Nitric oxide (NO) is involved in vasodilation, neurotransmission, and immunity in mammals (see Ref. 1 for review). It is produced by a family of nitric-oxide synthases (NOS), which are encoded by three distinct genes in the mammalian genome. These genes are expressed throughout development as well as in the adult animal. Their transcriptional regulation is highly complex; a range of alternative promoters, splice sites, and polyadenylation signals is used to generate families of transcripts and proteins from each chromosomal gene (see Refs. 2–4 for review).

In Drosophila NO has been shown to be involved in imaginal disc development, synaptogenesis, formation of retinal projection pattern, response to hypoxia, and behavioral responses (see Ref. 5 for review). The gene for Drosophila NO synthase, dNOS, is located on the second chromosome at cytological position 32B (6). The major product of the gene, dNOS1, encodes a protein that bears a strong resemblance to all three NOS isoforms of mammals, with the highest homology to neuronal NOS (nNOS) (6). Several attempts to clone putative orthologs of mammalian endothelial or inducible forms of NOS (eNOS or iNOS) from various Drosophila cDNA or genomic libraries have not revealed any other NOS loci in the fruit fly’s genome.2 The recently released complete sequence of the Drosophila genome (7) has further confirmed our conclusion that the dNOS locus represents the only gene for NO synthase in Drosophila.

To explore the role of NO in fruit fly physiology and development, we sought to determine the structure of the Drosophila NOS gene to search for alternative transcripts and proteins and to investigate the role of possible DNOS isoform diversity in NO signaling. This paper analyzes the Drosophila NOS locus and identifies multiple transcripts that code for a family of DNOS proteins. dNOS mRNA isoforms are expressed throughout Drosophila development. We have found that some of these mRNAs encode truncated DNOS polypeptides, and we have gone on to show that these truncated DNOS proteins can form heterodimers with the full-length DNOS1 and strongly inhibit its enzymatic activity when co-expressed in cultured cells. Together, our observations suggest that the diversity of products encoded by the dNOS gene may have a direct impact on NO activity and NO signaling in Drosophila.

EXPERIMENTAL PROCEDURES

Isolation of the dNOS Genomic Clones—A Drosophila ADASH genomic library (5 × 10^6 plaques) was screened with probes corresponding to different regions of the dNOS1 cDNA (6). The four longest overlapping phage clones covering the entire dNOS gene were isolated and mapped using HindIII and EcoRI restriction enzymes (New England Biolabs). Restriction fragments were subcloned into plBluescript II KS (Stratagene) and sequenced by the Cold Spring Harbor Laboratory DNA sequencing facility. Phage purification, hybridization, and cloning steps were performed using the standard methods (8).

Isolation of RNA Samples for Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Experiments—Drosophila embryos from 0 to 24 h after fertilization were collected together as described previously (9), dechoronated with 50% bleach, and subjected to total RNA isolation.

Yuri Stasiv, Michael Regulski, Boris Kuzin, Tim Tully, and Grigori Enikolopov

From Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received for publication, June 1, 2001, and in revised form, August 21, 2001

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
tion using TRIZol reagent (Life Technologies). Larvae enriched in 3rd instar were collected essentially as described (9). Total RNA from larvae and adult flies was isolated with TRIZol. RNA samples were treated with DNase I (Roche Molecular Biochemicals) to remove traces of genomic DNA. Poly(A)⁺ RNA was isolated using oligo(dT)-cellulose chromatography (Pharmacia Biotech). The obtained RNA samples were amplified by RT-PCR and primers from exons 12 (sense, 5'-ACTTGGCTGAAAGGCCGACG-3' and 5'-AGCCGAGCAGTGCCTCCACTTTGCTCC-3' and antisense, 5'-CAATCCATGCTCGGAAGACTC-3') and an antisense oligonucleotide 5'-TTTCCCTAATAGCCGCGAAGGAGG-3' (nested for exon 1b) to the sequence of either the influenza virus hemagglutinin (HA) epitope (dNOS1-HA and dNOS3-HA constructs) or the FLAG epitope (dNOS4-FLAG, dNOS5-FLAG, and dNOS6-FLAG constructs) followed by a stop codon. These were subcloned into the mammalian expression vector pCG, which uses a strong cytomegalovirus promoter to direct transcription of the transgene (12). 293 embryonic human kidney cells (293 cells) were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% calf serum (Life Technologies) in the presence of 100 units/ml penicillin and 100 units/ml streptomycin. The plasmids were transfected into 293 cells using lipofectamine (Life Technologies). The same antibody was used to detect the expression of the transgene product. The 150 μl reaction mixture containing 20 μl (20–50 μg) of soluble protein extract, 50 μl Hepes (pH 7.4) 2 mM MgCl₂, 2.5 μM L-arginine, 1 mM NADPH, 20 μM tetrahydro-L-biotin, 10 μg/ml calmodulin (CaM), 2 μl of L-D-histidine (2.04 TBq/mmol, 55.0 Ci/mmol) was incubated for 30 min at 25 °C. The reaction was stopped and processed to determine the extent of conversion of [L-3H]arginine to [L-3H]citrulline. Protein concentration in the extracts was determined using BCA reagent system (Pierce), and the results were used to normalize the assays. To compare the expression of the dNOS1-HA construct across the co-transfection experiments, equal amounts of protein extracts from the transfected cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting as described (14) using the 12CA5 monoclonal antibody (15) against HA epitope at a final concentration of 10 μg/ml. The same antibody was used to detect the expression of the dNOS1-HA construct. The M2 monoclonal antibody against FLAG epitope (Stratagene) was used at a final concentration of 1 μg/ml to check the expression levels of dNOS4-FLAG, dNOS5-FLAG, and dNOS6-FLAG constructs. Immunoblots were developed using the ECL detection system software (version 1.7, Applied Biosystems). Standard curves for the 10-fold serial dilutions of each examined ampiclon were obtained using the above quantitative PCR approach. The resulting experimental data for each transcript were plotted on a corresponding standard curve to determine the amount of the specific dNOS isoform in a given RNA sample.

**Transfection, NOS Activity Assay, and Immunoblotting—Expression constructs for enzymatic activity assays contained the protein-coding regions of interest flanked by SpeI and EcoRV sites.** The expression plasmids were transfected into embryonic 293 cells using lipofectamine (Life Technologies). The same antibody was used to detect the expression of the transgene product. The 150 μl reaction mixture containing 20 μl (20–50 μg) of soluble protein extract, 50 μl Hepes (pH 7.4) 2 mM MgCl₂, 2.5 μM L-arginine, 1 mM NADPH, 20 μM tetrahydro-L-biotin, 10 μg/ml calmodulin (CaM), 2 μl of L-D-histidine (2.04 TBq/mmol, 55.0 Ci/mmol) was incubated for 30 min at 25 °C. The reaction was stopped and processed to determine the extent of conversion of [L-3H]arginine to [L-3H]citrulline. Protein concentration in the extracts was determined using BCA reagent system (Pierce), and the results were used to normalize the assays. To compare the expression of the dNOS1-HA construct across the co-transfection experiments, equal amounts of protein extracts from the transfected cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting as described (14) using the 12CA5 monoclonal antibody (15) against HA epitope at a final concentration of 10 μg/ml. The same antibody was used to detect the expression of the dNOS1-HA construct. The M2 monoclonal antibody against FLAG epitope (Stratagene) was used at a final concentration of 1 μg/ml to check the expression levels of dNOS4-FLAG, dNOS5-FLAG, and dNOS6-FLAG constructs. Immunoblots were developed using the ECL detection system software (version 1.7, Applied Biosystems). Standard curves for the 10-fold serial dilutions of each examined ampiclon were obtained using the above quantitative PCR approach. The resulting experimental data for each transcript were plotted on a corresponding standard curve to determine the amount of the specific dNOS isoform in a given RNA sample.

**Transfection, NOS Activity Assay, and Immunoblotting—Expression constructs for enzymatic activity assays contained the protein-coding regions of interest flanked by SpeI and EcoRV sites.** The expression plasmids were transfected into embryonic 293 cells using lipofectamine (Life Technologies). The same antibody was used to detect the expression of the transgene product. The 150 μl reaction mixture containing 20 μl (20–50 μg) of soluble protein extract, 50 μl Hepes (pH 7.4) 2 mM MgCl₂, 2.5 μM L-arginine, 1 mM NADPH, 20 μM tetrahydro-L-biotin, 10 μg/ml calmodulin (CaM), 2 μl of L-D-histidine (2.04 TBq/mmol, 55.0 Ci/mmol) was incubated for 30 min at 25 °C. The reaction was stopped and processed to determine the extent of conversion of [L-3H]arginine to [L-3H]citrulline. Protein concentration in the extracts was determined using BCA reagent system (Pierce), and the results were used to normalize the assays.
translated DNA or cDNAs from various developmental stages. After we deposited the DNA or cDNAs, we performed 1 h at room temperature, either 20 µl of the anti-FLAG M2-agarose affinity gel or 25 µl of the protein G-Sepharose beads, which were pre-absorbed for 1 h with 10 µl of the HA polyclonal antibody (Upstate Biotechnology), were added to the duplicate samples. After a further incubation for 3 h at room temperature, bound immune complexes were washed three times with a buffer containing 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl. Immunoprecipitated proteins were eluted from the agarose or Sepharose beads by boiling and then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS
Isolation of the Drosophila Nitric-oxide Synthase Gene—We used the dNOS1 hybridization probe to isolate several overlapping bacteriophage clones from a Drosophila ADASH genomic library. These clones encompass a continuous 50-kilobase (kb) region of genomic DNA and contain the entire dNOS1 coding sequence (Fig. 1). Four overlapping genomic clones were mapped, and their restriction maps were found to correspond to the maps of the dNOS gene in the Drosophila genome as determined by probing Southern blots with dNOS1 cDNA fragments. Drosophila genomic blots were hybridized under both high and low stringency conditions, and no other NOS-like genes were revealed (data not shown). We searched for other NOS-related genes in Drosophila using PCR with degenerate primers corresponding to a variety of conserved regions of mammalian NOS cDNAs (data not shown). This search failed to identify novel NOS-related genes when tested on genomic DNA or cDNAs from various developmental stages. After we deposited the dNOS gene sequence into the GenBank™ data base, the total Drosophila genome sequence was released (7), analysis of which confirmed our conclusion that there is only one chromosomal locus encoding dNOS.

The major functional product of the dNOS gene, the full-length protein DNS1, reveals structural motifs similar to those of mammalian NOS enzymes (6). It consists of an oxygenase domain at the amino terminus and a reductase domain at the carboxyl terminus, connected by a CaM binding region.

Structural Organization of the Drosophila Nitric-oxide Synthase Gene—We mapped the exons in genomic DNA and determined precise exon-intron boundaries by hybridization and sequencing of the overlapping genomic subclones using oligonucleotides from different parts of the dNOS1 cDNA. Introns were also sequenced in their entirety except for introns 1b, 3, 5–11, and 14. Our estimates of intron sizes (based on restriction mapping, Southern blot analysis, and PCR amplification of genomic DNA) (Table I) were confirmed by analysis of the complete Drosophila genome sequence (7). The dNOS gene is composed of 19 exons dispersed over 34 kb of Drosophila genomic DNA. The location and size of exons and introns are shown in Fig. 1 and in Table I. Exons range in size from 67 to 1142 base pairs (bp), and introns range from 0.1 to 6.0 kb. The exon-intron boundaries of the dNOS gene are shown in Table II. All of them conform to the known GT/AG donor/acceptor rule (16). Translation initiation and termination sites for the dNOS1 are located in exons 2 and 19, respectively. The potential polyadenylation signals AATAAA were found 148 nucleotides (nt) and 226 nt downstream of the stop codon TAA in exon 19. The distribution of the coding sequences among dNOS exons reveals a striking similarity with the human nNOS gene as well as with the mosquito Anopheles stephensi NOS (AsNOS) gene (17, 18). Seven dNOS exons (exons 5–9 and 12–13) are identical in size to exons 6–10 and 14–15, respectively, of the human nNOS gene and are followed by introns of the same type (as determined by the position of a splice junction within a codon) (Table I). Similarly, seven dNOS exons (exons 4–7, 10, 12, and 13) have the same size as the homologous mosquito AsNOS exons (exons 2–5, 7, 10, and 11, respectively), with the intron type being conserved as well (Table I).

Evolutionary conservation is found mainly in the region extending from the heme-binding site to beyond the CaM-binding site. This region in mammalian nNOS is responsible for homodimerization and for catalytic conversion of L-arginine into L-citrulline and NO (19). In contrast, much of the reductase domain of the Drosophila enzyme is encoded by a single 1142-nt-long exon 16 (Table I), whereas the homologous region in the human nNOS gene is dispersed among eight exons (exons 19–26).

Identification of Multiple Alternatively Spliced Transcripts of the dNOS Gene—In the original report on the cloning of the dNOS cDNA, two transcripts, dNOS1 and dNOS2, were identified (6). dNOS1 encodes a full-length protein, whereas dNOS2 has an in-frame deletion of 315 nt. To investigate the variety of alternative isoforms of dNOS mRNA, we screened cDNA libraries from Drosophila adult heads and larvae and performed 5′-RACE with RNA from embryos, larvae, and adult flies. This search resulted in identification of eight novel dNOS transcripts, which differ both in their 5′-untranslated regions (UTRs) and in their coding regions. We have termed these isoforms dNOS3–dNOS10.

We observed four different 5′-ends of the dNOS transcripts (Fig. 2A), designated as exons 1a, 1b, 1c, and 1d. Each variant of exon 1 splices to the common exon 2, which contains the translation initiation codon ATG (preceded by stop codons in all three translation reading frames). None of these alternative exons contain the optimal consensus sequence for the initiation of translation, suggesting that all dNOS isoforms encode proteins with identical amino termini.
Transcriptional Complexity of the Drosophila NOS Gene

Intron type O indicates a splice junction between codons, type I indicates a splice junction after the first nucleotide of a codon, and type II indicates a splice junction after the second nucleotide. Amino acids with interrupted codons were assigned to the exon containing two of the three nucleotides.

### TABLE I

| Exon | Size | Amino acids | Features | Intron | Size | Type |
|------|------|-------------|----------|--------|------|------|
| 1a   | 343  | 5′-UTR, dNOS7 | —        | 1a     | 6.0  |      |
| 1b   | 232  | 5′-UTR, dNOS1, dNOS4- dNOS6 | —        | 1b     | 5.2  |      |
| 1c   | 254  | 5′-UTR, dNOS8 | —        | 1c     | 5.3  |      |
| 1d   | 298  | 5′-UTR, dNOS2 | —        | 1d     | —    | —    |

Two splice junctions are found in intron 1. Exon 2 is 201 nt long, and it is spliced in the common exon 2 (Fig. 2B). The 5′-end of dNOS3, residues 24 (Q 11LQ6LQ3KAQTQ3), but lacks the entire reductase domain of the full-length DNOS1 as 1d, starts 298 nt upstream of exon 2 and continues without splicing into the common exon 2 (Fig. 2B). All of the presumptive donor/acceptor splice sites conformed to the GT/AG consensus (Table II). Numerous attempts to extend the various cDNA sequences farther upstream using 5′-RACE with the series of primers specific to different variants of exon 1 did not identify any novel 5′-ends other than those described above. This indicates that these exons may represent true transcription initiation sites, suggesting that the dNOS gene may contain at least four alternative promoters. Importantly, the variations in exon 1 do not appear to affect the amino termini of the encoded proteins.

Furthermore, alternative splicing of the primary transcript changes the coding capacity of several dNOS isoforms. These changes include single and multiple exon deletions, single and multiple exon insertions, and alternative usage of splicing sites within an exon (Fig. 3B).

Isomorf dNOS3 was isolated from a Drosophila 3rd instar larvae cDNA library. It is an example of a single exon deletion within the protein-coding region; dNOS3 lacks the 115-nt-long exon 3 (Fig. 3B). This creates a frameshift in the mRNA and introduces a stop codon TGA 40 nt downstream of the new splice junction between exons 2 and 4. The resulting open reading frame (ORF) is 642 nt long (each of three cDNA clones examined contained the entire ORF of dNOS3 plus the 5′- and 3′-UTRs) and codes for a protein of 214 amino acids (aa) with a calculated molecular mass of 23 kDa. The first 200 aa of DNOS3 are identical to those of DNOS1, and they are followed by 14 aa (RFPARRRPPVARAL) unique to DNOS3 at the carboxyl terminus. The DNOS3 protein contains the glutamine (Gln)-rich sequence of DNOS1, residues 24–52 (Q11LQ6LQ3KAQTQ3), but lacks the entire reductase domain of the full-length DNOS1 as...
well as the heme and CaM binding regions (Fig. 3, A and B), which have been shown to be crucial for dimer formation and NOS catalytic activity (see Ref. 20 for review). The dNOS3 RNA was detected in larvae only.

Isoform dNOS4, identified after RT-PCR of Drosophila larval RNA, is an example of an intron retention or exon insertion. A 109-nt-long intron 13 (designated as exon 14a) is not excised from the primary transcript (Fig. 3B). The preceding ORF continues for 63 nt into exon 14a until it is terminated by a TAA stop codon. Using RT-PCR we cloned the dNOS4 cDNA that extends from exon 1b to exon 19. This transcript has an ORF of 2271 nt, and it encodes a protein of 757 aa with a calculated molecular mass of 84 kDa. This truncated protein contains the heme- and CaM-binding sites but lacks the entire reductase domain (Fig. 3, A and B). The first 736 aa of DNO4 are identical to those of DNO1; in addition, DNO4 carries a unique 21-aa peptide (encoded by exon 14a) at the carboxyl terminus (VSTPPKDHELINGLGPAAF). The dNOS4 transcript is expressed throughout Drosophila development in embryo, larva, and imago (adult fly) (Fig. 3C).

The dNOS5 and dNOS6 transcripts were initially identified by RT-PCR of embryonic RNA. We cloned the cDNA for both isoforms using RT-PCR with primers from exons 1b and 19. Both transcripts contain exon 14a as does dNOS4. In addition, each of them has an insertion of an alternative exon into the coding region between exons 12 and 13. The entire 732-nt-long intron 12 (now designated as exon 13a) is retained in the dNOS5 transcript (Fig. 3B). The preceding ORF is extended by 12 nt into exon 13a before it terminates with a TGA codon. The dNOS5 ORF is 2115 nt long and encodes a protein of 705 aa (predicted molecular mass of 79 kDa) that carries a unique carboxyl-terminal peptide (VSSTTPKDHELINGLGPAAF). Similar to dNOS4, both DNOS5 and DNOS6 proteins are truncated immediately after the CaM-binding site and, thus, do not possess the reductase domain (Fig. 3, A and B). dNOS5 and dNOS6 transcripts are expressed during all Drosophila developmental stages examined (Fig. 3C).

Another alternative transcript, dNOS7, was only found in RNA samples from late larvae. The ORF of dNOS7 is identical to the above-described ORF of dNOS4. However, these two transcripts utilize different variants of the non-coding exon 1, exon 1a in the dNOS7 sequence versus exon 1b in the dNOS4. We did not find dNOS7 RNA in embryos and adult flies (Fig. 2B). Exon 1a is apparently present in other dNOS transcripts as well, because we were able to detect expression of RNA(s) containing exon 1a, which is spliced to exon 2, during all Drosophila developmental stages examined (Fig. 2B).

The dNOS8, which uniquely contains exon 1c, was found in larval RNA. Although we did not identify an ORF for the dNOS8 isoform, we did not find exon 1c in any of the dNOS transcripts with splicing alterations within the coding region, suggesting that the dNOS8 transcript may encode the full-length protein.

Similar to the transcripts dNOS4, -5, -6, and -7, the splice variant dNOS9 contains exon 14a. However, in addition to the insertion of exon 14a, it also has a 1602-nt-long cassette deletion of three exons (exons 15–17) (Fig. 3B). We detected dNOS9 transcripts in RNA samples from Drosophila embryos and larvae but not adult flies. dNOS9-specific RT-PCR product (171 nt long) contained exons 14a, 14, and 18 only (Fig. 3D). We were unable to detect the above deletion of exons 15–17 by RT-PCR using a sense primer from exon 13b (dNOS8- and dNOS6-specific) in conjunction with an antisense primer from exon 18 (Fig. 3D). Moreover, RT-PCR using an antisense oligonucleotide from exon 14a and sense oligonucleotides from different exon 1 variants did not reveal any additional dNOS RNA isoforms (data not shown). Therefore, even though we did not isolate the entire protein-coding region of the dNOS9 transcript, we assume that the ORF of dNOS9 is identical to that of dNOS4 or dNOS7.

An identical cassette deletion of exons 15, 16, and 17 was found in the dNOS10 transcript (Fig. 3B). The amplified 266-bp-long RT-PCR product (Fig. 3D) included exons 12, 13, 14, and 18 but not exon 14a as did dNOS9. Deletion of the 1602 nt did not cause a frameshift in the dNOS10 protein-coding sequence. Although we did not clone the full-length dNOS10 cDNA, RT-PCR data suggest that the remaining dNOS10-coding region (upstream of exon 12) is identical to the corresponding part of dNOS1. Thus, the resulting dNOS10 ORF would encode a protein of 816 aa with a predicted molecular mass of 91 kDa. DNOS10 is shorter than the full-length Drosophila...
DNOS1 by 534 aa. It lacks all of the residues from position 760 to position 1293 of DNOS1; thus, DNOS10 does not possess a significant portion of the reductase domain including FMN-, FAD-, and part of the NADPH-binding sites. The splice junction site between exons 14 and 15 in the dNOS1 mRNA is located after the second nucleotide in serine 759 AGC codon. In the dNOS10 transcript this codon is mutated to AGG (encodes arginine 759) due to the direct splicing of exon 14 to exon 18. The remaining 815 aa of DNOS10 are identical to the 758 amino-terminal and 57 carboxyl-terminal residues of the
DNOS1. The dNOS10 transcript is expressed throughout Drosophila development (Fig. 3D).

Finally, we have characterized a 315-nt-long in-frame deletion in the previously described dNOS2 RNA. It is due to the removal of exons 8 and 9 from the primary dNOS transcript (Fig. 3B). dNOS2 encodes a protein that lacks 105 aa in the region between the heme binding and CaM binding sites (6).

Thus, the dNOS gene generates a family of transcripts that code for proteins ranging in length from 214 (DNOS3) to 1350 (DNOS10) aa. Most of these proteins lack the domains essential for NOS activity and may lack the ability to produce NO.

**dNOS Expression during Drosophila Development**—To investigate the expression of the dNOS gene in developing Drosophila, we used in situ hybridization and quantitative real-time RT-PCR. dNOS transcripts were not detectable by in situ hybridization in early embryos (although they were detected by RT-PCR in unstaged embryos). However, dNOS transcripts are abundantly present in the late larvae and early pupae. Fig. 4 shows in situ hybridization of the antisense dNOS probe (this probe recognizes all of the transcripts except dNOS9 and dNOS10) with imaginal discs from late 3rd instar larvae. In the second leg imaginal disc the most intense hybridization signal is seen in the tarsal segments with lower signal intensity in tibia, femur, and coxa. In the eye imaginal disc the signal is seen anterior to the morphogenetic furrow. In the wing disc a strong signal is seen in the regions of the dorsal and ventral hinges. Structurally, many of the specific dNOS RNA isoforms overlap each other, and this precludes the use of isoform-specific probes for in situ hybridization. However, it is possible to design specific primers for the detection of several individual dNOS isoforms by RT-PCR. We used a quantitative fluorescent approach to estimate the relative abundance of selected dNOS transcripts in embryo, larva, and imago. Calibration experiments demonstrated that real-time PCR correctly represents the differences between the samples with serial dilutions of dNOS template for each pair of primers used (data not shown). The sensitivity of this method was very high, enabling us to detect as little as a few cDNA molecules per sample. Extensive overlap between dNOS RNA isoforms prevents analysis of each individual RNA species (for instance, exon 14a is present in five different dNOS isoforms, see Fig. 3B). However, it is possible to estimate the combined expression level of all dNOS transcripts and individual content of the dNOS5 isoform. dNOS1 appeared to be the predominant species. It was expressed at high levels in embryos and imagos, with 4-fold lower levels in larvae (Fig. 5). Levels of the dNOS5 transcript in embryos were 1.5- and 3-fold higher than its levels in imagos and larvae, respectively (Fig. 5). Results obtained with quantitative RT-PCR support the notion of differential expression of individual NOS transcripts in developing Drosophila.

**Inhibition of the DNOS1 Enzymatic Activity by Truncated DNOS Isoforms**—Several of the newly identified transcripts of the dNOS gene encode proteins that are truncated at the carboxyl terminus in comparison to the DNOS1. These proteins (DNOS3, DNOS4, DNOS5, and DNOS6) lack the entire reduction domain, which is crucial for NO synthesis. However, they retain the amino-terminal portion of the protein, implicated in dimer formation in mammalian forms of NOS (except for the DNOS3, which lacks the heme binding region of the oxygenase domain and the CaM binding region) (see Ref. 20 for review). In addition, all of them contain a Gln-rich region. Similar Gln-rich domains have been shown to contribute to protein-protein interactions (21). This suggests that the truncated proteins may be incapable of producing NO but may be capable of forming complexes, e.g., heterodimers with the enzymatically active isoform DNOS1. Because the activity of NOS depends on homodimerization, we hypothesized that co-expressing DNOS1 with truncated DNOS isoforms may result in inhibition of NOS activity. Therefore, we generated constructs in which the cytomegalovirus promoter was used to drive expression of different DNOS isoforms with short peptide tags fused to their carboxyl termini (HA in case of DNOS1 and DNOS3 and FLAG in case of DNOS4-DNOS6) (Fig. 6A).

The dNOS1-HA construct was transfected into 293 cells, which have undetectable levels of the endogenous NOS activity. Cell-free extracts from the transfected cells were tested in the arginine-citrulline conversion assay to measure NOS activity. As expected, extracts from dNOS1-HA-transfected cells had substantial levels of enzymatic activity (constructs with or without the HA-tag yielded identical activities, data not shown), whereas extracts from cells transfected with the ex-
pression vector alone had negligible background NOS activity. When the truncated variants \textit{dNOS3}, \textit{dNOS4}, \textit{dNOS5}, and \textit{dNOS6} were individually transfected into 293 cells, no NOS activity was detectable (data not shown). In three independent experiments, when either \textit{dNOS4-FLAG}, \textit{dNOS5-FLAG}, or \textit{dNOS6-FLAG} was co-transfected with the \textit{dNOS1-HA} construct, the enzymatic activity of DNOS1 was strongly inhibited, to 5–15% of the original level (Fig. 6B). In contrast, co-transfected \textit{dNOS3-HA} (Fig. 6B) or unrelated control constructs alone was not detectable (data not shown). For the detection of expressed DNOS isoforms an equal amount of total protein (50 µg/lane) from cells transfected with different \textit{dnos} constructs was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using either HA antibody or FLAG antibody. The results are representative of three independent experiments.

Co-immunoprecipitation of Full-length DNOS1 and Truncated DNOS Proteins—The oxygenase domain of various NOS proteins contains the sequences responsible for the dimerization of the NOS polypeptide. To examine whether truncated DNOS proteins can form heterodimers with the enzymatically active full-length DNOS1, we co-expressed constructs coding for HA-tagged DNOS1 and for FLAG-tagged DNOS4, DNOS5, or DNOS6 (each of which retains the oxygenase domain) in 293 cells. We then used either the anti-HA or anti-FLAG antibodies for immunoprecipitation followed by immunoblotting to detect protein-protein interactions.

When HA-tagged DNOS1 is expressed alone it is immunoprecipitated by the anti-HA but not by the anti-FLAG antibody. Conversely, FLAG-tagged DNOS4, DNOS5, and DNOS6 can be immunoprecipitated by the anti-FLAG but not by the anti-HA antibody. However, if the full-length DNOS1-HA is co-expressed with DNOS4-FLAG, DNOS5-FLAG, or DNOS6-FLAG, it can be immunoprecipitated by the anti-FLAG antibody (Fig. 7), indicating that the full-length and truncated DNOS proteins form complexes. Conversely, FLAG-tagged truncated DNOS proteins can be immunoprecipitated by the anti-HA antibody if they are co-expressed with DNOS1-HA. These re-
The organization of the region that codes for the Drosophila nNOS domain is also highly similar to the organization of the oxygenase-coding region of the mosquito A. stephensi NOS gene (18). Seven of the dNOS exons are identical in size and intron type to the corresponding AsNOS exons (Table I). Exon 11 of the dNOS gene appears to be composed of two homologous exons (exons 8 and 9) in the mosquito gene, whereas two dNOS exons (exons 8 and 9) correspond to exon 6 of the AsNOS gene (Table I). The overall homology between the fly and mosquito proteins is 81% (with 69% of the aa identical), whereas the homology within oxygenase domains and CaM-binding sites reaches 88% (with 78% of the aa identical). Interestingly, there is no exon-intron conservation between these two insect NOS genes in their reductase domains. The structural similarity between the mosquito AsNOS and the human nNOS genes within their reductase-coding regions (17, 18) is higher than similarity between corresponding regions of the dNOS gene and its human counterpart (Table I). However, additional studies of NOS genes from various invertebrate and vertebrate species are needed to better characterize the evolution of NOS gene.

The dNOS gene demonstrates a remarkable degree of transcriptional complexity resembling that of mammalian nNOS (for review, see Ref. 2). Alternative transcription initiation sites combined with the alternative usage of splice sites generated a family of dNOS transcripts. We found four alternative variants of the first non-coding exon: 1a, 1b, 1c, and 1d. All of them are located within the 6-kb-long 5′-region of the dNOS gene upstream of exon 2. Most likely these exons begin at alternative transcription start sites; using 5′-RACE, we were unable to extend these exons farther upstream, and using RT-PCR, we were unable to detect dNOS transcripts in which exon 1 variants are spliced to each other. Exon 1b is common to several dNOS transcripts (dNOS1, 4, 5, and 6), whereas exons 1a, 1c, and 1d are present in the dNOS7, dNOS8, and dNOS3 isoforms, respectively. The fact that dNOS3 and dNOS8 transcripts are expressed only during the larval stage of Drosophila development (Fig. 2B) further supports the notion that alternative promoters are used to direct expression of the different dNOS RNAs in a tissue- and/or development-specific manner.

This mechanism of NOS regulation seems to be evolutionary conserved; similarly, complex splicing patterns were found in the 5′-regions of mammalian nNOS genes. For instance, in the human nNOS gene, nine differentially expressed variants of non-coding exon 1 were identified (31). It is unclear, though, whether these human nNOS transcripts have additional structural alterations (frameshifts, in-frame deletions, or insertions) within their coding region, similar to those we have found in the dNOS transcripts.

Alternative splicing affects the coding region as well as the 5′-UTR of the dNOS gene. Transcript dNOS2 is an example of a deleted coding exon (exon 3). This deletion leads to a frameshift and premature termination of the dNOS3 ORF. A transcript similar to the dNOS3 was found in the mosquito A. stephensi, where deletion of exon 2 (translation start codon in the AsNOS is located in exon 1) causes a premature termination of the ORF in the exon 2 mRNA (32).

Another transcript, dNOS10, has a novel type cassette in-
frame deletion of three consecutive exons (exons 15–17) that composes almost 40% of the dNOS-coding region. Thus, DNOS10 protein retains only 57 carboxyl-terminal aa of the reductase domain. No splicing alterations in this part of NOS gene have been found in other organisms. Several dNOS transcripts arise due to the insertion of extra exon(s) in their coding regions, resulting in the premature termination of their ORFs. The dNOS4 and dNOS7 transcripts contain an alternative exon 14a, whereas dNOS5 and dNOS6 RNAs have two extra exons, either 13a plus 14a or 13b plus 14a, respectively. Similar types of exon insertions that introduce premature stop codons have been found in mammalian NOS genes. Two alternative transcripts arise from the human nNOS primary transcript after the unusual splicing of intron 16 (33). In the nNOS+47 RNA, the first 47 nt at the very 5′-end of intron 16 are inserted into the coding region between exons 16 and 17. The nNOS+67 transcript has an extra 67 nt (derived from the central part of intron 16) inserted between exons 16 and 17. Both insertions introduce an in-frame stop codon. Although exon 16 of the human NOS gene is homologous to exon 14 of the dNOS gene (Table I), we were unable to detect any dNOS RNA species with insertions of an extra exon(s) downstream of exon 14. Thus, the fruit fly most likely does not have an RNA isoform corresponding to the mammalian nNOSµ transcript, which contains an in-frame 102-nt-long insertion between exons 16 and 17 and is expressed in various rat and human tissues (33, 34).

Alternative splicing of the mosquito AsNOS pre-mRNA causes an insertion of additional exon (174 nt long) between exons 11 and 12 in the exon 11′ transcript (32). An alternative AsNOS exon contains a stop codon, which results in a premature termination of translation 16 aa downstream of the novel splice junction. This product of the AsNOS gene resembles the dNOS4 transcript (Table I). Finally, the previously described transcript dNOS2 (6) has an in-frame cassette deletion of exons 8 and 9 (Table I) that is identical to the deletion of exons 9 and 10 found in the alternative transcript nNOS-2 of the human NOS gene (35).

Individual dNOS isoforms are differentially expressed in the developing Drosophila according to quantitative RT-PCR results. Although dNOS1 is a predominant RNA product in embryo, larva, and imago (Fig. 5), it is important to note that these experiments were performed with RNA pools representing various phases of each developmental stage. It is possible that individual dNOS transcripts are transiently induced at selected steps of the developmental cascade (e.g. before pupariation, when a strong increase in diaphorase staining is observed (22)).

The family of dNOS transcripts encodes a variety of DNOS-like proteins. Seven of them, DNOS3, -4, -5, -6, -7, -9, and -10 (but not DNOS2), lack either part of or the entire reductase domain, which leads to a loss of enzymatic activity. However, most of the truncated DNOS proteins (except for DNOS2 and DNOS3) retain almost the entire oxygenase domain, including the sites that are thought to be responsible for NOS homodimerization in mammalian cells. Thus, these truncated forms lack NOS enzymatic activity but may still retain their ability to dimerize. This notion is supported by our experiments, in which we were able to detect interaction between GAL4-DNOS4 hybrid proteins (DNOS4 was fused to GAL4 binding and to GAL4 activation domain) using a yeast two-hybrid system (data not shown). Moreover, this implies that the truncated DNOS proteins may not only form dimers with each other but also form heterodimers with the full-length DNOS1. In experiments with purified nNOS and iNOS homodimers, the flow of electrons during catalysis has been shown to occur from the flavins in the reductase domain of one subunit to the heme iron in the oxygenase domain of the other subunit (36, 37, 38). This suggests that heterodimers between the full-length and truncated NOS polypeptides will have diminished enzymatic activity. Indeed, rat NOS full-length polypeptide and a synthetic polypeptide lacking the reductase domain can form heterodimers in vitro, but these complexes show drastically decreased enzymatic activity (38). Furthermore, a fragment of eNOS that lacks the reductase domain can form complexes with the full-length eNOS when co-expressed in cultured cells and exhibits a strong dominant negative effect on eNOS activity (39). Thus, truncated DNOS proteins capable of forming heterodimers with DNOS1 may act as dominant negative inhibitors of NO production. This notion was confirmed in our experiments in which DNOS4, DNOS5, and DNOS6, each of which can form complexes with DNOS1, were able to strongly suppress NOS activity when co-expressed along with the DNOS1 (Figs. 6 and 7). This suggests that formation of heterodimers between the full-length DNOS1 and its truncated isoforms may be a basis for a mechanism of regulation of NO production in Drosophila. It will be interesting to determine whether truncated DNOS proteins are indeed synthesized in the fruit fly and whether they can serve to modulate NOS activity in vivo.

In summary, our results demonstrate that the dNOS locus in Drosophila generates a large family of transcripts, some of which code for truncated DNOS-like proteins. These proteins are capable of suppressing the enzymatic activity of the full-length DNOS1 protein, perhaps by disrupting the dimerization of DNOS1 molecules. Because such truncated NOS proteins have been postulated to exist in mammals, this novel regulatory function proposed for the truncated Drosophila NOS proteins may apply more widely for NOS regulation.

Acknowledgments—We thank Vladimir Scheinker and Naoki Nakaya (Cold Spring Harbor Laboratory) for invaluable help with NOS activity assay and quantitative RT-PCR. We are grateful to Givonid Ramanathan, Alberto Hazan, Rithwick Rajagopal, and Michael Verzi (all participants of the Summer Undergraduate Research Program at Cold Spring Harbor Laboratory) for technical assistance during different stages of this project. We are grateful to Julian Banerji for invaluable advice on the manuscript. We thank all members of Enikolopov lab for helpful discussions on the manuscript and their critical suggestions.

REFERENCES

1. Bredt, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
2. Wang, Y., Newton, D. C., and Marsden, P. A. (1999) Crit. Rev. Neurobiol. 13, 21–43
3. Förstermann, U., Boijsell, J. P., and Kleinert, H. (1998) FASEB J. 12, 773–790
4. Geller, D. A., and Billiar, T. R. (1998) Cancer Metastasis Rev. 17, 7–23
5. Enikolopov, G., Banerji, J., and Kozin, B. (1999) Cell Death Differ. 6, 956–963
6. Regulski, M., and Tully, T. (1995) Natl. Acad. Sci. U. S. A. 92, 9072–9076
7. Adams, M. D., Celinker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., et al. (2000) Science 287, 2185–2189
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Ashburner, M. (1989) Drosophila: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Frohman, M. A. (1995) Methods Enzymol. 218, 340–356
11. Israel, D. I. (1993) Nucleic Acids Res. 21, 2627–2631
12. Tanaka, M., and Herr, W. (1990) Cell 60, 375–386
13. Bredt, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 682–685
14. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 488–501
15. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) Mol. Cell. Biol. 8, 2109–2165
16. Mount, S. M., Burks, C., Hertz, G., Stormo, G. D., White, O., and Fields, C. (1992) Nucleic Acids Res. 20, 4262–4266
17. Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbuz, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994) J. Biol. Chem. 269, 33092–33090
18. Luckhart, S., and Rosenberg, R. (1999) Gene 232, 25–34
19. Klatt, P., Pleifer, S., Liet, B. M., Lehner, D., Glatter, O., Bachinger, H. P., Werner, E. R., Schmidt, K., and Mayer, B. (1996) J. Biol. Chem. 271, 7336–7342
20. Raman, C. S., Martasek, P., and Masters, B. S. (2000) The Porphyrin Hand-
book: Biochemistry and Binding, Activation of Small Molecules, Vol. 4, pp. 293–339, Academic Press, Inc., New York
21. Stott, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6509–6513
22. Kuzin, B., Roberts, I., Peunova, N., and Enikolopov, G. (1996) Cell 87, 639–649
23. Kuzin, B., Regulski, M., Stasiv, Y., Scheinker, V., Tully, T., and Enikolopov, G. (2000) Curr. Biol. 10, 459–462
24. Truman, J. W., De Vente, J., and Ball, E. E. (1996) Development 122, 3949–3958
25. Wildemann, B., and Bicker, G. (1999) J. Neurobiol. 38, 1–15
26. Gibbs, S. M., and Truman, J. W. (1998) Neuron 20, 83–93
27. Dow, J. A., Maddrell, S. H., Davies, S. A., Skaer, N. J., and Kaiser, K. (1994) Am. J. Physiol. 266, R1716–R1719
28. Nappi, A. J., Vass, E., Frey, F., and Carton, Y. (2000) Nitric Oxide 4, 423–430
29. Wingrove, J. A., and O’Farrel, P. H. (1999) Cell 98, 105–114
30. DiGregorio, P. J., Ubersax, J. A., and O’Farrell, P. H. (2001) J. Biol. Chem. 276, 1830–1937
31. Wang, Y., Newton, D. C., Robb, B. G., Kau, C. L., Miller, T. L., Cheung, A. H., Hall, A. V., Van Damme, S., Wilcox, J. N., and Marsden, P. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12150–12155
32. Luckhart, S., and Li, R. (2001) Insect Biochem. Mol. Biol. 31, 249–256
33. Larsson, B., and Phillips, S. C. (1998) Biochem. Biophys. Res. Commun. 251, 898–902
34. Silvagno, F., Xia, H., and Bredt, D. S. (1996) J. Biol. Chem. 271, 11204–11208
35. Fujisawa, H., Ogura, T., Kurashima, Y., Yokoyama, T., Yamashita, J., and Esumi, H. (1994) J. Neurochem. 63, 140–145
36. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998) J. Biol. Chem. 273, 18950–18958
37. Panda, K., Ghosh, S., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 23349–23356
38. Sagami, I., Daff, S., and Shimizu, T. (2001) J. Biol. Chem. 276, 30036–30042
39. Lee, C. M., Robinson, L. J., and Michel, T. (1995) J. Biol. Chem. 276, 27403–27406

Transcriptional Complexity of the Drosophila NOS Gene

by guest on July 24, 2018
http://www.jbc.org/Downloaded from

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
The *Drosophila* Nitric-oxide Synthase Gene (dNOS) Encodes a Family of Proteins That Can Modulate NOS Activity by Acting as Dominant Negative Regulators

Yuri Stasiv, Michael Regulski, Boris Kuzin, Tim Tully and Grigori Enikolopov

*J. Biol. Chem.* 2001, 276:42241-42251.
doi: 10.1074/jbc.M105066200 originally published online August 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105066200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 15 of which can be accessed free at
http://www.jbc.org/content/276/45/42241.full.html#ref-list-1