activity in three patient kidney carcinoma samples and in a bladder carcinoma cell line (RT112MMC), all of which appear to only express the T at nucleotide position 609 as determined by sequencing analysis and restriction diges-

tion with HindII. Regardless, the present work suggests that the function of the substituted nucleotide remains unclear since a large percentage of the population examined appear to be heterozygous at this nucleotide and, as seen in Table III, C/T heterozygotes have widely differing DT-diaphorase activity. Using the Wilcoxon rank-sum test, the five wild-type C cell strains also demonstrated a significant difference in DT-diaphorase activity from the five C/T cell strains at the 0.05 level.

Two models are proposed for the role of the substituted T nucleotide at position 609. The first model is that the presence of the T is a polymorphism present in the population which has no functional role and no effect on DT-diaphorase activity. The second model is that the T is a missense mutation which plays a role in altering DT-diaphorase activity. Post-transcriptional regulation of DT-diaphorase activity may occur, such as decreased expression of the C-containing allele, destabilisation of the mRNA or the formation of an altered protein. Future work will require a close examination of both these models to determine how DT-diaphorase activity is regulated and the impact of this regulation.

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