Nuclear NonO/p54nr Protein Is a Nonclassical Carbonic Anhydrase*

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The growing carbonic anhydrase (CA) gene family includes 11 enzymatically active isozymes in mammals. Each of them has a characteristic cellular and subcellular distribution pattern. In this report, we demonstrate for the first time a nuclear protein with CA activity. A polypeptide recognized by CA II antibodies was purified from several rat tissues using CA inhibitor affinity chromatography. This polypeptide of apparent 66 kDa mass was characterized using amino acid sequencing and CA activity measurements. It appeared to be identical to nonO/p54nr, a previously cloned and characterized DNA and RNA binding nuclear factor. Recombinant nonO generated in baculovirus bound to the CA inhibitor affinity chromatography matrix and revealed detectable CA activity (25 units/mg). Hansson’s histochemical staining of rat lymph nodes followed by light and electron microscopy showed nuclear CA activity in lymphocytes, suggesting that the nuclear nonO protein is catalytically active in vivo. These results demonstrate that a previously known transcription factor is a novel, nonclassical CA. Through its CA activity, the nonO may function in the maintenance of pH homeostasis in the nucleus.

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that are responsible for the reversible hydration of carbon dioxide in a reaction: 

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^- \]

The enzyme is present in almost all organs where it participates in ion, fluid, and acid-base balance. Eleven active isozymes have been characterized, including cytoplasmic (CA I, CA II, CA III, and CA VII) (1, 2), mitochondrial (CA VA and CA VB) (3), secreted (CA VI) (4), and membrane-associated (CA IV, CA IX, CA XII, and CA XIV) (5–9) forms. The range of the specific activity of these isozymes is quite large, with CA II having the highest (10) and CA III the lowest activity (11). All isozymes are expressed in some normal tissues. Two recently characterized transmembrane proteins, CA IX and CA XII, have been linked to oncogenesis, and their overexpression has been observed in malignant tumors (7, 8, 12–15).

The present study was designed to identify the CA isozyme(s) expressed in the interstitial cells of testis, which are known to contain CA activity (16). Immunocytochemistry, using anti-CA II antibodies showed strong signals in normal and tumor-derived Leydig cells. Interestingly, these antibodies recognized a previously unknown 66-kDa polypeptide in Western blots of testis and Leydig tumor cells. Since an apparent molecular mass of 66 kDa had not been reported for any known CA isozyme, this protein was considered as a promising candidate for a novel CA. However, amino acid sequencing of the purified protein revealed identity with nonO/p54nr, a novel, nonclassical carbonic anhydrase, as the enzyme activity is higher than determined for CA III and CA VA.

**Experimental Procedures**

**Antibodies**—The polyclonal antibodies against human and rat CA II have been produced and characterized earlier (19, 20). Generation and use of rabbit antiserum against 6-azathiochrome S-transferase-nonO fusion protein was described in Yang et al. (17). The following secondary antibodies were used for the immunohistochemistry: fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG (Sigma), tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit IgG (Dakopatts), and biotinylated swine anti-rabbit IgG (Dakopatts).

**Cell Culture**—LC-540, Leydig tumor cells (CCL-43; American Type Culture Collection, Manassas, VA) were grown in Eagle’s minimum essential medium supplemented with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 1.0 mM Earle’s balanced salt solution, and 10% fetal bovine serum in a humidified atmosphere of 5% CO2, 95% air at 37 °C on 75-mm plastic bottles. The cells were grown 3 days to confluency, trypsinized, and centrifuged at 1000 rpm for 10 min after which the cells were used for Western blotting.

**Histological Staining of CA Activity Followed by Light and Electron Microscopy**—The rat lymph nodes were stained for CA activity by the method of Hansson (21), in which the cells were used for Western blotting.

**Immunofluorescence**—LC-540 cells grown on plastic chamber slides for microscopy for 3 days were rinsed with 0.1 M phosphate-buffered saline (PBS), pH 7.4, and fixed with 4% neutral-buffered formaldehyde for 15 min. Then they were rinsed with PBS and subjected to immunofluorescence staining that consisted of the following steps: 1) pretreatment of the cells with 0.1% bovine serum albumin in PBS (BSA-PBS) for...
40 min and rinsing in PBS, 2) incubation for 1 h with primary antibodies diluted 1:100 in 0.1% BSA-PBS, and 3) incubation with 1:100 diluted fluorescent secondary antibodies in 0.1% BSA-PBS. The cells were washed three times for 10 min after the incubation steps. All incubations and washings were done in the presence of 0.05% saponin. The cells were viewed by a Leitz Aristoplan epifluorescence microscope (Wetzlar, Germany).

Samples of the human testis (n = 3) were obtained together with routine histopathological specimens taken during surgical operations for prostate cancer. The procedures were carried out according to the provisions of the Declaration of Helsinki, and informed consent was obtained from each patient. Testis and lymph node specimens were obtained from adult rats of Sprague-Dawley strain. Each tissue sample was divided into several small pieces. The specimens were fixed in Carnoy’s fluid (absolute ethanol + chloroform + glacial acetic acid 6:3:1) for 6 h at 4 °C. The samples were then dehydrated and embedded in paraffin wax in a vacuum oven at 58 °C, and sections of 5 μm were placed on gelatin-coated microscope slides. The immunohistochemical staining was performed using the biotin-streptavidin complex method employing the following steps: 1) pretreatment of the sections with undiluted cow colostral whey for 40 min and rinsing in PBS, 2) incubation for 1 h or overnight (testis samples) with the primary antiserum diluted 1:100 in 1% BSA-PBS, 3) incubation for 1 h with biotinylated swine anti-rabbit IgG diluted 1:300 in 1% BSA-PBS, 4) incubation for 30 min with peroxidase-conjugated streptavidin (Dako; Denmark) diluted 1:600 in PBS, and 5) incubation for 2 min in DAB solution containing 9 mg of 3,3’-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 ml of PBS + 5 μl of 30% H2O2. The sections were washed three times for 10 min in PBS after incubation steps 2, 3, and 4. All the incubations and washings were carried out at room temperature, and the sections were finally mounted in Permount (Fisher). The stained sections were examined and photographed with a Leitz Aristoplan microscope (Wetzlar, Germany).

**Immunofluorescent Staining of CAs**—Rat tissues were homogenized by Ultra-Turrax homogenizer and sonicated in ice-cold 0.1 M Tris-SO4 buffer, pH 8.7, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM o-phenanthroline as protease inhibitors. The cell and tissue homogenates were centrifuged at 100,000 g for 30 min. The supernatants were recovered and subjected to affinity purification. The inhibitor affinity chromatography was performed using the carboxymethyl Bio-Gel A coupled to p-aminomethylbenzenesulfonamide as described (22).

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting**—Samples of homogenized rat testis (20 μg), LC-540 cells (20 μg), or eluted protein from inhibitor affinity chromatography (2 or 10 μg) were analyzed by SDS-PAGE under reducing conditions according to Laemmli (23). All the reagents for SDS-PAGE were from Bio-Rad or Sigma. The electrophoreses were performed in a Mini-Protein electrophoresis unit (Bio-Rad) using a 10% acrylamide separating gel and a 4% acrylamide stacking gel. The proteins were transferred electrophoretically from the gel to a polyvinylidene difluoride membrane (Millipore; Bedford, MA). After the transblotting, the sample lanes were first incubated with TBST buffer (50 μg/ml Tris-HCl, pH 7.5, bound protein was eluted with 1 ml of 0.5 M sodium perchlorate). The sections were finally mounted in Permount (Fisher). The stained sections were examined and photographed with a Leitz Aristoplan microscope (Wetzlar, Germany).

**Protein Sequence Analysis**—The protein sequencing for tryptic-digested polypeptides was carried out with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) followed by analysis with ProFound and PeptideSearch programs. The work was performed in the HHMI Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

**Production and Purification of Recombinant nonO**—The coding region of nonO with a six-histidine tag at the N terminus was amplified by polymerase chain reaction with Pfu DNA polymerase (Stratagene, La Jolla, CA) and cloned into the transfer vector of the BAC/BAC system (Invitrogen, Carlsbad, CA). BACs containing the gene were transferred into DH10BAC and transfected into s9 insect cells (9 × 106 cells). Large scale protein expression was generated by infecting the nonO-containing virus at high multiplicity of infection into insect cells incubated at 27 °C for 72 h. Infected cells were harvested, resuspended in HK buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 15 mM imidazole, and protease inhibitor; Roche Molecular Biochemicals), lysed by sonication, and then centrifuged at 5500 rpm for 30 min. The supernatants were recovered and subjected to affinity purification. The inhibitor affinity chromatography was performed using the carboxymethyl Bio-Gel A coupled to p-aminomethylbenzenesulfonamide as described (22).

**Carbonic Anhydrase Assay**—CA activity from recombinant nonO protein was determined as described previously (24). The protein was dialyzed against the following buffer at 4 °C: 50 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 250 mM imidazole, protease inhibitor). The eluted protein was dialyzed against buffer D and stored at −70 °C in aliquots. The purity of the preparations was estimated by SDS-PAGE to exceed 90%.

**Binding of nonO Protein to CAs**—The binding of nonO protein to CAs was measured using a radiometric assay. A 100 μl aliquot of 200 μM nonO protein, in the presence or absence of 10 μM Ca2+, was incubated with 100 μl of 200 μM CAs for 1 h at 25 °C. The reaction was stopped by adding 50 μl of 0.5 M Tris-HCl at pH 7.5. The reaction mixture was extracted with an equal volume of chloroform, and the organic phase was discarded. The residual was then incubated with nickel nitrilotriacetic acid beads (Qiagen, Valencia, CA) for 30 min at 25 °C. The CA beads were washed three times with buffer and centrifuged at 5000 rpm for 30 min. The supernatant was assayed for CA activity by measuring the amount of carbonate released from NaH14CO3 at pH 7.5.

**FIG. 1.** Immunohistochemical staining of human (A) and rat testes (B, C, and F) and rat Leydig cell tumor-derived LC-540 cells (D and E) using polyclonal antibodies (A, B, D-F) or normal rabbit serum (C). Both anti-human CA II (A and D) and anti-rat CA II (B and E) antibodies show positive staining in the nuclei (arrows) and cytoplasm of Leydig cells. Control staining of rat testis using normal rabbit serum in place of the primary antibody remained unstained.

**FIG. 2.** Western blot of rat testis and LC-540 cells using anti-CA II antibody. 20 μg of protein was applied per lane. In both blots anti-CA II antibody recognizes a 66-kDa polypeptide in addition to the 30-kDa polypeptide of CA II present in the testis.
buffer, pH 7.5. Unbound and bound proteins were analyzed by SDS-PAGE followed by Western blotting.

RESULTS

Anti-CA II Antibody Shows both Nuclear and Cytoplasmic Staining in Leydig Cells—Fig. 1, A and B, shows immunohistochemical staining of human and rat testis using anti-human and anti-rat CA II antibodies. Strong cytoplasmic and nuclear signals were localized to the interstitial cells. Control immunostaining of rat testis using normal rabbit serum instead of the anti-CA II antibodies is shown in Fig. 1 C. Double immunostaining of rat testis using anti-human CA II and anti-rat P450c17 (a marker of testosterone synthesis) antibodies showed positive signals in the same cells (Fig. 1 F), indicating that the CA expression is confined to the testosterone-producing Leydig cells. Immunocytochemical staining of Leydig cell tumor-derived cells (LC-540) using anti-CA II antibodies also indicated that these cells contain CA immunoreactivity (Fig. 1, D and E). The control immunostaining of LC-540 cells using normal rabbit serum showed no positive reaction (data not shown).

Anti-CA II Antibody Recognizes a 66-kDa Polypeptide in Testis and Leydig Tumor Cells—Immunoblots from homogenates of rat testis and LC-540 cells were performed to confirm the specificity of the immunostaining results. Anti-rat CA II antibodies recognized a prominent polypeptide migrating at 66 kDa in both blots. In addition, a 30-kDa polypeptide of CA II was visible in the testis (Fig. 2).

Affinity-purified CAs from Rat Tissues Revealed an Apparent 66-kDa Polypeptide—To determine the distribution pattern of the apparent 66-kDa protein and its binding to CA inhibitor affinity chromatography matrix, soluble proteins from several rat tissues were subjected to affinity purification and analyzed by Western blotting. The strongest signal was detected in the liver, colon, ovary, and spleen (Fig. 3 A). In all other tissues examined the signal became apparent after longer exposures (data not shown). In another set of experiments (Fig. 3 B) both anti-human and anti-rat CA II antibodies revealed a strong 66-kDa signal in addition to the 30-kDa polypeptide of CA II in affinity-purified proteins from rat lymph nodes.

Amino Acid Sequencing Indicates That the 66-kDa Polypeptide Is Identical to nonO/p54nrb—To identify the apparent 66-kDa protein observed in the Western blots, the corresponding band was isolated from a polyvinylidene difluoride membrane after transblotting and subjected to protein sequencing. The sequence data obtained from MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) was analyzed using ProFound and PeptideSearch data bases, which revealed that the protein was identical to murine nonO and its human orthologue, p54nrb. Although the predicted mass of nonO/p54nrb is 55 kDa, its mobility in SDS-PAGE was previously estimated at 65 kDa. Thus the 66-kDa protein of nonO/p54nrb corresponds to the conventional ubiquitous form. The percentage coverage of the known sequence for this protein was 33% as shown in Fig. 4.

Recombinant nonO Protein Specifically Binds to CA Inhibitor-coupled Affi-Gel-10 Column—The results in Fig. 5 A show that recombinant nonO alone as well as in presence of human CA II bound to the p-aminomethylbenzenesulfonamide-coupled Affi-Gel-10 column. High molecular weight polypeptides for nonO seen in the bound fraction represent larger sizes of re-
coupled Affi-Gel-10 column (A) between the nonO protein and CA II (that the anti-nonO antibody recognizes the recombinant nonO protein anti-nonO (2) similar to CA VI. All other CA isozymes were 10- to 100-fold nonO was higher than determined for CA III and VA and was 25 units/mg. The comparison of specific activities of different activity assay. The specific CA activity of recombinant nonO was almost completely prevented the binding of both nonO and CA II to the column. In the panels B and C, 7 µg of recombinant nonO protein alone (lane 1) or together with 7 µg of purified human CA II (lane 3) and 10 µg of affinity-purified CAs from rat lymph node (lane 2) were subjected to SDS-PAGE followed by Western blotting using anti-nonO (B) or anti-CA II (C) antibodies. The results in panel B show that the anti-nonO antibody recognizes the recombinant nonO protein (lanes 1 and 3) and CA II (lane 3). Anti-CA II antibody shows a cross-reaction with polypeptides of 66 and 53 kDa similar to nonO protein in the partially purified CAs from rat lymph node (lane 2). The results in panel C show that anti-CA II antibody cross-reacts with the recombinant nonO protein (lanes 1 and 3). A strong reaction with CA II protein is seen (lane 3). A polypeptide similar to CA II is present in the partially purified CAs from rat lymph node (lane 2). The wide bands of nonO and CA II in lane 2 of panels B and C, respectively, are due to high salt concentration in the protein sample, which generally diffuses the polypeptide mobility in SDS-PAGE.

- Acetazolamide  
  - Non O Bound  
  - CA II Bound  
+ Acetazolamide  
  - Non O Flow Through  
  - CA II Flow Through
  
**Fig. 5.** Binding of recombinant nonO protein to CA inhibitor-coupled Affi-Gel-10 column (A) and immunological similarity between the nonO protein and CA II (B and C). Panel A shows that the recombinant nonO protein alone as well as in the presence of purified human CA II binds to affinity column, which was analyzed by Western blot using a mixture of anti-CA II and anti-nonO antibodies. 2 mM acetazolamide completely prevents the binding of both nonO protein and CA II to the column. In the panels B and C, 7 µg of recombinant nonO protein alone (lane 1) or together with 7 µg of purified human CA II (lane 3) and 10 µg of affinity-purified CAs from rat lymph node (lane 2) were subjected to SDS-PAGE followed by Western blotting using anti-nonO (B) or anti-CA II (C) antibodies. The results in panel B show that the anti-nonO antibody recognizes the recombinant nonO protein (lanes 1 and 3) and CA II (lane 3). Anti-CA II antibody shows a cross-reaction with polypeptides of 66 and 53 kDa similar to nonO protein in the partially purified CAs from rat lymph node (lane 2). The results in panel C show that anti-CA II antibody cross-reacts with the recombinant nonO protein (lanes 1 and 3). A strong reaction with CA II protein is seen (lane 3). A polypeptide similar to CA II is present in the partially purified CAs from rat lymph node (lane 2). The wide bands of nonO and CA II in lane 2 of panels B and C, respectively, are due to high salt concentration in the protein sample, which generally diffuses the polypeptide mobility in SDS-PAGE.

**TABLE I**

| Isozyme | Specific activity |
|---------|------------------|
| CA I    | 200              |
| CA II   | 2000–3000        |
| CA III  | 1–5              |
| CA IV   | 3000             |
| CA VA   | 5                |
| CA VI   | 30               |
| CA IX   | 2000             |
| CA XII  | 500              |
| CA XIV  | 2000             |

PAGE followed by Western blotting using anti-nonO or anti-CA II antibodies (Fig. 5, B and C). Anti-nonO serum recognized the recombinant nonO protein (panel B, lanes 1 and 3) and also showed a weak reaction with CA II (lane 3). Anti-nonO antibody recognized polypeptides of 66 and 53 kDa, similar to nonO protein in the partially purified CAs from rat lymph node (lane 2). The results in Fig. 5C show that anti-CA II antibody cross-reacted with the recombinant nonO protein as shown in lanes 1 and 3. A strong reaction with CA II protein was seen as expected (lane 3). A polypeptide similar to CA II was seen in the partially purified CAs from rat lymph node (lane 2). From these results we conclude that anti-nonO antibodies cross-react with CA II and vice versa, suggesting an immunological similarity between these proteins. The failure to detect CA II (panel B, lane 2) and nonO (panel C, lane 2) was due to low amount of CA II and nonO protein in the partially purified CA preparation obtained from rat lymph nodes. In addition, the immunological cross-reactivity between α-nonO and CA II as well as α-CA II and nonO is much weaker than the specific binding of these antibodies (compare with lane 3 of panels B and C). The poor immunological cross-reactivity between these two proteins resulted in nondetectable CA II and nonO in panels B (lane 2) and C (lane 2), respectively.

Anti-CA II and Anti-nonO Antibodies Stained the Nuclei in Lymphocytes, and These Cells Express Nuclear CA Activity—Both anti-CA II and anti-nonO antibodies stained lymphocytes in the rat lymph node (Fig. 6). The immunoreaction for the nonO protein was confined to the nuclei (B), whereas anti-CA II serum showed positive signal in both nucleus and cytoplasm (A). The control immunostaining of the rat lymph node using normal rabbit serum instead of the primary antibody was negative (Fig. 6C). These results suggest that lymphocytes may contain both nonO and CA II proteins.

Frozen sections of rat lymph node were stained for CA activity using Hansson’s histochemical method and analyzed by light and electron microscopy. Fig. 6, D and E, shows that much of the black reaction product is localized to the nuclei of lymphocytes, indicating that these cells express nuclear CA activity. 10 µM acetazolamide completely blocked the reaction as shown in Fig. 6F.

**DISCUSSION**

As a first step to identify the CA isozyme(s) expressed in Leydig cells, we used antibodies against CA II in conjunction with immunohistochemical methods and Western blotting. Interestingly, anti-CA II antibody repeatedly recognized an apparent 66-kDa polypeptide, which was previously thought to be a dimeric form of CA II (19). Since reduction by β-mercaptoethanol did not dissociate this polypeptide and the same molecular weight band was obtained by CA inhibitor affinity chromatography from several tissues, the 66-kDa polypeptide was considered as a promising candidate for a novel CA isozyme. Amino acid sequencing revealed, however, that it was previ-
ously cloned and characterized as nonO/p54αβ, a nuclear protein shown to bind both RNA and DNA and be implicated in transcription and pre-RNA splicing (17, 18). This finding is provocative because no other class of mammalian proteins except CAs has been shown to bind specifically to a CA inhibitor affinity chromatography matrix and to contain CA catalytic activity.

The present results showed significant immunological cross-reactivity between CA II and nonO. A polyclonal anti-rat CA II antibody recognized recombinant nonO in Western blots. Conversely, polyclonal anti-nonO serum raised against a bacterial glutathione S-transferase-nonO fusion protein cross-reacted with purified CA II. One explanation for this cross-reactivity is the minor sequence homology found between CA II and nonO (Fig. 4).

In addition to the immunological similarity with CA II, the nonO protein was found to be an enzymatically active CA. Its specific activity is similar to CA VI2 and higher than determined for CA III (11) and CA VA.2 Since these isozymes have been reported to participate in various physiological processes, we can predict that the level of CA activity in nonO is also physiologically meaningful. The amino acid sequence of nonO predicted from its cDNA shares no structural elements required for conventional CA activity. All conserved histidines, which are involved in zinc binding and heretofore have been considered essential for CA activity, are absent in nonO. Interestingly, Lesburg et al. (26) demonstrate that CA activity and zinc binding capacity of CA II is retained when His-119 is substituted with glutamine. A polyglutamine stretch, Q-29–Q-38, in the nonO protein is a potential site for Zn2+ binding and could be a potential site for the CA activity of nonO. It will be of interest to explore the tertiary structure of nonO and compare it with other CAs. This could give some clues to explain the nature of the CA activity in the nonO protein.

NonO is a nucleic acid-binding protein that shares significant sequence identity with NonA, a Drosophila optomotor protein of unknown function (17, 18), and with PSF, a mammalian splicing factor (18). Several lines of evidence indicate a direct or indirect role for nonO in transcription. First, Yang et al. (27) show that nonO enhances the binding of some conventional sequence-specific transcription factors (Oct-2, E47) to their recognition sites, suggesting that nonO protein might be a positive co-activator. Second, nonO has been independently implicated as a transcription factor in at least three systems. The murine intracisternal A particle provirus elements are expressed at low levels in undifferentiated F9 embryonic carcinoma cells but highly when F9 cells are induced to differentiate into ectoderm. Intracisternal A particle up-regulation re-

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2 A. Waheed and W. S. Sly, unpublished observation.
quires a 60-kDa DNA-binding protein (28, 29) whose identity has been established as nonO (30). Hepatic and intestinal transcription of the human apolipoprotein A-II gene requires several nuclear activities at the apolipoprotein A-II promoter (31). One of these turns out to be nonO. NonO binds and appears to transactivate the core promoter of the hepatocyte growth factor gene. In these cases, identity was established by protein purification followed by in vitro transcription. The third line of evidence is provided by heteromeric analyses. Three known transcription factors have been found in complexes with nonO. Hallier et al. (32) identify nonO by microsequencing as a partner for Spi-1, a DNA target-specific transactivator involved in erythocyte differentiation. Dr. John Hassell (Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada) identified nonO in the same manner as a 65-kDa protein copurifying with the Ets-domain partner, PEA3 (33). PEA3 is a developmentally-regulated transcriptional activator of unknown function primarily expressed in brain and epididymis. We demonstrated that the respective recombinant proteins interact but without obvious consequence for PEA3 DNA binding (27). Finally in a standard two-hybrid screen of a gal4 activation domain-fused cDNA library, we isolated a third partner, the retinoic X receptor (RXR) chain of the nuclear hormone receptor superfamily. Preliminary observations indicate that nonO can repress nuclear hormone mediated transcriptions through interaction with its DNA binding domain.

Our results provide both physiologic and an expanded significance for the CA activity of nonO in transcriptional regulation. We propose that a common and fundamental property underlies this unique diversity. As an active CA, nonO might participate in pH-dependent events occurring in the nucleus. These might include protein transport, ion gradients, or apoptosis.

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