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Molecular and biochemical characterization of *dNOS*: A *Drosophila* Ca^{2+}/calmodulin-dependent nitric oxide synthase

(signal transduction/RNA isoforms)

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ABSTRACT **Nitric oxide (NO) is an intercellular messenger involved with various aspects of mammalian physiology ranging from vasodilation and macrophage cytotoxicity to neuronal transmission. NO is synthesized from L-arginine by NO synthase (NOS). Here, we report the cloning of a *Drosophila* NOS gene, *dNOS*, located at cytological position 32B. The *dNOS* cDNA encodes a protein of 152 kDa, with 43% amino acid sequence identity to rat neuronal NOS. Like mammalian NOSs, DNOS protein contains putative binding sites for calmodulin, FMN, FAD, and NADPH. DNOS activity is Ca^{2+}/calmodulin dependent when expressed in cell culture. An alternative RNA splicing pattern also exists for *dNOS*, which is identical to that for vertebrate neuronal NOS. These structural and functional observations demonstrate remarkable conservation of NOS between vertebrates and invertebrates.**

Nitric oxide (NO) is synthesized by NO synthases (NOSs) during conversion of L-arginine to L-citrulline (for reviews, see refs. 1 and 2). Biochemical characterization of NOSs has distinguished two general classes: (*i*) constitutive, dependent on exogenous Ca^{2+}/calmodulin, and (*ii*) inducible, independent of exogenous Ca^{2+}/calmodulin. Analyses of cDNA clones have identified three distinct NOS genes in mammals (3–6): neuronal, endothelial, and macrophage. The neuronal and endothelial NOSs are constitutive, and the macrophage NOS is inducible. The nomenclature for these different isoforms used here is historical, as it is clear now that one or more isoforms can be present in the same tissue (7).

As a diffusible, free-radical gas, NO is a multifunctional messenger affecting many diverse aspects of mammalian physiology (for review, see ref. 8), such as regulation of vascular tone, macrophage-mediated cytotoxicity, and cell–cell interactions in the nervous system, including synaptogenesis and apoptosis during development of the rat central nervous system (9) and during neuronal cell differentiation (10). NO also appears to be involved with long-term potentiation in hippocampus and with long-term depression in cerebellum, two forms of synaptic plasticity that may underlie behavioral plasticity (11–15). Consistent with these cellular studies, inhibition of NOS activity may disrupt learning (refs. 16–19, but see refs. 20 and 21).

Many of the above results are based on pharmacological studies using inhibitors of NOS or donors of NO. Interpretations of such studies usually are limited because the drugs interact with more than one target and cannot be delivered to specific sites. A molecular genetic approach can overcome these problems, however, by disrupting a specific gene, the product of which may be one of the drug's targets. Recently, such an approach has been attempted in mice via generation of a knockout mutation of the neuronal NOS (nNOS) (22). While nNOS mutants appeared fully viable and fertile, minor defects in stomach morphology and hippocampal long-term potentiation were detected (22, 23). Taken together, the above reports suggest roles for NO in developmental and behavioral plasticity of the vertebrate central nervous system.

In this paper, we report the molecular characterization of a *Drosophila* NOS gene, *dNOS*.† The primary structure and activity of DNOS protein is strikingly similar to the class of constitutively expressed mammalian NOSs, the activities of which are regulated by transient changes in intracellular Ca^{2+} concentration. We also have detected a splicing variant of the *dNOS* transcript which is identical to that described for the nNOS. This evolutionary conservation of structure and function between *Drosophila* and vertebrate NOSs suggests a universality of NOS function underlying neuronal plasticity.

MATERIALS AND METHODS

Library Screens. We have screened 6×10^4 plaques of a *Drosophila* λDASH genomic library with the 1.3-kb *Bgl* II fragment of rat nNOS cDNA (nt 3282–4573) under low-stringency conditions of 40% formamide as described (24). Fifty positive phage plaques were purified and grouped on the basis of inter se hybridization results. The contig containing the 2.4R fragment of *dNOS* comprised 15 phage clones. Regions of cross-hybridization to the rat probe were identified and subcloned, and three of them were sequenced. The other two did not contain sequences similar to any protein in the data base. Approximately 5×10^5 plaques of *Drosophila* head cDNA library (a gift from P. Salvaterra; Beckman Research Institute, City of Hope, Duarte, CA) were screened with the 2.4R fragment isolated from phage clone λ8.11 by using standard conditions. Eight positive phage plaques were identified. All phage purification and cloning steps were done by following standard methods (25). cDNA fragments were inserted into Bluescript (Stratagene) and sequenced on both strands with Sequenase 2.0 (United States Biochemical).

Cell Culture Assay of DNOS Activity. The expression construct for activity assays contained *dNOS* cDNA (with an *Xba* I restriction site engineered immediately upstream of the ATG initiation codon) inserted into the *Xba* I and *Sma* I restriction sites of the pCGN vector (26). Human 293 embryonic kidney cells were transfected with 15 μg of either the *dNOS* construct or vector DNA precipitated with calcium phosphate as described (27). Cells were collected 2 days later, and protein extracts were prepared as described (25). The enzymatic assay for NOS activity was done essentially as described (28). A 100-μl reaction mixture containing 25 μl (50–100 μg) of soluble protein extract, 50 mM Hepes (pH 7.4), 1 mM dithiothreitol, 0.8 mM $CaCl_2$, 1 mM NADPH, 10 μg of calmodulin per ml, 2

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Abbreviations: NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; RT-PCR, reverse transcription–PCR; ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U25117).

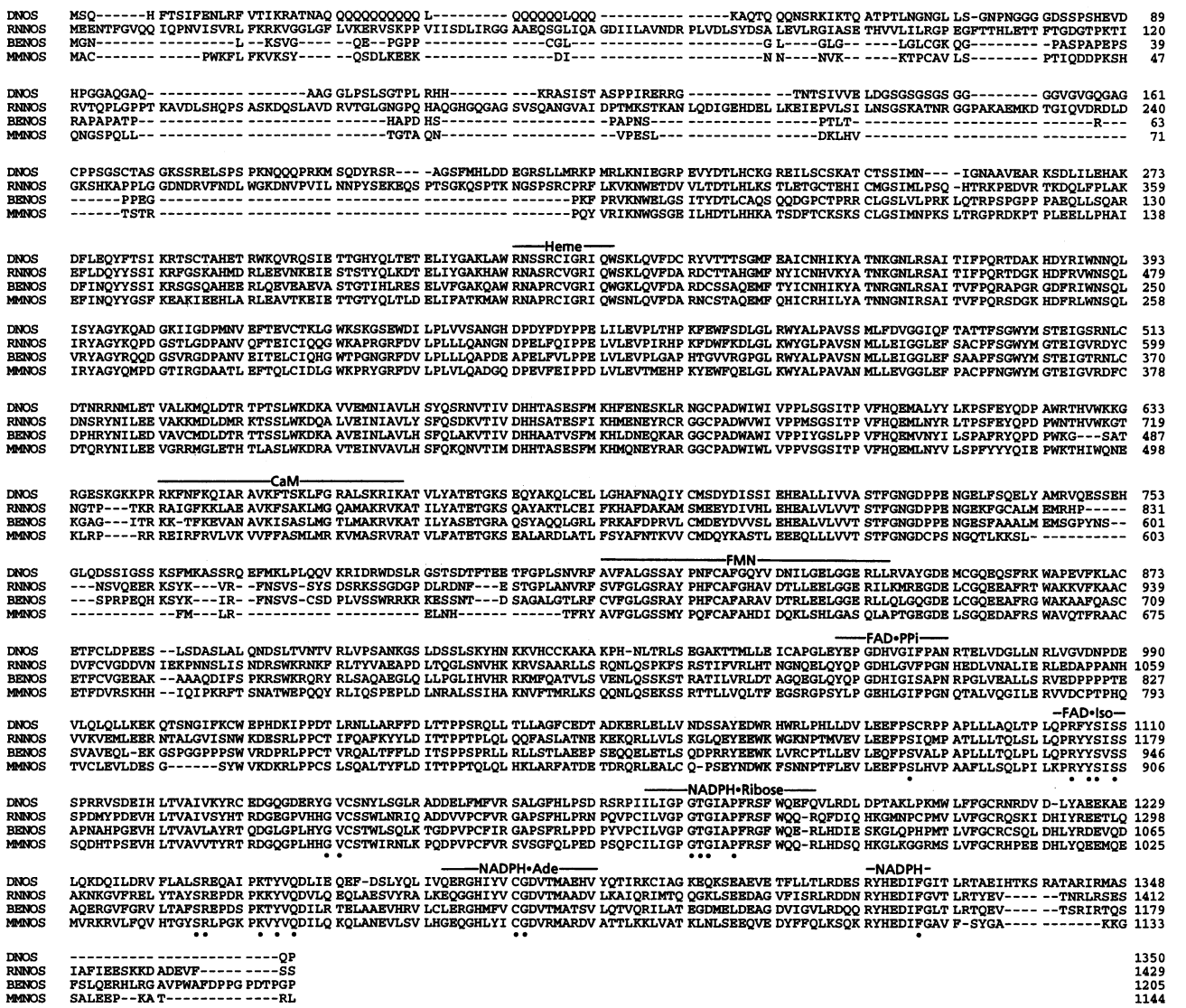

FIG. 1. Comparison of the deduced amino acid sequences of DNOS and mammalian NOSs. Numbering starts at the first methionine in each ORF. Putative cofactor-binding sites for heme, calmodulin (CaM), FMN, FAD pyrophosphate (FAD·PPi), FAD isoalloxazine (FAD·Iso), NADPH ribose (NADPH·Ribose), NADPH adenine (NADPH·Ade), and the C-terminal conserved sequence necessary for NADPH binding (33) are overlined. Amino acids that have been proposed as contacts with FAD and NADPH on the basis of the crystal structure of ferrodoxin–$NADP^+$ reductase (34) are conserved at equivalent positions (●). DNOS, *Drosophila* NOS; RNNOS, rat nNOS (3); BENOS, bovine endothelial NOS (5); and MMNOS, mouse macrophage NOS (6). Sequence alignment performed by GENEWORKS 2.3 (IntelliGenetics).

μl of L-[^{3}H]arginine (35.7 Ci/mmol; 1 Ci = 37 GBq; NEN), and 50 mM L-valine was incubated for 60 min at 37°C. The reaction was stopped and processed as described (28).

Detection of DNOS Protein in Cell Extracts. Anti-DNOS antibodies were raised against a glutathione *S*-transferase fusion protein containing the 97 N-terminal amino acids of the *dNOS* open reading frame (ORF) (0.29-kb *Eam*1105I–*Sac* I fragment of *dNOS* cDNA) expressed from pGEX-KG (29) in *Escherichia coli* strain BL21 (DE3) and purified over a glutathione-Sepharose column (Pharmacia) as described (30). Immunization of rabbits and serum preparation were done by Hazleton Research Products (Denver). SDS/7.5% PAGE (25 μg of protein per lane) and Western blots were done by following standard methods (25). The DNOS protein was detected by using a 1:500 dilution of rabbit serum and anti-rabbit alkaline phosphatase conjugate (Promega).

RNA Analysis. Heads and bodies of adult flies were separated on sieves. Total RNA was isolated as described (31). Poly(A)$^+$ RNA selection and Northern blot analysis [10 μg of poly(A)$^+$ RNA per lane] were done by following standard methods (25). The blot was hybridized with random-primed *dNOS* cDNA (10^6 cpm/ml), washed in 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 65°C and exposed to x-ray film for 72 h. Each reverse transcription–PCR (RT-PCR) reaction was started with 30 ng of poly(A)$^+$ head RNA, 90 ng of the bottom primer (nt 1793–1817 of *dNOS* cDNA) and 5 units of *rTth* DNA polymerase (Perkin–Elmer) in the MJ Research Minicycler as follows: 95°C for 1 min, 67°C for 45 sec, and 70°C for 13 min. The second stage, with 90 ng of the top primer (nt 1374–1399), was carried out for 35 cycles as follows: 94°C for 45 sec, 63°C for 45 sec, and 70°C for 90 sec. Products of the reaction were resolved on a denaturing 8% polyacrylamide gel, cloned, and sequenced.

RESULTS

Identification of *dNOS* cDNA. To identify candidate *Drosophila* NOS homologs, a fragment of the rat nNOS cDNA (3) was hybridized at low stringency to a phage library of the *Drosophila* genome. The rat cDNA fragment encoded the binding domains of FAD and NADPH (amino acids 979–1408), which are cofactors required for NOS activity and,

therefore, were expected to be conserved in fruit fly homologs. Several *Drosophila* genomic clones were identified by the rat probe and were classified into eight contigs. Sequence analysis of three restriction fragments from these genomic clones revealed one (2.4R) with high sequence similarity to mammalian NOSs. The deduced amino acid sequence of the ORF encoded within the 2.4R fragment indicated 40% sequence identity to the rat nNOS and binding sites for FAD and NADPH.

The 2.4R DNA fragment then was used to probe a *Drosophila* adult head cDNA library, and eight clones were isolated. Restriction analysis indicated that all contained identical inserts and, thus, defined a predominant transcript expressed by this *Drosophila* gene. One clone (c5.3) was sequenced in both directions. The 4491-bp cDNA contained one long ORF of 4050 bp. The methionine codon initiating this ORF was preceded by ACAAG, which is a good match to the translation start consensus sequence (A/CAAA/C) for *Drosophila* genes (32). Conceptual translation of this ORF yielded a protein of 1350 amino acids with a molecular weight of 151,842.

Primary Structure of DNOS Protein. Comparison of the amino acid sequence of this deduced *Drosophila* protein (DNOS) with sequences of mammalian NOSs revealed that DNOS is 43% identical to nNOS, 40% identical to endothelial NOS, and 39% identical to macrophage NOS. It also revealed similar structural motifs in DNOS (Fig. 1*A*). The C-terminal half of the DNOS protein contains regions of high similarity corresponding to the presumptive FMN-, FAD-, and NADPH-binding sites. Amino acids thought to be important for making contacts with FAD and NADPH in mammalian NOSs (6) are conserved in DNOS. The middle section of DNOS, between residues 215 and 746, showed the highest similarity to mammalian NOSs: it is 61% identical to the neuronal isoform and 53% identical to endothelial and macrophage isoforms. This region contains the presumptive heme- and calmodulin-binding sites in mammalian NOS enzymes. The heme-binding site was proposed to be centered around C-415 in the nNOS (35, 36), and this cysteine residue has been shown to be important for heme binding (37). The sequence surrounding the corresponding cysteine (C-329) in DNOS is well conserved. The region located between residues 643 and 671 has the characteristics of a calmodulin-binding domain (basic, amphiphilic α-helix) (38). The amino acid sequence between these two sites is very well conserved among all four NOS proteins, suggesting the location of functionally important domains, such as binding sites for arginine and tetrahydrobiopterine or a dimerization domain (39, 40). DNOS also has a protein kinase A consensus site (41) (at S-287) in a position similar to that in neuronal and endothelial NOSs (3, 4).

The 214-amino acid N-terminal region of DNOS shows no obvious similarity to its equivalent portion of nNOS or to the much shorter N-terminal domains of endothelial and macrophage NOSs. This region of DNOS contains an almost uninterrupted homopolymeric stretch of 24 glutamine residues. Such glutamine-rich domains, found in many *Drosophila* and vertebrate proteins, have been implicated in protein–protein interactions that regulate the activation of transcription (42, 43). Thus, this domain of DNOS might be involved with protein–protein interactions necessary for localization and/or regulation of DNOS activity.

The above sequence comparisons suggest that we have identified a *Drosophila* structural homolog of a vertebrate NOS gene. The order of the putative functional domains in the DNOS protein is identical to that of mammalian enzymes. Structural predictions based on several protein algorithms (Kyte-Doolittle and Garnier algorithms via GENEWORKS 2.3 software; IntelliGenetics) also indicate that general aspects of DNOS protein secondary structure (hydrophobicity plot, distribution of α-helixes and β-strands) from the putative heme-binding domain to the C terminus are similar to those of mammalian NOSs (data not shown). DNOS does not contain the internal deletion present in the macrophage NOS (6) (corresponding to residues 742–809 in DNOS), which suggests that it is more closely related to neuronal and endothelial NOSs. Similarly to the nNOS, DNOS has the N-terminal domain larger than that of other NOS proteins. Unlike the endothelial NOS (4, 5), DNOS does not have an N-terminal myristoylation site. Like all described mammalian NOSs, DNOS does not contain a transmembrane domain (as defined by Kyte-Doolittle).

DNOS Expressed in Cell Culture Has NOS Activity. To establish that our putative DNOS protein functions as a NOS, the *dNOS* cDNA was expressed transiently in human 293 embryonic kidney cells, which have been used routinely in studies of mammalian NOSs (3, 44). Protein extracts prepared from *dNOS*-transfected cells contained a 150-kDa polypeptide, which was recognized by a polyclonal antibody raised against the N-terminal domain of DNOS (Fig. 2*A*, lane 293 + dNOS). This immunoreactive polypeptide was the size expected for DNOS and was absent from cells transfected with the pCGN vector alone (lane 293 + vector).

Extracts made from *dNOS*-transfected human 293 embryonic kidney cells showed significant NOS activity, as measured by the conversion of L-arginine to L-citrulline (28) (0.1276 ± 0.002 pmol·mg^{-1}·min^{-1}; Fig. 2*B*, group B). This level of NOS activity was 20-fold higher than that of an extract of cells transfected with the pCGN vector alone (0.0062 ± 0.002 pmol·mg^{-1}·min^{-1}; Fig. 2*B*, group A). (In a parallel experiment, the specific activity of rat nNOS expressed from the same vector in human 293 embryonic kidney cells was 3.0 ± 0.02 pmol·mg^{-1}·min^{-1}; n = 4.) DNOS activity was dependent on exogenous Ca^{2+}/calmodulin and on NADPH, two cofactors

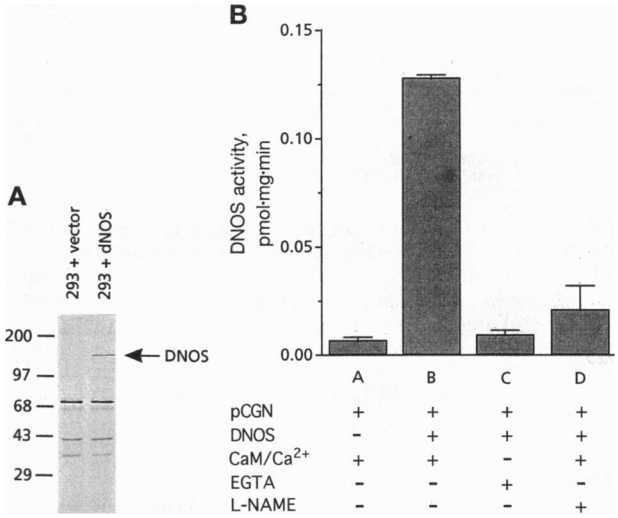

FIG. 2. Expression of DNOS enzymatic activity in human 293 embryonic kidney cells. (*A*) Western blot analysis of protein extracts from cells transfected with vector alone (lane 293 + vector) or with *dNOS* cDNA construct (lane 293 + dNOS). A total of 25 μg of soluble protein extract was resolved by electrophoresis through a 7.5% polyacrylamide gel, transferred to nitrocellulose membrane, and treated with anti-DNOS antibody. The arrow indicates the position of the DNOS protein. Positions of molecular mass markers (in kDa) are shown on the left. (*B*) DNOS enzyme activity measured by conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Enzymatic activity was detected in cells transfected with the *dNOS* cDNA construct (groups B–D) but not in cells transfected with pCGN vector alone. It is presented as specific activity (pmol of citrulline per mg·min^{-1}). DNOS activity also was measured in the presence of 1 mM EGTA without exogenous Ca^{2+} or calmodulin (CaM) (group C), or in the presence of 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (group D). n = 4 reactions per group.

necessary for activity of constitutive mammalian NOSs (28, 45). DNOS activity was reduced 90% by the Ca^{2+} chelator EGTA (Fig. 2*B*, group C). Also, 500 μM *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5), a calmodulin antagonist which inhibits activity of nNOS (28), diminished DNOS activity 82% (0.0222 ± 0.001 pmol·mg^{-1}·min^{-1}; n = 2) (data not shown). In the absence of exogenous NADPH, DNOS (or nNOS) activity was reduced 20% (0.1061 ± 0.011 pmol·mg^{-1}·min^{-1}; n = 4 for DNOS; 2.7935 ± 0.033 pmol·mg^{-1}·min^{-1}; n = 2 for nNOS). DNOS activity also was blocked by inhibitors of mammalian NOSs (46). N^{ω}-nitro-L-arginine methyl ester (L-NAME) reduced DNOS activity 84% (Fig. 2*B*, group D), and 100 μM N^{ω}-monomethyl-L-arginine acetate produced a complete block (0.0001 ± 0.0002 pmol·mg^{-1}·min^{-1}; n = 2) (data not shown). These enzymatic data demonstrate that DNOS is a Ca^{2+}/calmodulin-dependent NOS.

An Alternative Splicing Pattern of NOS Transcripts Is Conserved Between Vertebrates and Flies. Northern blot analysis indicated a 5.0-kb *dNOS* transcript that was expressed (preferentially) in adult fly heads but not bodies (Fig. 3*A*). More sensitive RT-PCR experiments, however, detected a similar *dNOS* message in poly(A)$^+$ RNA from fly bodies (data not shown). nNOS genes from mice and humans, however, have been shown recently to produce two alternatively spliced transcripts, the shorter of which yields a protein containing a 105-amino acid in-frame deletion (residues 504–608 in mouse or rat nNOS) (48). RT-PCR amplification of *Drosophila* head mRNA revealed two DNA fragments: a 444-bp fragment corresponding to the vertebrate long form and the 129-bp fragment corresponding to the vertebrate short form (Fig. 3*B*). Conceptual translation of the 129-bp sequence indicated a splicing pattern identical to that for the mammalian nNOS gene (Fig. 3*C*), thereby confirming the existence of a short NOS isoform in *Drosophila*.

DISCUSSION

Our discovery of a NOS homolog in *Drosophila* provides definitive proof that invertebrates produce NO and, as suggested by recent reports (see below), most likely use it for intercellular signaling. Our data also suggest that a NOS gene was present in an ancestor common to vertebrates and arthropods, implying that NOS has existed for at least 600 million years. Thus, we expect NOS genes to be prevalent throughout the animal kingdom.

Consistent with this view are existing histochemical data. NOS activity has been detected in several invertebrate tissue extracts, including *Drosophila* brain (49–53). Applications of NOS inhibitors or NO-generating substances have been shown to modulate the activity of buccal motoneurones in *Lymnaea stagnalis* (52) and the oscillatory dynamics of olfactory neurons in procerebral lobe of *Limax maximus* (54). NADPH-diaphorase staining, a relatively specific indicator of NOS protein in fixed vertebrate tissue samples (55, 56), also has suggested the presence of NOS in *Drosophila* brain (53). Our molecular cloning of *dNOS* considerably strengthens the notion that this NADPH-diaphorase staining reflects, at least in part, the presence of NOS.

Intriguingly, the presence of identical alternative transcripts for *dNOS* and nNOS genes suggests a conserved function. Presently, this putative *in vivo* function is unknown. A recombinant mouse molecule, which corresponds, in part, to the shorter splice form, does not show NOS activity in cell culture (57). Since NOS molecules must dimerize to function (40, 58), we speculate that the shorter splice form may block activity of the long form in a "dominant-negative" fashion through dimerization. Alternatively, since this molecule retains the NADPH-diaphorase activity (57), its function might be to create other products, like superoxide (59). Generation of transgenic flies carrying an inducible *dNOS* short-form may help resolve this issue.

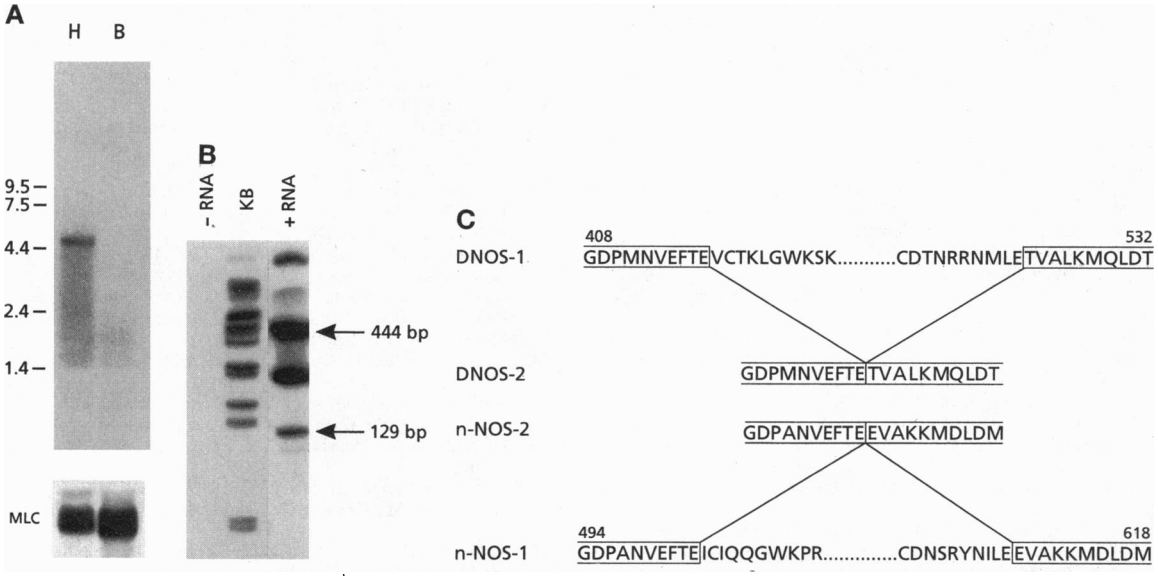

FIG. 3. *dNOS* transcripts in adult *Drosophila*. (*A*) Northern blot analysis of *dNOS* expression shows a 5.0-kb *dNOS* transcript present in heads. Each lane contained 10 μg of poly(A)$^+$ mRNA isolated from *Drosophila* heads (H) or bodies (B). The Northern blot was hybridized with the *dNOS* cDNA. Positions of size markers (in kb) are shown on the left. The blot was overprobed with a myosin light chain (MLC) probe (47) to normalize for RNA concentration. (*B*) The *dNOS* gene expresses two alternatively spliced mRNA species. RT-PCR reactions were performed on poly(A)$^+$ mRNA isolated from *Drosophila* heads, and the products were resolved on an 8% polyacrylamide gel. Arrows indicate the positions of DNA fragments of expected sizes: the 444-bp long-form fragment and the 129-bp short-form fragment (lane, + RNA). Other bands present in this lane are artifacts from heteroduplexes that failed to denature. Poly(A)$^+$ mRNA was omitted from the control reaction (lane, − RNA), which otherwise was done in identical conditions. Size markers (kb ladder) are shown in the middle lane (KB). (*C*) Alignment of deduced amino acid sequences of two protein isoforms of DNOS and mouse nNOS implies conservation of the splicing pattern between these two genes. The upper half of the figure shows the relation between two conceptual *Drosophila* NOS proteins, DNOS-1 and DNOS-2, corresponding to the longer and shorter RT-PCR products, respectively. The lower half of the figure shows the relationship between corresponding regions from two protein isoforms of the mouse nNOS, n-NOS-1 and n-NOS-2. Numbers indicate positions of amino acid residues relative to the first methionine in the respective ORFs.

Our data indicate that DNOS protein is a homolog of constitutively expressed vertebrate NOS enzymes which are regulated by transient changes in the concentration of intracellular Ca^{2+}. Further biochemical characterization of the DNOS protein may reveal closer functional homology to either the nNOS or the endothelial NOS. Preliminary data also suggest the presence of other NOS homologs in the *Drosophila* genome. Characterization of these candidate genes should contribute further insights to NOS function in fruit flies and to the evolution of NOS in vertebrates and invertebrates.

Identification of this well-conserved NOS gene in *Drosophila* is yet another example of the universality of molecular mechanisms involved with signaling and plasticity. Over the last 20 years, evidence has accumulated to reveal homologous genes involved in different aspects of neuronal plasticity between vertebrates and invertebrates. These include developmental plasticity in adults (60–62), synaptic plasticity (60, 63), and behavioral plasticity (64, 65). Recently, memory formation in fruit flies has been dissected genetically into component phases, just as has been done pharmacologically in vertebrates (66, 67). Molecular genetic experiments then demonstrated that long-term memory formation in *Drosophila* can be modulated by manipulating the expression of different isoforms of a cAMP-response-element-binding protein (CREB) transcription factor (68, 69). Overexpression of a repressor isoform disrupts long-term memory, while overexpression of the activator isoform enhances long-term memory. Similarly in mice, disruption of the *CREB* gene function blocks long-term memory (70). Thus, we anticipate that study of genetic disruptions of NOS function in fruit flies will confirm and extend our understanding of developmental and behavioral plasticity.

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