Analysis of mRNA Transcripts from the NAD(P)H Oxidase 1 (Nox1) Gene

EVIDENCE AGAINST PRODUCTION OF THE NADPH OXIDASE HOMOLOG-1 SHORT (NOH-1S) TRANSCRIPT VARIANT

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Recent reports indicate that NAD(P)H oxidase 1 (Nox1) mRNA undergoes alternative splicing, producing a short transcript (NOH-1S) encoding a novel H+ channel. Although the H+ transport properties of NOH-1S-transfected cells resemble those of many cells, the production of a NOH-1S protein was never documented. We characterized Nox1 transcripts in colon-derived cells and present evidence that mRNA splicing does not produce NOH-1S; rather, NOH-1S appears to be an artifact of template switching during cDNA synthesis. The NOH-1S transcript was not observed by Northern blotting, despite claims of its abundance based on RNase protection assays. The shortened cDNA was generated by avian myeloblastosis virus reverse transcriptase, but not by thermally stable reverse transcriptase under conditions that produce full-length Nox1. Analysis of shortened cDNAs detected NOH-1S sequence and other variants that differ at the alleged splice junction site. Although no appropriate RNA splicing sites were found within Nox1 to account for NOH-1S formation, we found repetitive sequence elements bordering the deleted region, which could promote intramolecular template switching during cDNA synthesis. Template switching was confirmed in vitro, where the deleted cDNA was generated by avian myeloblastosis virus reverse transcriptase from a synthetic, full-length Nox1 RNA template. A survey of the expressed sequence tags database suggests that similar switching phenomena occur between repetitive elements in other Nox family transcripts, indicating such cloning artifacts are common. In contrast, genuine RNA splicing does account for another Nox1 transcript lacking the entire exon 11, which is abundant in colon cells but encodes a protein incapable of supporting superoxide production.

Reactive oxygen species (ROS)1 have diverse roles in various biological processes including host defense, mitogenesis, hormone biosynthesis, apoptosis, and fertilization (1, 2). Currently, our understanding of ROS production and function is based largely on studies of phagocytic cells, where ROS act as antimicrobial agents during phagocytosis. In phagocytic cells, the ROS precursor, superoxide, is produced by the NADPH oxidase enzyme complex (2). The phagocytic NADPH oxidase (phox) consists of the membrane-bound cytochrome b558, several cytosolic factors (p47phox, p67phox, p40phox), and the small GTPase Rac2. Cytochrome b558 is a complex of two proteins: the flavin and heme-binding glycoprotein gp91phox and p22phox.

Recently, several novel gp91phox homologs have been discovered (3–9), suggesting that enzymes similar to the phagocytic NADPH oxidase are present in a variety of tissues, including colon, kidney, thyroid gland, testis, and lymphoid organs (3–9). Although several studies have demonstrated ROS production by these Nox family gp91phox homologs, their exact physiological functions remain unknown.

Superoxide production is accompanied by a robust intracellular H+ production resulting from oxidation of NADPH to NADP+ and H+ and from the activation of the NADPH-regenerating hexose monophosphate shunt. Phagocytic cells have at least three mechanisms for removal of H+: Na+-H+ exchange, H+-ATPase, and electrogenic H+ transport (10). The mechanism of electrogenic H+ transport in phagocytes is still debated; some evidence suggests that gp91phox could act as a proton translocator (11–13), although the existence of other pathways is likely (14–16). Henderson et al. (11, 17) demonstrated that cells transfected with gp91phox or the N-terminal portion of this protein acquire arachidonate-dependent H+ transport activity. This arachidonate-dependent activity appears to involve three histidine residues within the third transmembrane domain of gp91phox; these features are conserved in other Nox family homologs.

The notion that gp91phox functions as a proton channel was supported further by studies on the gp91phox homolog Nox1, where a putative alternatively spliced product (NOH-1S; NADPH oxidase homolog-1 short; GenBank accession no. NM013954) was shown to have H+ channel activity (18). That work, representing the first report of cloning of a mammalian H+ channel, suggested the H+ transport function could be dissociated from NADPH oxidase activity as a distinct molecular entity. The sequence encoding NOH-1S, originally derived from the database of expressed sequence tags (dbEST), was proposed to result from an unusual intra-exonic splicing event, involving only a portion of exon 5 spliced to the exon 14 sequence. The putative product, which was not detected at the protein level, consists of four transmembrane domains and a short cytoplasmic tail; the recombinant protein, when expressed ectopically in HEK 293 cells, exhibited the properties of voltage-gated H+ channels. These results agreed with pre-
vious observations on the truncated, N-terminal fragment of gp91<sup>phox</sup> (17).

In this study, we characterized Nox1 mRNA transcripts in normal colon and cancerous colon epithelial cells, where the Nox1 gene is expressed abundantly. We show that the mRNA transcript for NOH-1S is not synthesized from the Nox1 gene but is instead an artificial product of cDNA synthesis. We attribute this result to the phenomenon of intramolecular template switching between repetitive sequence elements during cDNA synthesis. Our observations indicate that although the dbEST represents a rich source of novel transcript variants, these data should be viewed cautiously because of the frequent occurrence of such cloning artifacts. Furthermore, we demonstrate that the Nox1 gene encodes another genuine spliced variant of Nox1 that is abundant in colon cells but is not functional because of the absence of exon 11-encoded sequence.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All cell culture reagents were obtained from Invitrogen, unless indicated otherwise. Caco2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Hyclone Laboratories), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HT29 cells were maintained in McCoy’s 5a medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human HEK 293 cells (ATCC) were maintained in Eagle’s minimum essential medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 100 μM nonessential amino acids. COS-7 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, and streptomycin.

**Northern Blot Analysis**—Total RNA was prepared from Caco2 and HT29 cells (15 μg), separated electrophoretically on a 1% agarose formaldehyde gel, and transferred to nylon membranes. Membranes were probed with labeled human Nox1 cDNA fragments from the corresponding genomic regions (Oncor Biosciences) in Quickhyb hybridization solution (Stratagene) following standard hybridization protocols. The β2P-radiolabeled probe was prepared with a Prime-It RmT random primer labeling kit (Stratagene). In other experiments, pre-made human gastrointestinal Northern blots (Clontech) were probed under the same conditions as detailed above.

**Reverse Transcription (RT)-PCR**—For RT-PCR experiments, 1 μg of mRNA from Caco2 cells, HT29 cells, or normal human colon (Invitrogen) was transcribed into cDNA using an oligo(dT) primer and the avian myeloblastosis virus (AMV) reverse transcriptase-based cDNA cycle<sup>TM</sup> kit (1 h at 42 °C) or using the ThermoScript<sup>TM</sup> RT-PCR kit (1 h at 69 °C; Invitrogen). PCRs were performed with a High Fidelity PCR kit (Invitrogen) using the following Nox1-specific primers: primer 1, 5’-ATGGGAACTGGGTGGTTAAC-3’; primer 2, 5’-CAGCTGTTGTTATATGGGGA-3’; primer 3, 5’-GCTGTCCACTCGGATA-AGACC-3’; primer 4, 5’-TCAAAATTTTCCTTTGTTGA-3’. The cDNA templates were denatured at 94 °C for 4 min followed by 35 cycles of amplification (94 °C for 50 s, 55 °C for 50 s, and 72 °C for 2 min). The PCR products were cloned into pCR2.1-TOPO or pCR4-TOPO TA-cloning vectors (Invitrogen), and randomly selected clones were sequenced with vector-specific primers.

**In Vitro Transcription**—A 2267 bp-long fragment of the human Nox1 (colon) cDNA was amplified with primers 1 and 3 and cloned into pCR4-TOPO TA-cloning vector. RNA was transcribed with 30 units T7 RNA polymerase (MBI Fermentas) using 1 μg of linearized template DNA from this construct. After 1 h at 37 °C, the template DNA was digested by DNase I (Roche Applied Science), and the RNA was purified by the RNeasy Mini Kit (Qiagen). RT-PCR was performed as detailed above, using the cDNA cycle<sup>TM</sup> kit (Invitrogen) for cDNA synthesis at 42 °C and primers 1 and 2 for the PCR.

**RNase Protection Assays**—RNase protection assays were performed as described by Bánsí et al. (18) with minor modifications. A radiolabeled 3’-terminal NOH-1S cDNA fragment was synthesized from a BanI-digested NOH-1S cDNA (M.A.G.E. clone 900560, Research Genetics) using T3 RNA polymerase (MBI Fermentas) and [α-<sup>32</sup>P]UTP (Amersham Biosciences). The resulting transcript produces a 407-base probe, of which 374 bases correspond to the 3’-portion of NOH-1S (starting at nucleotide 241 relative to the start ctron). Synthetic, full-length Nox1 mRNA was transcribed and purified as above and used to anneal to the NOH-1S probe at 42 °C for 16 h. The resulting hybrids were treated with RNase A and T1 mixtures for 30 min at 37 °C using the RPA III<sup>TM</sup> RNase protection assay kit (Ambion). The protected products were analyzed by autoradiography after electrophoresis on 6% acrylamide/urea/TBE gels (Invitrogen).

**Database Searches**—Database searches were performed in the dbEST and the unfinished high throughput genomic sequences using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

**Cell Transfections and Superoxide Production Assays**—For transfection studies, the complete coding sequence of human Nox1, Nox1v, p51<sup>phox</sup>/Nox1c, p51<sup>phox</sup>/Nox1-a (19), and p22<sup>phox</sup> were cloned into pcDNA3.1 plasmid (Invitrogen). Cells were seeded at 400,000 cells/well (HEK 293 cells) or 120,000 cells/well (COS-7 cells) in 6-well dishes 24 h prior to transfection. HEK 293 cell transfections were performed in serum-free medium using 9 μl of FuGENE 6 (Roche Applied Science) prepared in complexes with plasmid DNAs (0.8 μg of Nox1, Nox1v, or pcDNA3.1 (control) plasmid, plus 0.8 μg each of p41<sup>phox</sup>/Nox1-a and p51<sup>phox</sup>/Nox1 plasmids), using the manufacturer’s suggested protocol. The cells were fed at 5 h post-transfection, followed by harvesting 72 h post-transfection. COS-7 cell transfections were as above, but also included p22<sup>phox</sup> pcDNA3.1 plasmid DNA, using 0.6 μg of each plasmid. These cells were assayed 48 h after transfection. Trypsinized cells were assayed for phorbol 12-myristate 13-acetate-stimulated superoxide release by chemiluminescence methods using the Diogenes reagent, as described previously (19). For Nox protein detection by Western blotting, the Nox1 or Nox1v cDNAs were cloned into the pcDNA3.1 V5 His TOPO plasmid vector (Invitrogen). COS-7 cells were transfected with 1 μg of Nox1, Nox1v, or pcDNA3.1 (control) plasmid, along with 1 μg of p22<sup>phox</sup> plasmid. The cells were harvested 48 h post-transfection using cell scrapers and the membrane fractions were probed by Western blotting using mouse monoclonal anti-V5 peptide antibody (Invitrogen), according to manufacturer’s protocols.

**RESULTS**

**Exonic Organization of the Nox1 Gene**—Based on a genomic DNA database search, Bánsí et al. (18) concluded that the NOH-1/Nox1 gene was located on Xq12 and consisted of 14 exons. They indicated that the first 13 exons are of comparable size and are homologous to the 13 exons of gp91<sup>phox</sup> and that the 14th exon, containing 99 nucleotides, was unique to the Nox1 gene. They suggested that the generation of NOH-1S involved splicing of an intra-exonic site in exon 5 with the sequence in exon 14, which would encode a unique C-terminal portion of NOH-1S. Our reevaluation of genomic and corresponding cDNA sequences deposited in the human genomic database indicates that the Nox1 gene encompasses only 13 exons. Fig. 1a provides a revised exon map of Nox1 encompassing 99 nucleotides, was unique to the Nox1 gene. They suggested that the generation of NOH-1S involved splicing of an intra-exonic site in exon 5 with the sequence in exon 14, which would encode a unique C-terminal portion of NOH-1S. Our reevaluation of genomic and corresponding cDNA sequences deposited in the human genomic database indicates that the Nox1 gene encompasses only 13 exons. Fig. 1a provides a revised exon map of Nox1, which was deduced from a BLAST-based alignment of the full-length cDNA sequence with a contiguous genomic Nox1 DNA sequence (GenBank<sup>TM</sup> accession number HsX 11808). In this revised genomic map, the last exon, exon 13, consists of 741 nucleotides and includes a 3’-terminal 99 nucleotides that previously represented exon 14 (18). These observations indicate that the putative NOH-1S transcript would involve the use of intra-exonic splice donor and splice acceptor sites within exon 5 and exon 13, respectively.

RNA splicing is a precisely regulated process involving strict adherence to the “GT-AG” rule (20, 21), even in those relatively rare examples where intra-exonic splicing occurs (22, 23). We screened exon 5 and exon 13 of the Nox1 gene for suitable intra-exonic splice donor and acceptor sites corresponding to the proposed splice sites in NOH-1S. Using splice site predictions by neural network analysis (www.fruitfly.org/seq_tools) with low donor and acceptor site cutoff scores (0.40), we were unable to locate any appropriate splice sites corresponding to the proposed regions of NOH-1S.

**Demonstration of Nox1 Expression by Northern Blot Analysis**—The Nox1 gene is expressed abundantly in the colon and to a lesser extent in the uterus, the prostate, and proliferating vascular smooth muscle cells (3). To characterize the transcript originating from the Nox1 gene, we studied Nox1 mRNA expression by Northern blot analysis on a gastrointestinal
mRNA panel; various isolated portions of the colon were represented, as well as RNAs from two colorectal carcinoma cell lines (Caco2 and HT29). The cDNA probe encompassed a major 5'-fragment (409 nucleotides) of the Nox1 mRNA and included most of the NOH-1S sequence (Fig. 1A). Fig. 1B shows that one major band of ~2.5 kb, corresponding to the full-length Nox1 mRNA transcript, was recognized in all lanes, although no signal was detected at the expected size for NOH-1S (~0.50 kb). We observed that Nox1 expression could be significantly induced by treating Caco2 cells with 1α,25-dihydroxyvitamin D₃ or interferon-γ (24); however, the NOH-1S message RNA was still not detected in induced Caco2 cells. These results indicate that the NOH-1S mRNA species was either absent or was below the detection limits of the Northern blot method, although the RNase protection experiments by Bánsfi et al. (18) suggested that NOH-1S and NOH-1L are expressed in comparable amounts in normal colon and colon carcinoma lines.

Detection of NOH-1S by RT-PCR—To examine the possibility of low level NOH-1S expression (i.e., below Northern analysis detection limits), we used RT-PCR to amplify NOH-1S transcripts from colon RNA. Using AMV reverse transcriptase and primers corresponding to the 5'- and 3'-regions of the NOH-1S cDNA (primers 1 and 3), as described previously (18), we were able to amplify products corresponding to the expected size of NOH-1S mRNA (Fig. 2A). We also amplified similar sized products from Caco2 and HT29 cells (data not shown). After randomly sequencing selected clones derived from these PCR products, we discovered several novel sequences with extensive variations within the alleged intron-exonic spliced region of NOH-1S (Fig. 2B), whereas the sequences flanking the alleged splice site were virtually identical. Specifically, we identified five unique sequence variations (Fig. 2B), including several clones that matched the EST clone AA493362 (originally described as NOH-1S). Repeated sequencing of both DNA strands excluded the possibility that the sequence variations were caused by sequencing errors. Interestingly, a nucleotide BLAST search in dbEST using NOH-1S as a query sequence identified an additional sequence variant (AW450431; Fig. 2B, sequence 6) that was identical to NOH-1S except for the region indicated in Fig. 2B. Note that several of these sequences (sequences 2–4) contain longer stretches of exon 5 sequence than was observed in the NOH-1S transcript (sequence 1), indicating that these sequence variations were not caused by low fidelity transcriptional errors during amplification of the NOH-1S cDNA.

These data, together with the negative Northern blot result, strongly suggest that NOH-1S is not a novel splice variant of Nox1 but represents an artifact of the cDNA cloning process. In these experiments, we performed the cDNA synthesis at 42 °C using the AMV reverse transcriptase enzyme, as originally described (18). To examine the possibility that NOH-1S is a product of inaccurate cDNA synthesis, we used a different reverse transcriptase for cDNA synthesis which performs at higher temperatures. Using the heat-stable ThermoScript™ reverse transcriptase system for cDNA synthesis at 60 °C, we could not amplify the NOH-1S from colon RNA preparations, although the full-length, 2.3-kb-long Nox1 cDNA was obtained (using two different primer pairs) in good yield despite its considerably larger size (Fig. 2C). Thus, NOH-1S cDNA appears to be a unique product of AMV reverse transcriptase. These experiments also establish that errors in the PCR amplification were not responsible for the generation of the NOH-1S product.

Replicative Sequence Elements and Their Role in NOH-1S cDNA Synthesis—Although exons 5 and 13 do not contain suitable splice sites that account for NOH-1S formation by RNA splicing, we observed more than one repetitive sequence element at the boundaries of the deleted region, which would promote intramolecular template switching during cDNA synthesis (25) and can account for the heterogeneity of the Nox1-derived short forms shown in Fig. 2B. The basis for such a template switching mechanism is illustrated in the cases of two deleted Nox1 transcripts that were already deposited in dbEST (Fig. 3A). To explore directly the possibility of intramolecular template switching, we cloned a 2.2-kb portion of the full-length Nox1 cDNA, produced a synthetic mRNA template in vitro with T7 RNA polymerase, and then synthesized the cDNA at 42 °C using AMV reverse transcriptase. After PCR amplification, we identified two high yield products (Fig. 3B): the original full-length Nox1 cDNA copy and a product that corresponds to the size of NOH-1S. Sequencing of the short PCR product revealed a NOH-1S sequence variant corresponding to sequence 2 (Fig. 2B). This experiment shows that NOH-1S can be derived spontaneously from the full-length Nox1 transcript in the absence of any splicing machinery and proves that NOH-1S cDNA formation involves intramolecular template switching between repetitive sequence elements within the RNA template.

GenBank™ searches of the dbEST revealed that internally deleted forms of other gp91phox homologs were also deposited in this database. Using the cDNAs of mouse NADP(H) oxidase 4/renal oxidase (Nox4/Renox) and the rat dual oxidase 2/thymidine oxidase 2 (Duox2/Thox2) as query sequences, we identified internally deleted forms of both sequences (AW107048, AW012126, and AW259939 for NOX4 and AW920088 for
Duox2). Because a partial sequence of the mouse Renox (Nox4) gene was already deposited in the high throughput genomic sequences database, we confirmed that these deletions in the Nox4 cDNA also occurred within exons and in the absence of appropriate splice signals, arguing against an alternative RNA splicing mechanism. We examined the specific deleted regions in full-length Nox4 and Duox2 cDNA sequences, and, interestingly, both sequences also contain repetitive elements within the boundaries of the deleted regions (Fig. 3C, boxed sequences).

RNase Protection Assays Using Synthetic RNA—RNase protection was the only other experimental approach used besides RT-PCR to support the model of RNA splicing as the basis for NOH-1S synthesis (18). Because we showed that cDNA synthesis by AMV reverse transcriptase spontaneously generates deleted Nox1 products from full-length templates (Fig. 3B), we

**FIG. 2.** RT-PCR amplification of short and long Nox1 transcripts. A, AMV reverse transcriptase (42 °C protocol; see “Experimental Procedures”) generates only a short cDNA product from colon RNA corresponding in size with that of NOH-1S (right lane). The Nox1-specific primers are primers 1 and 3. Left lane, migration of 100-bp markers. B, alignment of several short, Nox1-derived cDNA sequences reveals variability in the proposed splice junction site of NOH-1S. The black arrows indicate the end of the sequence matching exon 5; the white arrows indicate the beginning of the sequence corresponding to exon 13. C, high temperature RT-PCR (60 °C) using ThermoScript™ reverse transcriptase (See “Experimental Procedures”) generates only a large Nox1 cDNA product from human colon mRNA. The Nox1-specific primers are 1 and 3 in lane a and 1 and 2 in lane b. The results of PCR represent at least five experiments.
reevaluated the RNase protection assay described by Bánfi et al. (18). In these experiments, we prepared a radiolabeled riboprobe from the 3'-end of NOH-1S, similar to this previous study, and examined the RNase protection patterns resulting from hybridization to the synthetic, full-length RNA transcribed in vitro from Nox1 cDNA. Fig. 4 shows that hybridization of this single, undeleted transcript provides RNase protection resulting in a mixture of two major fragments: one that corresponded to hybridization of the probe up to the boundary of the deletion site (235 nucleotides), which was predicted for detection of the full-length transcript, and another larger fragment (309 nucleotides) corresponding to the intact NOH-1S sequence, which would require formation of a stable looped structure allowing hybridization between complementary terminal sequences (Fig. 4). These results indicate that the NOH-1S probe used in earlier RNase protection studies does not distinguish between the full-length and the deleted (spliced) Nox1 transcripts and, therefore, cannot be used to detect this deleted variant. The Nox1 Gene Encodes an Exon 11-spliced Variant, Nox1v, Which Is Catalytically Inactive—As noted above, we detect one major band of ~2.5 kb in Northern blots of colon RNA using a cDNA probe corresponding to the 5'-end of the Nox1 open reading frame (Fig. 1), although Northern blotting does not distinguish between transcripts that differ slightly in size. Therefore, we used RT-PCR to analyze large Nox1 gene transcripts. Using primers aligned to the 5'- and 3'-ends of the Nox1 open reading frame (primers 1 and 4), we detect two different PCR products that are close in size (1.7 and 1.55 kb) in normal colon and in two colon carcinoma cell lines (Fig. 5A). After cloning and sequencing the PCR products, we confirmed that the larger sequence is NoxI, whereas the smaller product, lacking 147 nucleotides, is identical to the sequence reported previously as NAD(P)H oxidase homolog 1 long variant, NOH-1Lv (GenBank accession no. NM013955; 18). Because the short variant of Nox1 (NOH-1S) represents a cDNA synthesis artifact, we suggest designating this long Nox1 variant as Nox1v (consistent with the new nomenclature system for NAD(P)H oxidases). The human Nox1 gene was already sequenced as a part of the human genome project, therefore we determined that the 147 nucleotides absent in Nox1v corresponds precisely with exon 11 of the Nox1 gene, which is flanked by appropriate RNA splice donor and acceptor sequences. Thus, unlike the NOH-1S product, Nox1v appears to represent a genuine spliced variant that lacks a complete exon. Protein sequence comparison of Nox1 and Nox1v revealed that the exon 11-encoded sequence (residues 433–482) contains a highly conserved motif (135-FYWICRE441) shared by other Nox enzymes (Fig. 5B). In view of the abundance of the deleted Nox1v transcript in colon cells and the presumed importance of this conserved motif in NADPH ribose binding in gp91phox and related oxidases (3, 18,
we examined the catalytic activities of Nox1 and Nox1v expressed in transfected HEK 293 and COS-7 cells (Fig. 6). Recent studies indicate that Nox1 functions as a multicomponent oxidase complex similar to the phagocytic oxidase (19, 28, 29). Nox1 activity is relatively low when transfected into a variety of cell hosts, unless it is coexpressed in the presence of cofactors detected in colon epithelial cells (p41\textsubscript{nox1}/Noxo1 and p51\textsubscript{nox1}/Noxa1), which are homologous to the cytosolic phox proteins (19, 28, 29). Other work showed that p22\textsubscript{phox} also contributes to higher Nox1 activity, consistent with the ability of p22\textsubscript{phox} to interact with Noxo1 (29). Fig. 6A shows that HEK 293 cells transfected with the Nox1-based multicomponent system including Noxo1 and Noxa1 exhibit robust oxidative responses to phorbol 12-myristate 13-acetate stimulation, whereas cells transfected with Nox1v along with the same cofactors show no significant ROS release in response to stimulation, similar to cells transfected with control (empty) pcDNA3.1 vector. Western blotting was performed on transfected COS-7 cells to determine whether the deleted Nox1v protein was detectable in these oxidase-deficient cells. Because neither Nox1 nor Nox1v was detected using several commercial and custom Nox1 antipeptide antibodies, both the Nox1 and Nox1v cDNAs were constructed to fuse their C-terminal coding sequences with the V5 peptide epitope. The epitope-tagged fusion proteins were produced in COS-7 cells by cotransfection with p22\textsubscript{phox}-pcDNA. Western blotting of the membrane (particulate fraction) proteins from these transfected cells confirmed that both the Nox1 and Nox1v isoforms were detectable with the expected molecular masses (Fig. 6B), despite the inability of Nox1v to support any detectable ROS generation. Both fusion proteins generated a similar pattern of breakdown products, although the Nox1v form appears to exhibit lower stability. These findings confirm a critical role for exon 11-encoded sequence in oxidase function, showing that in the absence of this sequence the expressed Nox1v spliced isoform is a non-functional protein.

**FIG. 5.** Detection of exon 11 alternative splicing in Nox1 cDNA products. **A**, exon 11 spliced variant (Nox1v) is detected in RT-PCR products derived from normal colon (NC), Caco2, and HT29 cell RNA. The Nox1-specific primers are 1 and 4. **B**, alignment of highly conserved portions of Nox family exon 11-encoded amino acid sequences with proposed NADPH ribose binding sequences in cytochrome p450 reductase (CPR) and nitric oxide synthase (iNOS) (based on Refs. 26 and 27). Sequence similarities are less clear between the proposed NADPH ribose binding sequences of Nox proteins and the corresponding sequence of FNR. The first residue number in each sequence is indicated on the left.

**FIG. 6.** Nox1v fails to reconstitute ROS generation in transfected cell models. **A**, chemiluminescence assays of superoxide production in cells transfected with Nox1, Nox1v, or pcDNA (control) vector, along with plasmids encoding Nox1-supportive cofactors, Noxo1-α and Noxa1, as described under “Experimental Procedures.” Upper graph, activities of transfected HEK 293 cells assayed 72 h after transfection. Lower graph, activities of COS-7 cells assayed 48 h after transfection, using the same vectors described above plus p22\textsubscript{phox}-pcDNA. Data represent the mean values of triplicate assays ± S.E. Similar results were obtained in two other independent transfection experiments. **B**, Western blotting of Nox1 and Nox1v V5 fusion proteins in membrane fractions of transfected COS-7 cells. Left panel, anti-V5 immunoblotting of Nox1 and Nox1v, with sizes of full-length proteins indicated by arrowheads. Right panel, total protein (Ponceau S) staining of the same blot confirms comparable loading. Migration of molecular mass standards is indicated on right in kDa.
DISCUSSION

In this study, we characterized mRNA transcripts derived from the Nox1 gene and show that Nox1 does not encode a H+ channel described previously as NOH-1S. Several independent results were obtained that refute all previous evidence suggesting NOH-1S is a natural product of mRNA splicing. First, NOH-1S was not detected by Northern blotting of colon cells, a site of high Nox1 expression, despite the suggestion of its relative abundance based on RNase protection assays (18). Second, the deleted NOH-1S could only be generated from colon cell RNA when using AMV reverse transcriptase at low temperatures, whereas only the large Nox1 transcripts were synthesized when using a reverse transcriptase (ThermoScript™) that performs at higher temperatures. Third, the shortened Nox1-derived cDNAs produced by AMV reverse transcriptase were heterogeneous at the alleged splice site. More important, the shortened cDNA was also generated artificially using full-length, synthetic Nox1 RNA as a template. Thus, it appears that NOH-1S is derived by a template switching process that is unique to low temperature cDNA synthesis by AMV reverse transcriptase resulting in a deletion in the transcribed sequence. Finally, we showed that hybridization with full-length Nox1 RNA provides protection to a deleted NOH-1S antisense probe against RNase degradation; thus, the probe does not distinguish between full-length and deleted transcripts. This result, as well as those showing spontaneous deletions during cDNA synthesis from the full-length template cast considerable doubt on the validity of the evidence presented for a NOH-1S mRNA splicing mechanism. These results were consistent with the formation of hybrids between the full-length and the complementary, deleted (NOH-1S) strands, which are likely stabilized by repetitive sequences present on the boundaries of the deleted sequence.

In addition to these findings, several structural features of the Nox1 gene are incompatible with an mRNA splicing mechanism. First, based on the corrected Nox1 genomic map, the proposed NOH-1S splicing requires intron-exonic sites at both the splice donor (exon 5) and acceptor sites (exon 13). Second, there are no suitable splice sites within the proposed intron-exonic splice boundaries that conform to the GT-AG rule. A recently compiled database of known mammalian splice site sequences, with more than 22,000 entries (21), confirms that 98.71% contain the canonical GT-AG junctions; the remainder have one of two other splice site pairs (GC-AG and AT-AC), which were also not observed near the proposed splice boundaries of NOH-1S. Third, in general, the Nox family of gp91phox homologs is highly conserved with minimal interspecies variations within coding sequences. For example, the human and the rat Nox1 proteins are 80% identical, whereas the 3‘-untranslated regions are poorly conserved. In the case of NOH-1S, this protein would appear to be unique to the human species because we could not identify any “hidden” reading frame in the rat Nox1 mRNA which encodes an amino acid sequence homologous to the C-terminal portion of human NOH-1S. Fourth, the Nox1 transcript, like most eukaryotic mRNA molecules, contains an AAUAAA polyadenylation signal 17 nucleotides upstream from the polyadenylated 3‘ terminus. In the proposed spliced NOH-1S transcript of Bánfi et al. (18), this polyadenylation signal is used instead as part of the C-terminal coding region of NOH-1S, whereby the UAA within this signal represents the proposed stop codon. These theoretical considerations based on our revision of the Nox1 genomic map also argue strongly against the feasibility of a NOH-1S RNA splicing mechanism.

Database searches with the seven Nox family cDNAs indicate that spontaneous deletions within cDNA sequences deposited in unedited sequence tag databases (i.e., dbEST) are not unique to Nox1 and are more common than is generally appreciated. These searches revealed internal deletions in the cDNAs of mouse Nox4/Renox and rat Duox2/Tho2. In both cases, the generation of these deleted products can be explained by template switching during cDNA synthesis by reverse transcriptase because these cDNAs share several remarkable structural features with NOH-1S and other well characterized transcripts that undergo artificial template switching (25). These features include the occurrence of deletions within intron-exonic sites, the absence of appropriate splice signals, the production of multiple, but imprecise, splice variants (sometimes resulting in frameshifts or missense mutations), as well as the presence of repetitive sequence elements flanking the deletion sites. Our analysis of multiple products derived by this process suggests that the repetitive sequence elements have a critical role in promoting the template switching process by enabling the newly synthesized strand to reanneal upstream to other homologous sequence elements. In cases where multiple deleted cDNAs were detected (as with Nox1), the presence of multiple, minor repeats or imperfect repeats accounted for these heterogeneous transcripts. These observations suggest that sequences derived from these rapidly growing sequence databases, such as dbEST, should be interpreted cautiously because these sequences frequently contain internal deletions resulting from template switching, in addition to other cloning, transcription, PCR amplification, and sequencing errors. One consequence of such errors is that calculations of protein diversity based on EST analysis could lead to overestimations of the frequency of alternative splicing in the human genome. Furthermore, template switching could generate unrecognized artifacts in RT-PCR protocols frequently applied in the diagnostic analysis of aberrant mRNAs.

Although RNA splicing does not account for NOH-1S synthesis, we confirmed that RNA splicing does generate significant amounts of another Nox1 transcript variant detected in normal colon and in two colon carcinoma cell lines (Fig. 5A), which we refer to as Nox1v (formerly NOH-1Lv (18)). The Nox1v transcript lacks the entire 11th exon, which encodes exactly 49 amino acids (residues 433–482). A BLAST search using this sequence revealed that the N-terminal portion of this sequence (135FYWICR441) is highly conserved in the Nox family (Fig. 5B). The corresponding sequence in gp91phox (441YWLCR477) was proposed to represent a NADPH ribose binding motif, based on similarities with sequences of other flavoenzymes, notably cytochrome P450 reductase and nitric-oxide synthase (26, 27). However, these features were not evident in the crystallographic structure of ferrodoxin NADP+ reductase (FNR; 30). Thus, the absence of crystallographic information on the NADP+-bound form of FNR, together with the lack of similarities between these regions of FNR and Nox proteins (Fig. 5B), have made the assignment of the NADP+/ribose-binding residues in Nox proteins less certain than other, more conserved nucleotide binding motifs shared with FNR-related flavoproteins (26, 27, 31). Because of these uncertainties, we directly compared the abilities of Nox1 and Nox1v to support ROS production in two transfected cell models that coexpressed p22phox and cytosolic factors (Nox1 and Nox1v) shown to support maximum activity of Nox1 (19, 28, 29). These experiments showed that the exon 11-deleted Nox1v transcript does not encode a functional oxidase, despite its abundant expression in colon cells. These observations confirm a critical role for exon 11-encoded sequence in the oxidative function of Nox1 and provide further support for the proposed role of this conserved motif in NADPH ribose binding.

H+-extruding mechanisms have an important role in the pH
homeostasis of phagocytic cells because phagocytes produce a significant amount of H⁺ during superoxide production (10). Voltage-operated H⁺ channels represent one major route of H⁺ removal; however, a genuine H⁺ channel protein has not been identified at the molecular level. One proposed candidate for the H⁺ channel function in phagocytic cells is gp91phox (11–13); however patch clamp experiments on gp91phox-deficient neutrophils (16), monocytes (14), and COS-7 cells expressing the complete oxidase enzyme (32) question the contribution of gp91phox to whole cell H⁺ currents. Thus, while the precise roles of gp91phox and related Nox proteins in proton transport are debated (33–36), the hunt for additional genes encoding genuine H⁺ channels should continue.

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