Alternative Splicing as the Basis for Specific Localization of tNOX, a Unique Hydroquinone (NADH) Oxidase, to the Cancer Cell Surface†

Xiaoyu Tang,‡ Zengsui Tian,§ Pin-Ju Chueh,§ Ssuhen Chen,§ Dorothy M. Morré,‡ and D. James Morré*,§

Departments of Medicinal Chemistry and Molecular Pharmacology and of Foods and Nutrition, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT: A novel hydroquinone and NADH oxidase with protein disulfide−thiol interchange activity (designated ENOX2 or tNOX), associated exclusively with the outer leaflet of the plasma membrane at the surface of cancer cells and in sera of cancer patients, is absent from the surface of noncancer cells and from sera of healthy individuals. Full-length tNOX mRNA is present in both normal and tumor cells but appears not to be expressed in either. Our research suggests alternative splicing as the basis for the cancer specificity of tNOX expression at the cell surface. Four splice variants were found. Of these, the exon 4 minus and exon 5 minus forms present in cancer cell lines were absent in noncancer cell lines. In contrast to full-length tNOX cDNA, transfection of COS cells with tNOX exon 4 minus cDNA resulted in overexpression of mature 34 kDa tNOX protein at the plasma membrane. The exon 4 minus form resulted in initiation of translation at a downstream M231 initiation site distinct from that of full-length mRNA. With replacement of M231 by site-directed mutagenesis, no translation of exon 4 minus cDNA or cell surface expression of 34 kDa mature tNOX was observed. The unprocessed molecular mass of 47 kDa of the exon 4 minus cDNA translated from methionine 231 corresponded to that of the principal native tNOX form of the endoplasmic reticulum. Taken together, the molecular basis of cancer-cell-specific expression of 34 kDa tNOX appears to reside in the cancer-specific expression of exon 4 minus splice variant mRNA.

Our work has described a unique, growth-related family of cell surface hydroquinone or NADH oxidases with protein disulfide−thiol interchange activity referred to as ECTO-NOX (ENOX) proteins (for cell surface NADH oxidases) (*1*). One member of the ECTO-NOX family, designated ENOX2 or tNOX (for tumor-associated) is specific to cancer (*2*, *3*). The presence of the tNOX protein has been demonstrated in several human tumor tissues (mammary carcinoma, prostate cancer, neuroblastoma, colon carcinoma, and melanoma) (*4*). However, serum analyses suggest a much broader association with human cancer (*5*, *6*). NOX proteins are ectoproteins bound to the outer leaflet of the plasma membrane (*7*). A unique characteristic that distinguishes tNOX from other ECTO-NOX proteins is its sensitivity to inhibition by anticancer drugs. The NADH oxidase activity of tNOX was inhibited by adriamycin (*8*), the antitumor sulfonylurea LY181984 (*9*), capsaicin (*3*), the catechin (−)-epigallocatechin 3-gallate (*10*), and the synthetic anticancer isoflavene phenoxodiol (*11*). As is characteristic of other examples of ectoproteins (sialyl and galactosyl transferase, dipeptidylamino peptidase IV, etc.), the NOX proteins are shed. They appear in soluble form in conditioned media of cultured cells (*12*) and in patient sera (*5*, *6*). The serum form from cancer patients exhibits the same degree of drug responsiveness as the membrane-associated form. The tNOX of sera from cancer patients was inhibited by the same drugs and substances and to approximately the same extent as the plasma membrane form. Drug-inhibited tNOX activities are seen in sera of a variety of human cancer patients, including patients with leukemias, lymphomas, or solid tumors (prostate, breast, colon, lung, pancreas, ovarian, liver) (*5*, *6*). An extreme stability and protease resistance (*13*) may help explain the ability of tNOX proteins to accumulate in sera of cancer patients to readily detectable levels. In contrast, drug-responsive NOX activities have not been detected with sera of healthy volunteers (*5*, *6*).

The concept of a tNOX protein specific to the surface of cancer cells is supported by several lines of evidence. (1) A drug-inhibited tNOX activity was not observed from plasma membranes of nontransformed human and animal cells and tissues (*1*, *3*). (2) A drug-inhibited tNOX activity is below the limits of detection (0.1 to 0.01 that of cancer patients) in sera from healthy volunteers (*4*) or from patients with diseases other than cancer (cardiac, arthritis and other inflammatory diseases, gastric ulceration, emphysema, various nonmalignant blood disorders). This observation has relevance since tNOX proteins being shed into the circulation is a reliable indicator of cancer presence (*5*, *6*). (3) The tNOX proteins lack a transmembrane binding domain (*14*) and are released from the cancer cell surface by brief treatment at low pH (*13*). No drug-inhibited NOX activity was detected in similar preparations from noncancer cells. (4) The immunoreactive band at 34 kDa (the processed molecular mass of the cell surface form of tNOX) has not been observed

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* To whom correspondence should be addressed. Phone: (765) 494-1388. Fax: (765) 494-4007. E-mail: morre@pharmacy.purdue.edu.

‡ Department of Foods and Nutrition.

§ Department of Medicinal Chemistry and Molecular Pharmacology.

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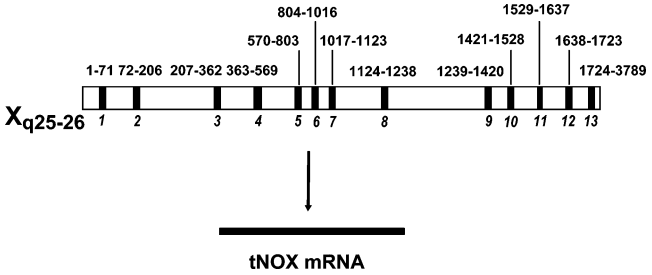

FIGURE 1: The tNOX gene located on chromosome X is composed of 13 exons.

by Western blot analysis or immunoprecipitation for non-transformed cells and tissues or sera from healthy volunteers or patients with disorders other than cancers as antigen sources (*4*, *15*, *16*) using several different tNOX antisera. Those antisera include (a) a monoclonal antibody (*4*), (b) a single-chain variable region fragment (ScFv)[1] recombinant antibody derived from DNA of the monoclonal hybridoma, (c) polyclonal antisera to expressed tNOX (*16*), and (d) polyclonal peptide antisera to the conserved adenine nucleotide binding region of tNOX (*14*).

tNOX cDNA has been cloned (GenBank accession no. AF207881; *17*). The derived molecular mass from the open reading frame was 70.1 kDa. Identified functional motifs include a quinone binding site, an adenine nucleotide binding site, and a CXXXXC cysteine pair as a potential protein disulfide−thiol interchange site on the basis of site-directed mutagenesis (*18*). On the basis of available genomic information (*19*), tNOX cDNA is comprised of 13 exons (Figure 1).

To elucidate the basis for the cancer-specific expression of cell surface tNOX, a series of RT-PCR, expression, Western blotting, and site-directed mutagenesis studies were carried out. The findings indicate that an exon 4 minus gene transcript of tNOX which arises as the result of an alternative splicing event unique to transformed cells is the source of the tNOX protein.

MATERIALS AND METHODS

Materials. General chemicals and reagents were from Sigma. DNA markers, Taq polymerase, and the random hexamer priming kit were from Promega (Madison, WI). SuperScript II reverse transcriptase, mammalian expression vector pcDNA3.1, and the calcium phosphate transfection kit were from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Ipswich, MA). Human multiple-tissue Northern blot was from Clontech (Palo Alto, CA). The Fast-Link DNA ligation kit was from Epicentre (Madison, WI). Cloned Pfu DNA polymerase and *E. coli* strain XL1-blue were from Stratagene (La Jolla, CA). The gel purification kit and plasmid DNA mini and midi purification kits were from Qiagen (Valencia, CA). Alkaline phosphatase-conjugated monoclonal anti-rabbit IgG was from Sigma (St. Louis, MO). Peptide antibodies PU02 and PU04 were generated by Covance Research Products, Inc. (Denver, PA). DNA sequencing was by the DNA Sequencing Center, Tufts University.

Total RNA Preparation. Approximately 10^6 cells of HeLa (human cervical carcinoma cells), BT-20 (human breast adenocarcinoma cells), and MCF-10A (human breast epithelial cells) were collected and washed twice with cold PBS, pH 7.4. Each cell pellet was resuspended in 300 μL of RNA preparation solution I (10 mM Tris−Cl, 0.15 M NaCl, 1.5 M $MgC1_2$, 0.65% NP-40, DEPC-treated). After centrifugation at 800*g* for 10 min, the upper phase was transferred to a new tube, and 200 μL of RNA preparation solution II [7 M urea, 1% SDS, 0.35 M NaC1, 10 mM EDTA, 10 mM Tris−Cl, DEPC (diethyl pyrocarbonate)-treated] was added. Then 400 μL of phenol/chloroform/isoamyl alcohol (50:50:1) was added, and the mixture was centrifuged at 15000*g* for 5 min. The clear phase that contained RNA was precipitated with 95% ethanol and dissolved in DEPC-treated water.

RT-PCR. A 20 μL reaction volume containing 1 μL of oligo$(dT)_{12-18}$ (500 μg/mL), 1 μL of 10 mM dNTP, 4 μL of 5× first-strand buffer, 2 μL of 0.1 M DTT, 5 μg of total RNA, and 1 μL of reverse transcriptase was used. The reaction mixture was incubated at 42 °C for 1 h, followed by heating at 70 °C for 15 min. The synthesized first-strand cDNA was amplified by PCR using primers 5′-CAGCCGATAACAGTAGAACTCTGA-3′ (forward) and 5′- CTGATTCCTCAGTTTCTTTTGTTTCTG-3′ (reverse). PCR products were separated on a 1% agarose gel and recovered using a gel extraction kit. Purified PCR products were sequenced.

Northern Blot. The cDNA probes used in the Northern blot were generated from PCR using a pcDNA3.1−tNOX plasmid. The probes were labeled prior to use with [α-^{32}P]-dCTP using a random hexamer priming kit (Promega). The probe used was 1.2 kb tNOX cDNA corresponding to the tNOX sequence from bp 680 to bp 1830. The human MTN (multiple-tissue Northern) blot was incubated with prehybridization solution at 65 °C for 6 h. Then a hybridization solution with either of the probes was added and the resulting solution incubated at 65 °C overnight. The MTN blot was washed and placed with film at −80 °C for autoexposure.

5′ Rapid Amplification of cDNA Ends (5′-RACE). Total RNA from BT-20 and MCF-10A cells was prepared by the guanidine isothiocyanate/acid−phenol method. Total RNA was quantified by Hitachi U-1100 UV spectrometry and stored at −20 °C in 30 μL of DEPC-treated water. A custom-designed primer, 109L, which is a 20 nt reverse primer matching the end of exon 2, 5′-GGTAAAATTGGTTGTCCGGC-3′, was used in the first-strand synthesis and the later PCR process. Tris−HCl (20 mM, pH 8.4), 50 mM KC1, 2.5 mM $MgC1_2$, 10 mM DTT, 100 nM cDNA primer 109L, 400 μM each dATP, dCTP, dGTP, and dTTP, 5 μg of RNA, and 200 units of Superscript II RT were used in the first-strand cDNA synthesis. After incubation at 42 °C for 1 h, the reaction was terminated by incubation at 70 °C for 15 min. Then RNase H was added to the mixture to digest the RNA in the RNA/cDNA hetero double strand. The digest was transferred to the G1assMAX spin cartridge and centrifuged at 13000*g* for 20 s. After three washes, the first-strand cDNA was purified and concentrated for the next step of terminal nucleotidyl transferase (TdT) tailing; several dC nucleotides were added at the 3′-end of the first-strand

[1] Abbreviations: ScFv, single-chain variable region fragment; PBS, phosphate-buffered saline; RT, reverse transcription; RACE, rapid amplification of cDNA ends; E4m, exon 4 minus form; E5m, exon 5 minus form; EGFP, enhanced green fluorescent protein.

cDNA. Using anchoring primers and the primer 109L, the cDNA was specifically amplified and resolved on a 1% agarose gel. It was further cloned into the T-vector and sequenced.

RT-PCR To Detect Full-Length, Exon 4 Minus, and Exon 5 Minus tNOX mRNA in Cancer and Noncancer Cells. Total RNA was prepared from 2×10^7 cells. The forward primer was at the beginning of exon 2: 5′-CCGATAACAGTAGAACTCTG-3′. The reverse primer was from the end of exon 8: 5′-CTGATTCCTCAGTTTCTTTTCTTTCTG-3′. Equal amounts of RNA were used within each experiment. PCR products were further cloned into the T-vector and sequenced.

RT-PCR To Detect Exon 4 Minus tNOX mRNA in Cancer and Noncancer Cells. Total RNA was prepared from 2×10^7 cells. As a forward primer, we used a 20-mer oligonucleotide at the junction of exon 3 and exon 5 with 15 nucleotides from exon 3 and 5 nucleotides from exon 5 as follows: 5′-TCCCTCCAAATCCAAGTTAC-3′. The reverse primer was from the end of exon 8: 5′-CTGATTCCTCAGTTTCTTTTCTTTCTG-3′. The expected product of 670 nucleotides was obtained. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as controls (640 nucleotides).

Expression of the Exon 4 Minus Form and Exon 5 Minus Form of tNOX in COS Cells. Plasmids carrying exon 4 minus and exon 5 minus tNOX sequences were prepared by replacing the full-length tNOX sequence in pcDNA3.1−full-length tNOX with the exon 4 minus or exon 5 minus tNOX sequence. This was accomplished by first amplifying by PCR the portion of the exon 4 minus form of tNOX (E4m tNOX) and of the exon 5 minus form of tNOX (E5m tNOX) cDNA as defined by the primers 5′-CCGATAACAGTAGAACTCTGAAC-3′ (forward) and 5′-GGCTGATTCCTCAGTTTCTTTTGTTTC-3′ (reverse). The PCR products were then ligated to pGEM-T Easy vector (Promega) for sequencing. The construct was digested with *Not*I. The digested products were separated on an agarose gel and extracted using a DNA extraction kit (Qiagen). The DNA then was doubly digested with *Ban*I and *Ase*I. The digested products also were separated on an agarose gel and extracted (fragment A). The pcDNA3.1−full-length tNOX was doubly digested with *Hin*dIII and *Bam*HI, and a fragment containing pcDNA3.1 and part of tNOX (fragment D) and fragment F were obtained. Fragment F was then doubly digested with *Ban*I and *Ase*I to get three fragments. There was a fragment between *Hin*dIII and *Ban*I (fragment B), a fragment between *Ban*I and *Ase*I (fragment G), and a fragment between *Ase*I and *Bam*HI (fragment C). These digested products were separated on an agarose gel and extracted. Finally, the four fragments (A, B, C, and D) were ligated using a Fast-Link kit (Epicentre). The resulting plasmid was used to transfect COS cells.

DNA sequences of the ligation products were confirmed by DNA sequencing. Expression of the exon 4 minus form of tNOX and the exon 5 minus form of tNOX was confirmed by SDS−PAGE with immunoblotting. Immunoblot analysis was with tNOX peptide antibody to the semiconserved adenine nucleotide binding domain (PU02). Detection used alkaline phosphate-conjugated anti-rabbit antibody.

COS cells (SV-40 transformed African monkey kidney cell line provided by Prof. E. Taparowsky, Purdue University) were plated 1 day prior to transfection at 4×10^5 cells per 100 mm dish. A 36 μL portion of 2 M $CaCl_2$ and 30 μg of pcDNA3.1−E4m tNOX or pcDNA3.1−E5m tNOX in 300 μL of sterile H_2O were slowly added dropwise to 300 μL of 2× HEPES-buffered saline at room temperature for 30 min. The transfection mixtures then were added dropwise to the medium containing the cells and the resulting mixtures incubated overnight at 37 °C. After overnight exposure to the DNA precipitate, the cells were washed twice with PBS, and 3 mL of 10% DMSO was added for 2.5 min. Then the 10% DMSO was removed, and the cells were fed with fresh medium for 2−3 days. For selection of stable transfectants, fresh medium containing 0.5 mg/mL G418 sulfate (Invitrogen) was added to the medium twice each week, and the cultures were maintained until colonies 2−3 mm in diameter were formed. A total of four colonies were selected and trypsinized individually followed by transfer into the wells of a 24-well plate and then into a 35 mm dish. The cells were harvested at ca. 80% confluency.

Site-Directed Mutagenesis. Methionines M220, M231, and M314 were replaced by alanines by site-directed mutagenesis according to Braman et al. (*20*). Numbered amino acids and nucleotide positions of splice variant products refer to numbers assigned to nucleotides and amino acids of the full-length transcript prior to sequence deletions (*17*).

Western Blot. Approximately 10^6 COS cells were transfected with pcDNA3.1, pcDNA3.1−tNOX, pcDNA3.1−exon 4 minus tNOX, or pcDNA3.1−exon 5 minus tNOX. Whole cell extracts were dissolved in 100 μL of 10 mM Tris−Cl and immediately loaded onto 10% SDS−PAGE gels. Resolved proteins were transferred onto a nitrocellulose membrane. After transfer, the nitrocellulose membrane was incubated with either PU02 or PU04 anti-tNOX antibody at 4 °C overnight. PU02 is a polyclonal peptide antibody to the tNOX C-terminus containing the putative adenine nucleotide binding motif (T589GVGASLEKRWKFCGFE605). PU04 is a polyclonal antibody to a tNOX peptide that includes a putative E394EMTE quinone binding site (EEMTECRREEEMEMSDDEIEEMTETK). The membrane was incubated with alkaline phosphatase-conjugated monoclonal anti-rabbit IgG at room temperature for 1 h. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) was used as the substrate. The membrane was placed into 5 mL of alkaline phosphate substrate buffer (100 mM Tris−HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) mixed with 33 μL of NBT and 16.5 μL of BCIP with shaking at room temperature.

Preparations of plasma membranes and internal cellular membranes free of plasma membranes were by aqueous two-phase partition of cell homogenates as described (*21*). The purity of the plasma membrane was determined to be >90% by electron microscope morphometry. The yield was approximately 20 mg of plasma membrane protein from 10^{10} cells.

Construction of Plasmids. To amplify the E4m tNOX coding region by PCR, oligodeoxyribonucleotide primers 5′-GATCTCGAGCTCAAGCTTGACCACACAATGCAA-3′ (forward) and 5′-ATCCCGGGCCCGCGGTACCGTCAGCTTCAAGCC-3′ (reverse) were used. The PCR products were digested with *Hin*dIII and *Kpn*I restriction endonucleases, and the DNA fragments were purified by agarose gel electrophoresis. The plasmid pEGFP-N1 was digested with

*Hin*dIII and *Kpn*I, and the backbone of the expression plasmid was gel-isolated. The two purified fragments were then joined to give pE4m−EGFP.

The 3′-end of the coding region of tNOX was fused to the Myc epitope from pCMV-Tag5A (Stratagene) to give pFL-tNOX−Myc. To amplify the full-length tNOX coding region by PCR, oligodeoxyribonucleotide primers 5′-GATCTCGAGCTCAAGCTTGACCACACAATGCAA-3′ (forward) and 5′-TTTATCGAGGTCGACGGTCAGCTTCAAGCCCTC-3′ (reverse) were used. The PCR products were digested with *Hin*dIII and *Sal*I restriction endonucleases, and the DNA fragments were purified by agarose gel electrophoresis. The plasmid pCMV-Tag5A was digested with *Hin*dIII and *Sal*I, and the backbone of the expression plasmid was gel-isolated. The two purified fragments were then joined to give pFL-tNOX−Myc.

Expression of Myc-Tag Full-Length tNOX in COS Cells. The COS cells were transfected with pFL-tNOX−Myc expression plasmids by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. COS cells at 90% confluency were transfected in 10 cm plates. The cells were harvested 48 h after transfection. The whole cell extracts and isolated microsomes were analyzed.

Expression of EGFP-Fused E4m tNOX in COS Cells. The E4m−EGFP expression plasmids were constructed using pEGFP-N1, which allowed expression of the enhanced green fluorescent protein fused at the C-terminus of E4m tNOX. *E. coli* cells were transformed with pE4m−EGFP, and the resultant plasmids were isolated. COS cells were transfected with pE4m−EGFP using calcium phosphate (Stratagene) transfection and DMSO shock according to the instructions furnished by the manufacturer.

Confocal Laser Scanning Microscopy. Cells were grown in Corning glass chambers and observed on a Bio-Rad MRC-1024 confocal microscope. Fluorescence of EGFP was excited using a 488 nm krypton/argon laser, and the emitted fluorescence was detected with a 515−540 band-pass filter. For tetramethylrhodamine concanavalin A, a 568 nm krypton/argon laser was used for excitation, and fluorescence was detected with a 590 nm band-pass filter. The cells were rinsed with cold PBS and treated with tetramethylrhodamine concanavalin A (10 μg/mL in PBS) for 2 min at 4 °C. After a final rinse with cold PBS, the cells were observed with a confocal microscope using the 60× oil immersion lens.

RESULTS

Full-Length tNOX mRNA Identical to That of Cancer Cells Exists in Human Noncancer Cells and Tissues. HeLa cells (human cervical carcinoma cell line) express the full-length mRNA and the functional 34 kDa tNOX protein. To investigate whether the full-length tNOX mRNA was cancer specific, human normal cell lines and tissues were examined separately by RT-PCR and Northern blot. In the Northern blot, commercially available human normal tissue mRNAs were probed with the tNOX cDNA sequence. Of the eight different human normal tissues examined, all exhibited detectable 3.8 kb full-length tNOX mRNA (Figure 2), although the levels of transcription varied. The membrane was reprobed with probes for β-actin to assess RNA integrity and loading. Sequencing results of RT-PCR products showed comparable full-length tNOX cDNA sequences in comparing both cancer and noncancer cell lines (*17*).

Full-Length 71 kDa tNOX Protein Not Translated. The tNOX cDNA open reading frame (ORF) is 1.83 kb's and translates into 610 amino acids. There was no naturally expressed 71 kDa tNOX (corresponding to the 610 amino acid open reading frame) observed in either cancer or noncancer cells (data not shown). The tNOX cDNA sequence has an unusually short 22 nt 5′-UTR. This may explain why translation may not start from the first ATG. On the other hand, it raises the question of whether there is additional sequence in the 5′-UTR and whether there was another ATG beyond the 5′-UTR end in cancer cells.

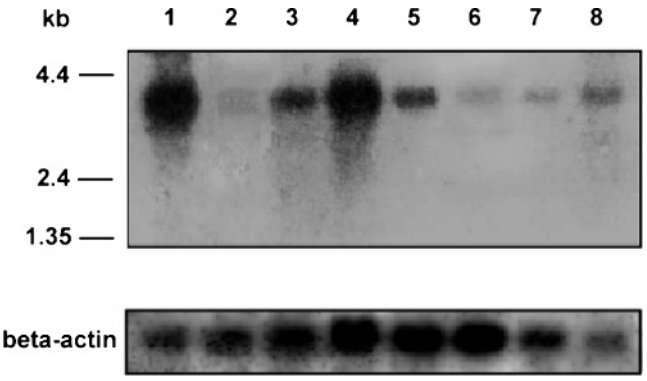

FIGURE 2: Northern blot analysis of normal human tissues probed by ttNOX (nucleotides 680−1830): lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, peripheral blood leukocytes. Probes for β-actin were used to assess RNA integrity and loading.

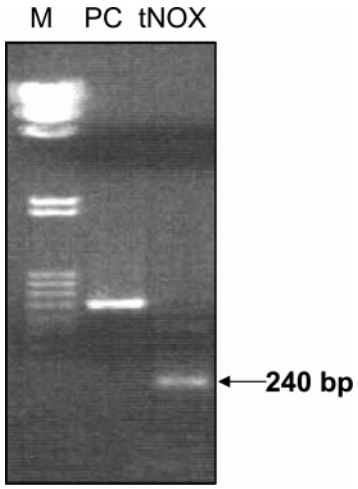

FIGURE 3: Verification of the 5′-terminus by 5′-RACE using an exon 2 3′-primer. The 240 bp band shown here corresponds to exon 1 plus exon 2 of tNOX as initially reported (*17*). PC = placenta control. M = λ DNA marker. The results revealed no additional sequence in the 5′-UTR.

5′-RACE Analysis for an Additional Upstream ATG. For 5′-RACE analysis, mRNA of noncancer human mammary MCF-10A cells was prepared following general mRNA preparation protocols. The ~240 bp band resulting from 5′-RACE corresponded to exon 1 and exon 2 of the tNOX gene (Figure 3). The result showed that the previously determined tNOX cDNA sequence was complete at the 5′-end with no additional sequence in the 5′-UTR. Results with mRNA of BT-20 human mammary cancer cells were similar.

Cancer-Specific Expression of tNOX. Plasma membranes purified from MCF-10A and BT-20 cells were compared by SDS−PAGE and Western blot analysis with monoclonal antibody to tNOX. The 34 kDa tNOX was present as a doublet (possibly due to small differences in the extent of glycosylation) in the plasma membranes of BT-20 cancer

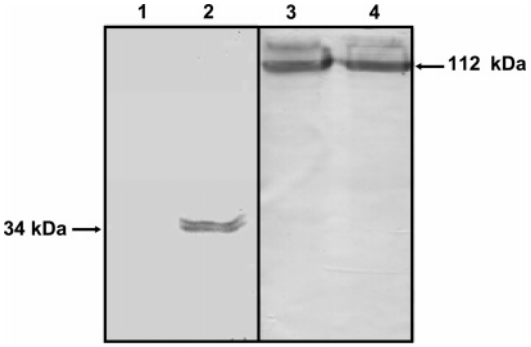

FIGURE 4: BT-20 (human mammary adenocarcinoma) and MCF-10A (noncancer mammary epithelia) plasma membranes (0.05 mg of protein per lane) analyzed by SDS−PAGE (10%) and by Western blot analysis with monoclonal antibody to tNOX with visualization using alkaline phosphatase-linked anti-mouse secondary antibody with BCIP and NBT as substrates. The 34 kDa protein (arrow) present in the plasma membranes of the BT-20 cells (lane 2) was below the level of detection from plasma membranes of MCF-10A cells (lane 1). Na^+,K^+-ATPase (112 kDa) detected by Na^+,K^+-ATPase monoclonal antibody was used as a loading control. Lane 3 is for plasma membranes of MCF-10A cells. Lane 4 is for plasma membranes of BT-20 cells.

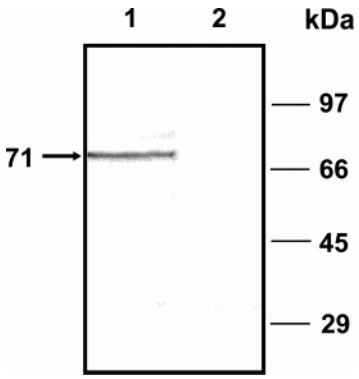

FIGURE 5: Full-length tNOX cDNA expressed a protein around 71 kDa under the control of a vector promoter in COS cells: lane 1, full-length tNOX cDNA in pcDNA3.1; lane 2, pcDNA3.1. All are COS cell transfectants. Antibody PU04, a peptide antibody to the quinone binding region on tNOX, was utilized.

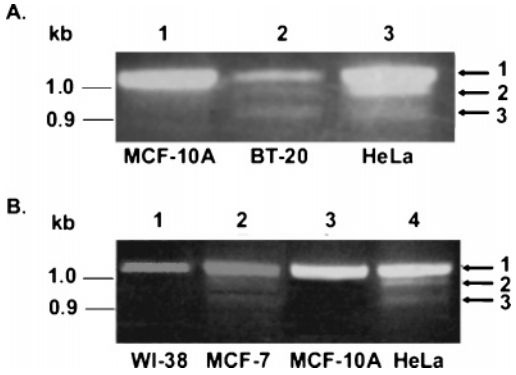

FIGURE 6: (A) RT-PCR of MCF-10A, BT-20, and HeLa cells showed, in addition to tNOX mRNA (band 1), an exon 4 minus form (band 2) and an exon 5 minus product (band 3) in both cancer lines BT-20 (lane 2) and HeLa (lane 3) but not in the noncancer MCF-10A (lane 1) cells. (B) RT-PCR of WI-38, MCF-7, MCF-10A, and HeLa cells showed an exon 4 minus form (band 2) and an exon 5 minus form product (band 3) in both MCF-7 (lane 2) and HeLa (lane 4) cancer cells but not in noncancer WI-38 (lane 1) and MCF-10A (lane 3) cells. The tNOX mRNA (band 1) was present in all four cell lines. Primers were at the beginning of exon 2 (nucleotides 72−91) and at the end of exon 8 (nucleotides 1212−1238). PCR products were cloned into the T-vector and sequenced.

cells but not in plasma membranes of noncancer MCF-10A cells (Figure 4). The plasma membrane marker Na^+,K^+-ATPase (112 kDa) was used as a loading control. Other matched pairs of normal and transformed pairs showing this specificity that we have examined include rat hepatocytes and hepatoma, human melanocytes and melanoma, and SV-40 transformed and nontransformed 3T3 cells.

Expression of Full-Length tNOX cDNA in Noncancer Cells Did Not Result in Expression of 34 kDa Processed tNOX Protein. As reported above, no naturally expressed 71 kDa tNOX protein was observed in cells of either cancer or noncancer cell lines. Therefore, experiments were conducted to determine whether the absence of the 71 kDa protein was due to rapid turnover or processing. Constructs of the full-length tNOX cDNA inserted into a mammalian expression vector pcDNA3.1-pcDNA3.1−full-length tNOX, were transfected into COS and MCF-10A cells (MCF-10A cells do not express tNOX protein, and COS cells exhibit only low levels of tNOX expression as has been demonstrated by Western blots and enzymatic assay of drug-responsive NOX activity). The transfectants and wild-type cells were collected and resolved on SDS−PAGE followed by Western blotting with PU04 antisera.

The results showed that the full-length tNOX cDNA was expressed as a 71 kDa protein under the control of the vector promoter in COS cells (Figure 5). However, there was no ca. 34 kDa processed form of the tNOX protein observed in the transfectants. This result and expression of the full-length tNOX cDNA under a vector promoter in MCF10A cells with a similar outcome (not shown) suggest that the 34 kDa processed form of tNOX protein observed on cancer cell membranes and in sera of cancer patients may not have been derived from the proposed 71 kDa full-length precursor. Therefore, the possibility of alternative splicing as a basis for the cancer-specific delivery of tNOX to the cancer cell surface was investigated.

Splice Variants of tNOX Were Found in Cancer Cells. In all cancer lines examined, RT-PCR revealed mRNAs of reduced molecular masses (Figure 6) which were below the limits of detection in noncancer cells. That these mRNAs arose by degradation was ruled out by direct sequencing. Rather, band 2 yielded a sequence corresponding to an exon 4 minus form, and band 3 yielded a sequence corresponding to an exon 5 minus form. Both splice variant forms were found in BT-20, MCF-7, and HeLa cells (cancer) but not in the noncancer MCF-10A (Figure 6A) or WI-38 (Figure 6B) cells. Primers used were at the beginning of exon 2 (nucleotides 72−91) and at the end of exon 8 (nucleotides 1212−1238). PCR products were cloned into the T-vector and sequenced.

Additional evidence for a cancer-cell-specific expression of exon 4 minus mRNA was provided using exon 4 minus-specific probes generated to the exon 4 minus-specific sequence at the splice juncture between exon 3 and exon 5. RNA preparations from HeLa human cervical carcinoma and BT-20 human mammary carcinoma cells clearly contained the expected 670 bp cancer-specific product indicative of exon 4 minus presence (Figure 7). MCF-10A human noncancer mammary epithelia and buffy coats (leukocytes and platelets from a normal volunteer) lacked the PCR product.

The presence of two additional splice variants was established by RT-RCR (Figure 8). These were identified

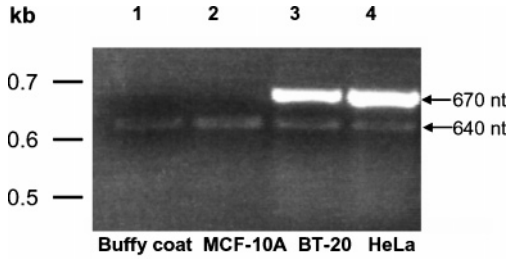

FIGURE 7: RT-PCR analysis of exon 4 minus mRNA using a forward primer at the junction between exon 3 and exon 5, with the reverse primer being the end of exon 8. A single cancer-specific dominant species of 670 nt's was generated. GAPDH primers were used as controls (640 nt's).

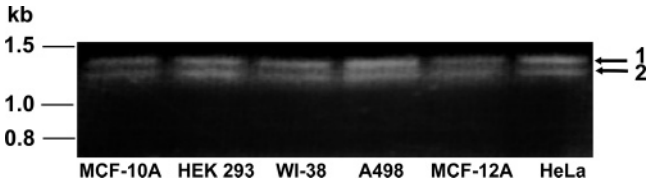

FIGURE 8: RT-PCR showed additional examples of alternative splicing with variations in exon 1. Primers used were nucleotides 191−215 of AK000353 and DBS 3′ (drug binding site of the tNOX 3′-end). AK000353 (band 1) and AL133207 (band 2) are shown.

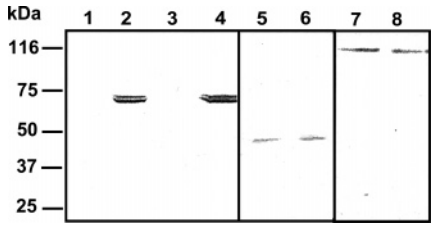

FIGURE 9: Expression of Myc-tagged full-length tNOX in COS cells identified by immunostaining with the anti-Myc antibody: lanes 1 and 5, pCMV-Tag5A vector transfected COS cells; lanes 2 and 6, pFL-tNOX−Myc transfected COS cells; lanes 3 and 7, microsomes of pCMV-Tag5A vector transfected COS cells; lanes 4 and 8, microsomes of pFL-tNOX−Myc transfected COS cells. β-Actin detected by actin monoclonal antibody (lanes 5 and 6) and Na^+,K^+-ATPase detected by Na^+,K^+-ATPase monoclonal antibody (lanes 7 and 8) were used as loading controls.

from the NCBI database (GenBank) as AK000353 (band 1) and AL133207 (band 2). No cancer specificity was observed as both variants were present in MCF-10A, MCF-12A, and WI-38 cells as well as in the cancer lines.

Expression of Myc-Tagged Full-Length tNOX in COS Cells. COS cells were transiently transfected with DNA encoding full-length tNOX fused with Myc tag and analyzed after 48 h (Figure 9). The whole cell extracts and microsomes exhibited bands corresponding to putative full-length tNOX plus the Myc tag. No bands at lower molecular mass were detected. This result confirmed that the full-length tNOX mRNA was incapable of generating the 34 kDa tNOX protein.

Expression of Exon 4 Minus and Exon 5 Minus Forms of tNOX in COS Cells. The exon 5 minus cDNA yielded an open reading frame for a deduced amino acid sequence for a protein of 532 amino acids with a predicted molecular mass of 60.8 kDa (Figure 10). The exon 4 minus tNOX transfectants exhibited a 62 kDa band (corresponding to E4m tNOX expressed from M1 under the control of the CMV promoter) and 47 and 34 kDa bands corresponding to proteins translated from M231 and fully processed tNOX, respectively, plus a 43 kDa band representing a possible processing intermediate

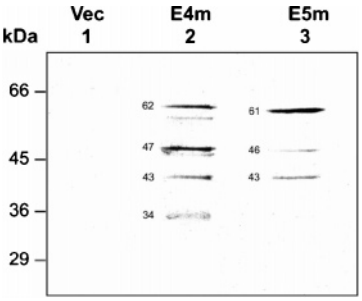

FIGURE 10: Expression of the exon 4 minus and exon 5 minus forms of tNOX mRNA in COS cells identified by immunostaining with the tNOX peptide antibody to the C-terminal adenine binding motif (PU02): lane 1, pcDNA3.1 vector; lane 2, exon 4 minus form of tNOX; lane 3, exon 5 minus form of tNOX.

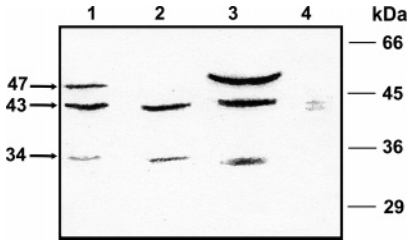

FIGURE 11: Western blot of plasma and internal membranes of the exon 4 minus transfectant and whole cell preparation of COS cells probed by a peptide antibody toward the quinone binding motif of the tNOX protein (PU04): lane 1, internal membranes of exon 4 minus variant transfectants; lane 2, plasma membrane of exon 4 minus variant transfectants; lane 3, whole cell preparation of exon 4 minus transfectants; lane 4, vector only transfected cells.

(*17*). Thus, with the exon 4 minus tNOX tranfectants, a 34 kDa protein that reacted with the tNOX antibody was obtained from the overexpression of a naturally existing mRNA in cancer cells. This result showed that the exon 4 minus splice variant mRNA is apparently capable of generating the 34 kDa tNOX protein.

Delivery of 34 kDa tNOX Protein to the Plasma Membrane. In addition to carrying out protein disulfide−thiol interchange, ECTO-NOX proteins function as terminal oxidases for plasma membrane electron transport (*1*, *3*). If the 34 kDa protein expressed from the exon 4 minus splice variant is to function as a terminal oxidase for plasma membrane electron transprot, it must reach the outer surface of the plasma membrane. To investigate the subcellular localization of the 34 kDa tNOX protein expressed in exon 4 minus COS transfectants, plasma membranes and internal membranes of the transfectants prepared by aqueous two-phase partition were resolved on SDS−PAGE followed by Western blot analysis (Figure 11). The Western blots of plasma membranes and internal membranes of the exon 4 minus transfectants along with whole cell preparations were probed by a peptide antibody toward the quinone binding motif of the tNOX protein. The internal membrane preparation (lane 1) exhibited the 47 and 43 kDa bands, plus a small but detectable amount of the 34 kDa protein presumably as a result of delivery of the 34 kDa processed form of the 47 kDa protein to the plasma membrane. The plasma membrane preparation (lane 2) exhibited the 43 and 34 kDa bands but not the 47 kDa band. The whole cell preparation (lane 3) exhibited all three (47, 43, and 34 kDa) bands, whereas none were present with vector alone (lane 4).

Mutation of Met 231 Blocked Expression of the Exon 4 Minus Splice Variant. In the previous overexpression experi-

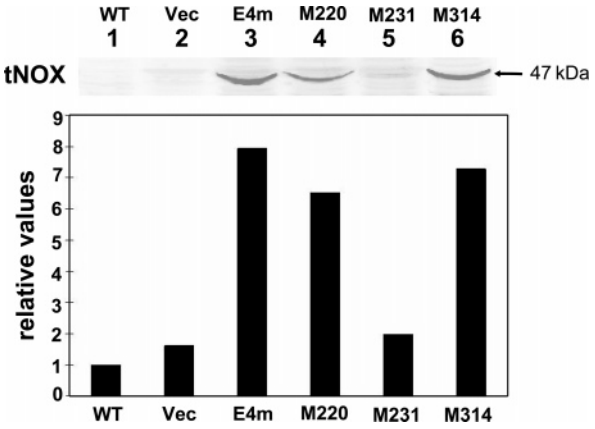

FIGURE 12: Site-directed mutagenesis validates exon 4 minus mRNA with initiation at M231 as the transcriptional template for tNOX. Replacement of Met 231 prevents the protein expression of exon 4 minus mRNA. Key: lane 1, wild-type COS cells; lane 2, COS cells transfected with pcDNA3.1 vector; lane 3, COS cells transfected with exon 4 minus cDNA in pcDNA3.1; lane 4, COS cells transfected with mutant M220A exon 4 minus cDNA in pcDNA3.1; lane 5, COS cells transfected with mutant M231A exon 4 minus cDNA in pcDNA3.1; lane 6, COS cells transfected with mutant M314A exon 4 minus cDNA in pcDNA3.1. The 47 kDa band is present in lanes 3, 4, and 6. Values of densitometry analysis were divided by the smallest value, and the division factors are plotted. The smallest value is calculated as 1.

ments, 47, 43, and 34 kDa proteins were observed in exon 4 minus splice variant transfectants. Expression of the 47 kDa protein would be consistent with utilization of a downstream Met as the initiation site during translation of the exon 4 minus mRNA (see the discussion of downstream translation initiation below). Possible downstream initiation sites include Met 220, Met 231, and M314. Mutagenesis experiments of the initiation sites of E4m showed that only with COS cells transfected with E4m mRNA in which Met 231 was replaced by alanine was a ca. 47 kDa band missing (Figure 12). Thus, initiation at Met 231 and subsequent processing of the 47 kDa protein and delivery to the plasma membrane of the 34 kDa active form of tNOX serve to explain the specific localization of tNOX at the cancer cell surface.

Subcellular Localization of E4m tNOX−EGFP and Full-Length tNOX−EGFP Fusion Proteins. To test further the hypothesis that cell surface tNOX is the result of expression of E4m mRNA, confocal microscopy was used to determine the subcellular localization of the E4m tNOX−EGFP and full-length tNOX−EGFP when expressed in COS cells. The constructs tagged with EGFP at the C terminus were expressed under the control of the cytomegalovirus promoter. Fluorescence microscopy revealed that the E4m−EGFP fusion protein was localized to the plasma membrane (Figure 13D−F). In contrast, a full-length tNOX−EGFP fusion protein was retained in internal membranes (Figure 13A−C). COS cells transfected with pE4m−EGFP in which Met231 was replaced by alanine (M231A-E4m−EGFP)

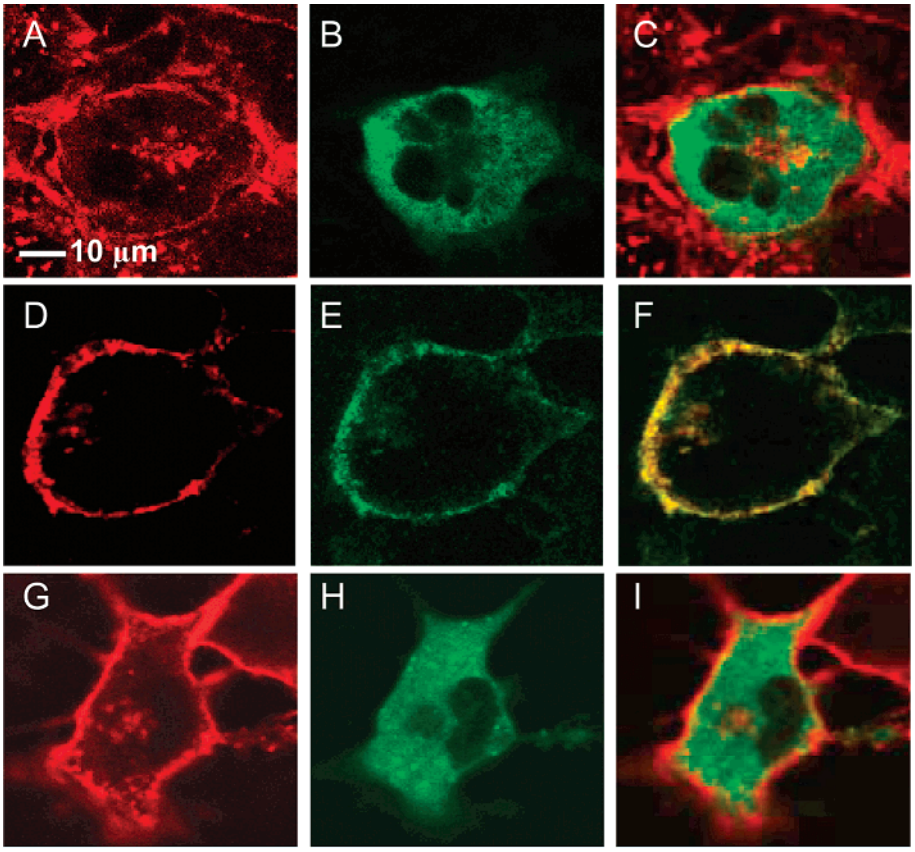

FIGURE 13: Confocal microscope images of full-length tNOX−EGFP transfected COS cells (A−C): the cell surface marker, tetramethylrhodamine concanavalin A, is in red (A); the EGFP fusion protein is in green (B); colocalization of the two (C). Confocal images of pE4m−EGFP transfected COS cells (D−F): the cell surface marker, tetramethylrhodamine concanavalin A, is in red (D); the EGFP fusion protein is in green (E); colocalization of the two appears as yellow (F). Confocal microscope images of M231A-E4m−EGFP transfected COS cells (G−I): the cell surface marker, tetramethylrhodamine concanavalin A, is in red (G); the EGFP fusion protein is in green (H); colocalization of the two (I).

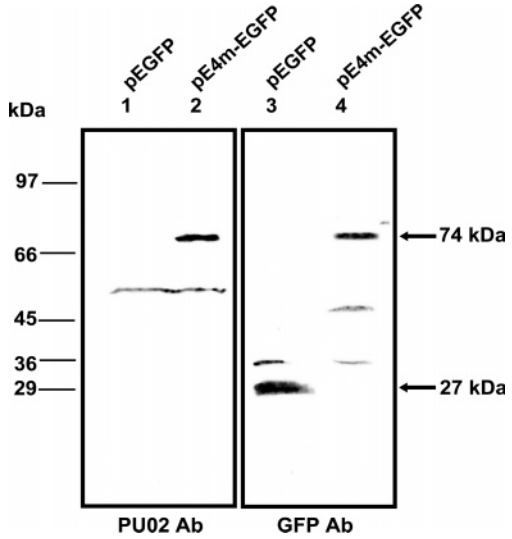

FIGURE 14: Expression of 27 kDa EGFP and of 74 kDa E4m−EGFP in COS cells. Immunostaining of EGFP and E4m−EGFP in COS cells identified by antibody PU02 (a polyclonal peptide antibody to the conserved adenine nucleotide binding region of tNOX, lanes 1 and 2) and anti-GFP antibody (lanes 3 and 4). Lanes 1 and 3 are for pEGFP vector transfected COS cells. Lanes 2 and 4 are for pE4m−EGFP transfected COS cells. The 74 kDa E4m−EGFP product was recognized by both antibodies, whereas with pEGFP vector alone only the 27 kDa product was revealed. The band at ca. 55 kDa was given by anti-rabbit second antibody alone.

failed to exhibit the EGFP fusion protein at the cell surface (Figure 13G−I), consistent with M231 as the cancer-specific E4m mRNA initiation site. With the M231A-E4m−EGFP cells, Western blot analyses revealed that only the 27 kDa green fluorescent protein was expressed to account for the GFP signal detected in Figure 13G−I. Similar results were observed when a stop codon was inserted at the end of tNOX preceding the EGFP sequence.

Immunoblot analysis of EGFP and E4m−EGFP Expressed in COS Cells. Proteins were analyzed by PAGE and Western blotting using the anti-GFP and PU02 (a polyclonal peptide antibody to the conserved adenine nucleotide binding region of tNOX). A 74 kDa band was seen in pE4m−EGFP transfected COS cells, consistent with the predicted molecular mass of the E4m tNOX (47 kDa) plus EGFP (27 kDa) fusion protein (Figure 14). There was no evidence from these gels with either antibody for the production of further processing intermediates of the GFP conjugates.

DISCUSSION

A 34 kDa tNOX protein found on the surface of cancer cells (*1*, *2*, *7*) is shed into the sera of cancer patients (*5*, *6*) as well as into the media of cultured cancer cells (*12*). A characteristic of the tNOX protein that distinguishes it from other ECTO-NOX proteins is its inhibition by the vanilloid capsaicin (*3*), the catechin (−)-epigallocatechin 3-gallate (*10*), the antitumor sulfonylureas (*9*), adriamycin (*8*), and the synthetic anticancer isoflavene phenoxodiol (*11*). tNOX is present in solid tumors, on the surface of cancer cells, and in sera of cancer patients but not in normal human tissues or on the surface of noncancer cells (*3*−*6*) or in sera of healthy volunteers. This study was undertaken to determine the mechanism for the cancer specificity of the 34 kDa cell surface tNOX.

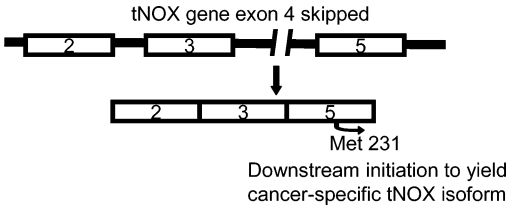

FIGURE 15: Summary diagram illustrating translation of the exon 4 minus tNOX mRNA from a downstream methionine (Met 231).

The presence of exon 4 minus mRNA was verified by Northern blot analysis. mRNA bands spanning the correct range of 3.5−3.9 kb's were obtained (full length of 3.8 kb's and exon 4 minus of 3.6 kb's) as an incompletely resolved doublet.

Two additional splice variants of tNOX were found. These were identified from the NCBI database (GenBank) as AK000353 (originally found from a hepatoma cell line) and AL133207 (gene located on chromosome X). Both have the same sequence as exon 2 to exon 8 of tNOX. AK000353 has a 234 bp extra sequence before exon 2 that is different from that of tNOX. AL133207 has a 348 bp extra sequence before exon 2 that differs from that of tNOX. Nucleotides 31−234 of AK000353 are the same as nucleotides 1−204 of AL133207. Neither appeared to be cancer specific (Figure 8).

Taken together, the findings suggest that an exon 4 minus splice variant of tNOX provides the basis for the cancer-specific appearance of the 34 kDa processed form of tNOX at the cell surface. Exon 4 minus tNOX mRNA was detected in all of the cancer cell lines thus far investigated but was below the limits of detection in noncancer cells. Site-directed mutagenesis and other evidence indicate that the generation of the 34 kDa cell surface tNOX is the result of downstream initiation at methionine 231 (encoded by nucleotides ATG in exon 5, Figure 15). Overexpression of the exon 4 minus transcript in noncancer cells generated the expected full-length protein of molecular mass 47 kDa, plus a 43 kDa processing intermediate and the fully processed 34 kDa form at the plasma membrane, all of which reacted with anti-tNOX antibodies.

As stated above, there is no evidence of physiological translation of the full-length tNOX mRNA despite its widespread transcription. Therefore, the significance of differences in full-length mRNA abundance (Figure 2) may not be functionally relevant. The cDNA sequence of tNOX revealed that the first ATG started at nt 23. There were only 22 nt's to form a 5′-UTR. This short 5′-UTR makes the first ATG an unlikely site for initiation of translation due to the potential restrictions on ribosome binding based on the current scanning model for initiation of translation in eukaryotes (*22*). Normally, the initiation complex forms around 21−24 nt's after recognition of the cap of mRNA and then begins to scan for the first Met codon. This "first AUG" rule holds for about 90% of the eukaryotic mRNAs that have been analyzed (*23*). However, the first Met typically is about 50−150 nt's downstream from the cap (*24*). Thus, with tNOX, the first Met at nt 23 may be too near to the cap to serve as a functional initiation sequence for translation especially as sequence elements in the 5′-UTR are often required as well for regulation of translation (*25*). The first methionine codon does not always function as the initiator

codon (*23*, *26*), and if the first ATG of full-length tNOX mRNA functioned as the initiator codon, the 5′-UTR of tNOX would be only 22 nucleotides, shorter than most functional 5′-UTRs (*27*). Thus, it is possible that the initiator codon would be further downstream. Downstream Kozak sequences (A/G)XXAUGG (*23*) are found at nt 167 (exon 2), nt 713 (exon 5), and nt 1163 (exon 8). A 5′-UTR length is usually smaller than 200 bp's because of the limitation of the scanning capacity of the ribosome (*23*). By deleting an upstream portion of the message through alternative splicing, it may be possible for the scanning ribosome to reach a downstream methionine codon to begin initiation to ultimately generate the cell surface 34 kDa form of tNOX. Alternatively, downstream initiation as a result of the deletion of exon 4 might occur through disruption of the secondary structure of tNOX mRNA in a region where secondary structure would normally hinder ribosome binding or block translation. Involvement of cancer-specific RNA binding proteins seems unlikely since transfection with exon 4 minus cDNA is sufficient to generate functional cell surface tNOX in noncancer cell lines which would lack the binding proteins.

As the processed tNOX is on the extracellular side of the plasma membrane (*1*,*7*), a functional translation initiation site would require a membrane insertion sequence to follow. The exon 4 minus splice variant results in a 207 bp deletion. There is no frame shift after the deletion of exon 4. The result is a 68 amino acid deletion plus one amino acid change. The net result is the deletion of one methionine codon at nt 542, and all downstream methionine codons are brought closer to the 5′-end. By facilitating downstream initiation at M231, the exon 4 minus alternatively spliced mRNA favors cancer-specific cell surface expression of the tNOX protein. Initiation at M231 has the advantage of there being both putative signal sequence and signal sequence cleavage sites. Initiation at M231 where a Kozak sequence occurs would result in a peptide sequence of 380 amino acids and a calculated molecular mass of 44.5 kDa. What is observed is a protein band with an apparent molecular mass on SDS−PAGE of 47 kDa, which after removal of the signal sequence and further processing would be expected to generate the functional 34 kDa tNOX protein that is present at the surface of cancer cells. This conclusion was supported by methionine to alanine replacements, which confirmed the 47 kDa protein as the expressed form of the exon 4 minus transcript with initiation at M231. The 43 kDa band likely represents the 47 kDa species after removal of a 29 amino acid sequence with characteristics of a signal peptide (*17*). The 34 kDa protein corresponds to the mature processed form of tNOX and appeared at the plasma membranes but was absent from internal membranes consistent with its translation as a 47 kDa peptide and subsequent processing. The 47 and 43 kDa proteins plus a small amount of the 34 kDa protein were found in the cell fractions containing internal membranes, whereas the plasma membranes contained only the 34 kDa processed form. Our evidence suggests that the full-length tNOX mRNA is not translated. Appearance of tNOX at the plasma membrane of cancer cells seems dependent upon the presence of exon 4 minus mRNA and thus would be expected to vary independently of the levels of the full-length tNOX message. However, processing does not seem to be an obligatory consideration for delivery to the plasma membrane. The exon 4 minus−EGFP construct, for example, was apparently delivered to the plasma membrane as the intact 74 kDa fusion protein as determined by fluorescence microscopy (Figure 13) without evidence of further processing (Figure 14). The enzymes of the detergent-solubilized Golgi apparatus from HeLa and to a somewhat less extent from MCF-10A cells efficiently process the 47 kDa tNOX to its mature molecular mass of 34 kDa. Processing differences may contribute but do not appear sufficient to explain the cancer-specific exon 4 minus-dependent expression of 34 kDa tNOX at the plasma membrane.

For the most part, alternative splicing has been divided into four general categories (*28*). The simplest form of alternative splicing is a choice to remove or not to remove an intron. Although this may appear only to depend on intron recognition, the choice can be viewed as one between alternative ways of defining exons. A second category is the alternative use of 5′ splice sites that will change the length of an exon by extending the 3′-border of the exon. A more complex, yet very frequent, mode of alternative splicing is a choice between exon inclusion and exon skipping. Our exon 4 minus form tNOX is an example of this. A well-studied example of exon skipping is the alternative splicing of fibroblast growth factor receptor-2 (FGF-R2) transcripts (*29*−*31*). In this example, a mutually exclusive choice is made between two exons, FGF-R2IIIb (IIIb) or FGF-R2IIIc (IIIc). The choice between IIIb and IIIc is typical of alternative splicing events that select one among two or more homologous exons; the other exon(s) are skipped (*28*). The choice between IIIb and IIIc is tissue specific. Epithelial cells and well-differentiated prostate tumors of epithelial origin (e.g., DT3 rat prostate tumors) include only IIIb, whereas mesenchymal cells or dedifferentiated prostate carcinomas (e.g., AT3 rat prostate tumors) include only IIIc (*30*). The IIIb/IIIc choice must be dictated by differences in the splicing machinery in different cells (i.e., DT3 vs AT3) and by unique cis-acting elements in FGF-R1 transcripts. It will be of interest to determine what mechanisms are employed for the cancer-specific exon 4 skipping that leads to expression and translation of tNOX exon 4 minus mRNA that appears to be permissive for delivery of mature tNOX protein to the cancer cell surface.

More than 100 genes exhibit altered pre-mRNA splicing in cancer (*32*). Several studies using bioinformatics methods have found potentially cancer-specific or cancer-associated splice variants (*33*−*35*). Several studies show specific alterations in the expression of splicing factors in cancer. Thus, expression of tNOX exon 4 minus mRNA may be reasonably expected to derive from cancer-specific alterations in splicing factors (*32*).

The expression of tNOX is not sufficient to cause cancer (*36*), but tNOX may be important to the unregulated cell growth that typifies cancer (*1*). Unlike constituitive CNOX proteins, tNOX is responsive to a variety of drugs and substances, all with anticancer activity that inhibits both hydroquinone and NADH oxidation (*3*). When tNOX activity is inhibited, cancer cells, once having divided, fail to enlarge to a size sufficient to divide again and undergo apoptosis (*1*).

A role for tNOX in maintaining growth of cancer cells in culture has been recently reported in experiments based on RNAi-mediated gene silencing (*37*). tNOX as the molecular target at the surface of cancer cells to explain the activity of

antitumor sulfonylureas (*9*) has been independently confirmed by Alonso et al. (*38*).

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