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Bradysia hygida (Diptera, Sciaridae) presents two eukaryotic Elongation Factor 1A gene homologues: partial characterization of the eukaryotic Elongation Factor 1A-F1 gene

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Bradysia hygida (Diptera, Sciaridae) presents two *eukaryotic Elongation Factor 1A* gene homologues: partial characterization of the *eukaryotic Elongation Factor 1A-F1* gene

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Abstract

Elongation factor 1A is a highly conserved protein that participates in translation. We report the occurrence of two genes homologous to the *eukaryotic Elongation Factor 1A* in *Bradysia hygida* and describe the partial cloning and characterization of the *B. hygida eukaryotic Elongation Factor 1A-F1* (*BheEF1A-F1*) gene. The pattern of *BheEF1A-F1* expression in the salivary gland at the end of the fourth larval instar was investigated using real-time PCR. The results showed that *BheEF1A-F1* expression levels are relatively constant at the time when rapid changes in protein synthesis occur in this tissue. *In situ* hybridization experiments coupled to Southern blot analyses showed that the *BheEF1A-F1* gene is located at position 3d of the A chromosome and a second gene homologous to e*EF1A* is located at position 6a of the X chromosome. Southern blot analyses showed that both the *BheEF1A-F1* gene and the second gene homologous to *eEF1A* constitute non-amplified genes. The present results contribute to the molecular characterization of a sciarid *eEF1A* gene.

Key words: Eukaryotic Elongation Factor 1A; *Bradysia hygida*; Polytene chromosomes; Sciaridae

Introduction

The salivary gland polytene chromosomes of Diptera present RNA puffs that are sites of intense transcription. Sciarid larvae also exhibit DNA puffs, which are sites of developmentally regulated gene amplification and transcription (1-3). Several studies have shown that sciarid DNA puff gene amplification and transcription, that occur at the end of the fourth larval instar, are processes regulated by the molting hormone ecdysone (for a review, see Ref. 4).

The expansion and regression of specific DNA puffs can be related to the developmentally regulated synthesis of groups of salivary gland polypeptides (5-8). These studies led to the suggestion that the biological role of gene amplification is to provide large amounts of salivary gland proteins that are employed in the construction of the cocoon in which the larvae pupate (5-8). This suggestion was confirmed by the demonstration that polyclonal antibodies raised against either the product of DNA puff B10 (the BhB10-1 protein) or against the product of DNA puff C4 (the BhC4-1 protein) detect these polypeptides both in salivary gland extracts and saliva of larvae at the time of DNA puff formation (9,10).

In addition to gene amplification and transcription, several other processes have been investigated using the Sciaridae family as a model, including mechanisms of telomere maintenance (11,12), programmed cell death (13), establishment of dorsoventral polarity (14), and the immune response (15). These studies contributed to the understanding of these processes and further strengthened the view that the study of non-model organisms can contribute to the understanding of processes of general occurrence in metazoans.

Elongation factor 1A (EF1A) is a protein involved in the GTP-dependent binding of an aminoacyl-tRNA to the empty A site of the ribosome. After the binding of the complex, the

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GTPase activity of EF1A is activated by the ribosome and GTP hydrolysis leads to the dissociation of EF1 from the tRNA, permitting the full entrance of the tRNA into the A site (for reviews, see Refs. 16,17). Eukaryotic EF1A (eEF1A) is the most abundant intracellular protein next to actin, comprising 1-3% of the total protein content in normal growing cells (18,19). The mRNA levels of *eEF1A* are not constant and an increase in *eEF1A* mRNA expression levels has been reported to occur in rapidly proliferating cells (19), whereas the down-regulation of *eEF1A1* contributes to the induction of caspase-independent cell death in tetraploid vertebrate cells (20).

The amino acid sequences of eEF1A are highly conserved between species and have been widely employed in studies assessing the phylogenetic relationships in insects both among species groups and genera within (sub)-families (reviewed in Ref. 21). Both the *Drosophila melanogaster* and *Apis mellifera* genomes contain two copies of the *eEF1A* gene, which encode two highly similar proteins, named eEF1A-F1 and eEF1A-F2 (22-24). Previous studies in *D. melanogaster* have suggested that *eEF1A-F1*, which is expressed throughout development, represents a housekeeping gene that is needed in all growing cells. In contrast, the expression of the *eEF1A-F2* gene peaks in the pupal stage, this being a gene that is expressed in a tissue-specific manner (23).

We describe here the molecular characterization of the *eukaryotic Elongation Factor 1A* gene of the sciarid *Bradysia hygida*. Our results indicate the occurrence of two genes that are homologous to the *eukaryotic Elongation Factor 1A* and describe the partial sequence of a cDNA coding for the *B. hygida* eukaryotic Elongation Factor 1A-F1 (BheEF1A-F1). We show that the *BheEF1A-F1* gene is located at position 3d of the A chromosome and its levels of expression in the salivary gland at the end of the fourth larval instar are relatively constant. To our knowledge, this is the first time the existence of two *eEF1A* paralogs is shown in a nematoceran Dipteran.

Material and Methods

Bradysia hygida

B. hygida was maintained in the laboratory at 20°C, and under these conditions has a life cycle of 36 days (6). From hatching to the pupal molt, the larvae go through four larval instars. The fourth instar begins on day 12 after hatching. On the 6th day of the fourth instar the eyespots appear (E1), which provide useful age markers. The E3 eyespot pattern coincides with the beginning of the process of gene amplification, when DNA puff anlage formation can be observed at several sites in the salivary glands polytene chromosomes. At the E7 eyespot stage, the first group of DNA puffs is partially open. The expansion of the second group of DNA puffs begins 12 h after the appearance of the E7 eyespot pattern (E7+12h). By the time the pupal molt begins (at E7+26h), all but one of the DNA puffs are already closed. Salivary glands were dissected from fourth-instar female larvae and kept in glycerol/ethanol (1:1) at -20°C until further processing.

RNA extraction

Total RNA was isolated from two pairs of salivary glands of larvae at the ages of E1, E7+4h and E7+16h. The salivary glands were washed twice in 70% ethanol to remove the glycerol and homogenized in 50 µL extraction buffer (4 M guanidine thiocyanate, 50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5% sarcosyl, 0.72% 2-mercaptoethanol). The samples were extracted once with 1 volume of phenol and a 0.2 volume of chloroform:isoamyl alcohol (49:1), followed by the addition of 1/10 of the volume of 2 M sodium acetate, pH 4.0, and 1 volume of isopropyl alcohol, and kept at -20°C for 16 h. Total RNA was recovered after centrifuging at 10,000 *g* for 30 min at 4°C, followed by two washes in 70% ethanol. After drying, the RNA was dissolved in water. Total RNA concentrations were estimated by absorbance at 260 nm, assuming that the absorbance of 40 µg/mL is 1.0.

RT-PCR, primer design and partial cloning of the *B. hygida eEF1A* cDNA

Total RNA samples were treated with RNase-free DNAse (Promega, USA), at a final concentration of 1 unit/µg RNA in DNAse buffer (40 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 1 mM $CaCl_2$) for 30 min at 37°C. After the addition of EGTA, pH 8.0 (final concentration 2 mM), DNAse was inactivated by incubating the reaction for 10 min at 65°C. A mixture of 1 µg total RNA, 0.25 µg oligo$(dT)_{15}$ (Promega) and 0.25 µg Random Primer (Promega) was incubated at 70°C for 5 min, and quickly chilled in ice, prior to the addition of Improm-II Reverse Transcriptase (Promega). Reverse transcriptase (RT) reactions were performed according to manufacturer instructions.

The CLUSTALW program (25) was used to build a nucleotide sequence alignment containing the *eEF1A* nucleotide sequences of *Rhynchosciara americana* (GenBank accession number X66131.1), *D. melanogaster* (NM_058027.3), *Anopheles gambiae* (XM_562379.2), *D. pseudoobscura* (XM_001361784.2), *Bombyx mori* (NM_001044045.1), *A. mellifera* (NM_001014993.1), *Aedes aegypti* (DQ440206.1), and *Manduca sexta* (AF234571.1), and polymerase chain reaction (PCR) primers were designed based on highly conserved regions. Primer sequences are as follows: eEF1A-forward 5'-CATCGGGATCCCATCAAGAACATG-3' and eEF1A-reverse 5'-GCAATGTCTGCAGTGTGGCAATC-3'. Restriction sites for *Bam*HI and *Pst*I (underlined) were introduced in the forward and reverse primers, respectively.

The PCR mixtures contained 1/10 of the product of each RT reaction, 100 pmol of each primer, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 2.5 units of Taq DNA Polymerase (Invitrogen, USA) and were amplified

employing the following protocol: 1 cycle of 5 min at 95°C, 40 cycles of 45 s at 95°C, of 90 s at 56°C, and of 90 s at 72°C, followed by a final 10-min extension at 72°C. An 824-bp DNA fragment was produced, gel purified, digested with *Bam*HI and *Pst*I and cloned into the PBSKS+ vector (Stratagene, USA).

Sequencing reactions and sequence analyses

Dideoxynucleotide sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Both strands were sequenced. The sequencing reactions were performed using M13 primers (forward and reverse) and two additional internal primers (eEF2-forward: 5'-CAACATGGGATCCTTGAAGGGATG G-3' and eEF3-reverse: 5'-CGGAAACGTTCTAGACGTT GAAACC-3'). The reaction products were analyzed on an ABI Prism® 3100 DNA Sequencer (Applied Biosystems). BLASTN (26) searches were performed using the NCBI non-redundant database and sequence analyses were performed using the BioEdit sequence alignment editor V7.0.9.0 (27).

Quantitative SYBR® Green assay

Five percent of the product of reverse transcription reactions (from 1 µg of total salivary glands RNA) was used in real-time PCR mixtures. Each reaction (final volume of 10 µL) contained 5 µL of DyNamo SYBR® Green qPCR mix (Finnzymes, Espoo, Finland), 0.3 µM of each primer and 1 µL template. Real-time PCR was performed in triplicate for all samples using the Mastercycler® ep *realplex* system (Eppendorf, Germany) and two independent experiments were carried out. Primers were designed in order to generate 100-bp PCR fragments for both *BheEF1A-F1* and the *28S* ribosomal RNA (internal control). Primer sequences were as follows: *BheEF1A-F1* (forward: 5'-GGTGATTGCAGTTGTT GCAT-3'; reverse: 5'-ATCAATTCTGCGCCTTTTGT-3') and *28S* ribosomal RNA (forward: 5'-ATTAGCGGGGAAAGAAG ACC-3'; reverse: 5'-GCACCCGAAGGTACACATCT-3'). The amplification reactions were carried out using the following protocol: 1 cycle of 5 min at 95°C, 40 cycles of 15 s at 95°C, of 15 s at 50°C, and of 20 s at 72°C. Analysis of the melting curves revealed that a single amplified product was generated in each reaction. The data were analyzed according to the Mastercycler® ep *realplex* User Manual (Eppendorf) and both baseline and cycle threshold (C_t = number of cycles needed for the fluorescence to reach a specific threshold level of detection) were set by the program. In order to determine the relative expression levels of *BheEF1A-F1*, the C_t values obtained for *BheEF1A-F1* were normalized against the values obtained for *28S* rRNA and expressed as a ratio between *BheEF1A-F1* and *28S* rRNA.

DNA extraction

B. hygida salivary glands were incubated in extraction buffer (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10% SDS, 1 mg/mL proteinase K) at room temperature for 30 min. After 2 phenol:chloroform extractions and 1 chloroform extraction, DNA was precipitated in ethanol and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentrations were determined by fluorometry using bisbenzimide (Hoescht 33258 from Sigma-Aldrich, USA).

Southern blot hybridization

Ten micrograms of salivary gland genomic DNA was treated with distinct restriction enzymes according to manufacturer instructions (New England BioLabs Inc., USA). After digestion, the DNA samples were treated with RNase A (20 µg, Sigma-Aldrich) for 30 min at 37°C, and run on 0.8% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), for 16 h at 22 V. Southern blotting was performed on nylon membranes (Amersham Hybond™ - N^+) according to membrane manufacturer instructions (GE Healthcare, UK). *BheEF1A-F1* cDNA fragment labeling, hybridization, post-hybridization washes, and detection were performed using the Amersham Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) according to manufacturer instructions.

***In situ* hybridization**

Larval salivary glands were dissected in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0). The anterior region of the salivary glands (S1 region) was removed and placed on a 2% gelatin-treated slide in a drop of 45% acetic acid for 2 min. A fresh drop of 45% acetic acid was added and the salivary glands were covered with a coverslip and tapped with a forceps in order to spread the chromosomes. The coverslip was removed after freezing in liquid nitrogen, and the slide immediately plunged into absolute ethanol kept at 0°C. The preparations were stored for up to 2 days in ethanol at 4°C before processing. Probes were labeled with digoxigenin-dUTP (Roche, Switzerland) by random primer reaction. *In situ* hybridization was performed according to the protocol of the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/about/methods/cytogenetics.html), with the following modifications: after incubation with anti-digoxigenin-POD (Roche, diluted 1:50) for 1 h, the preparations were washed three times in PBS-T containing 0.1% Triton X-100, for 5 min each time, followed by incubation with peroxidase-anti-peroxidase (Sigma-Aldrich, 1:100) for 2 h and washed again three times in PBS-T for 5 min each time. Enzyme activity was detected by incubating the slides with 0.5 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in PBS containing 0.01% hydrogen peroxide for 10 min. The slides were washed with PBS-T, stained with 0.05% toluidine blue and mounted in PBS-T/glycerol (1:1). The images were recorded using an Ortholux 2 Pol BK Leitz microscope (Leitz, Germany), equipped with a Leica EC3 camera system (Leica Microsystems, Germany) and were analyzed with the Las EZ version 1.4.0 software

(Leica Microsystems). Alternatively, the images were recorded using an Axioskop 40 microscope (Zeiss, Germany) equipped with an AxionCam MRc5 camera system (Zeiss) and were analyzed with the Axion Vision version 4.6 software (Zeiss).

Results and Discussion

Cloning of a *BheEF1A* cDNA fragment

In order to clone the *eEF1A* gene of *B. hygida*, we designed primers corresponding to highly conserved regions present in the nucleotide sequences of the e*EF1A* of 8 insect species (see Material and Methods). A single 824-bp RT-PCR product was obtained from RNA extracted from salivary glands at the late E7 stage. This 824-bp fragment was cloned and sequenced.

The nucleotide sequence of the cloned cDNA fragment was used in a similarity search performed with the BLASTN program (26). The sequence of the *BheEF1A* cDNA fragment presents high identity with the *eEF1A* mRNA sequences of different species (up to 87% identity with an *E* value <0.01). Furthermore, the cloned sequence presents 86% identity with the *eEF1A* of *R. americana*, the only sciarid *eEF1A* mRNA sequence currently available in the nucleotide database of the National Center for Biotechnology Information (NCBI).

The deduced amino acid sequence of the longest open reading frame, which spans the entire cloned cDNA fragment, is shown in Figure 1. Multiple alignments of the 266-amino acid deduced sequence with eEF1A amino acid deduced sequences of other insect species (Figure 1) revealed significant similarity (95, 93, 93, 91, and 89% identity) with the sequences of *D. melanogaster* (eEF1A-F1), *R. americana*, *A. mellifera* (eEF1A-F2), *D. melanogaster* (eEF1A-F2), and *A. mellifera* (eEF1A-F1), respectively. The cloned BheEF1A presents higher similarity to the eEF1A-F1 variant of *D. melanogaster* (95%) than to the eEF1A-F2 variant of *D. melanogaster* (91%). The sequence obtained was deposited in GenBank (accession number GQ119627) and named *eukaryotic Elongation Factor 1A-F1* of *B. hygida* (*BheEF1A-F1*), according to the nomenclature proposed in Ref. 28.

EF1A proteins consist of three structural domains: domain I or G domain, domain II, and domain III. Domain I (G domain) is approximately 200 residues long, is responsible for binding either GTP or GDP and is related to the GTPase domains of other G proteins. Domains II and III both contain approximately 100 residues and are β barrels. These domains are in the same relative orientation and might act as a single rigid unit during hydrolysis of GTP (16,17). EF1A domains I and II are both present in the BheEF1A-F1 deduced amino acid sequence (Figure 1). The consensus GTP-binding motif (NKMD) is found in domain I of BheEF1A between amino acids 55-58 (Figure 1). The observation of sequence conservation is not surprising, given the fact that recent structural studies have revealed that the structures of EF1A are highly conserved between archaea and bacteria (29).

BheEF1A-F1 expression at the end of the fourth larval instar of *B. hygida*

The end of the fourth larval instar of *B. hygida* has been previously characterized as a developmental time during which dynamic and abundant protein synthesis occurs in the salivary glands (6,7). After an initial period of nine days during which a characteristic set of polypeptides are synthesized, the salivary glands carry out two successive programs of specialized protein synthesis that last about 12 h each. The temporal coincidence between these two programs of protein synthesis and DNA puff formation has led to the suggestion that the newly synthesized proteins are the products of DNA puffs (6). In this context, we have investigated the pattern of expression of *BheEF1A-F1* mRNA at three different ages at the end of the fourth larval instar: E1 (first period of synthesis), E7+4h (second period of synthesis) and E7+16h (third period of synthesis).

BheEF1A-F1 mRNA relative expression levels were determined using real-time PCR. The C_t values obtained for *BheEF1A-F1* were normalized against the C_t values obtained for *28S* rRNA, which was employed as an internal control. Analysis of the dissociation curves suggested that a single *eEF1A* isoform is expressed at the end of the fourth instar. Real-time PCR results revealed that *BheEF1A-F1* expression occurs at similar levels in all ages analyzed (E1: 2.295 ± 0.07; E7+4h: 2.29 ± 0.19; E7+16h: 2.075 ± 0.47). Although the protein synthesis profiles undergo drastic changes at the end of the fourth larval instar (6) and a 28-fold increase in secretory protein synthesis occurs at age E7 (7), *BheEF1A-F1* expression levels at the ages examined showed no significant variations during the end of the fourth larval instar.

In *D. melanogaster*, the *eEF1A-F1* gene has been described as the housekeeping gene that gives rise to the elongation factor needed in all growing cells, whereas the *eEF1A-F2* gene is expressed in a developmentally regulated manner (23). The detection of constant *BheEF1A-F1* mRNA expression levels during the end of the fourth larval instar reinforces the suggestion that the cloned cDNA corresponds to the *eEF1A-F1* isoform and to the *eEF1A-F1* housekeeping gene. A similar situation has been described for the two human isoforms characterized (*eEF1A1* and e*EF1A2*). The *eEF1A1* isoform is highly expressed in tissues displaying elevated rates of protein synthesis and cell proliferation, whereas the *eEF1A2* isoform is expressed in tissues in which the majority of the cells are fully differentiated and are characterized by reduced or no cell division (30).

Genomic characterization of the *BheEF1A* gene

In order to determine the cytogenetic location of the *BheEF1A-F1* gene, we performed *in situ* hybridization in

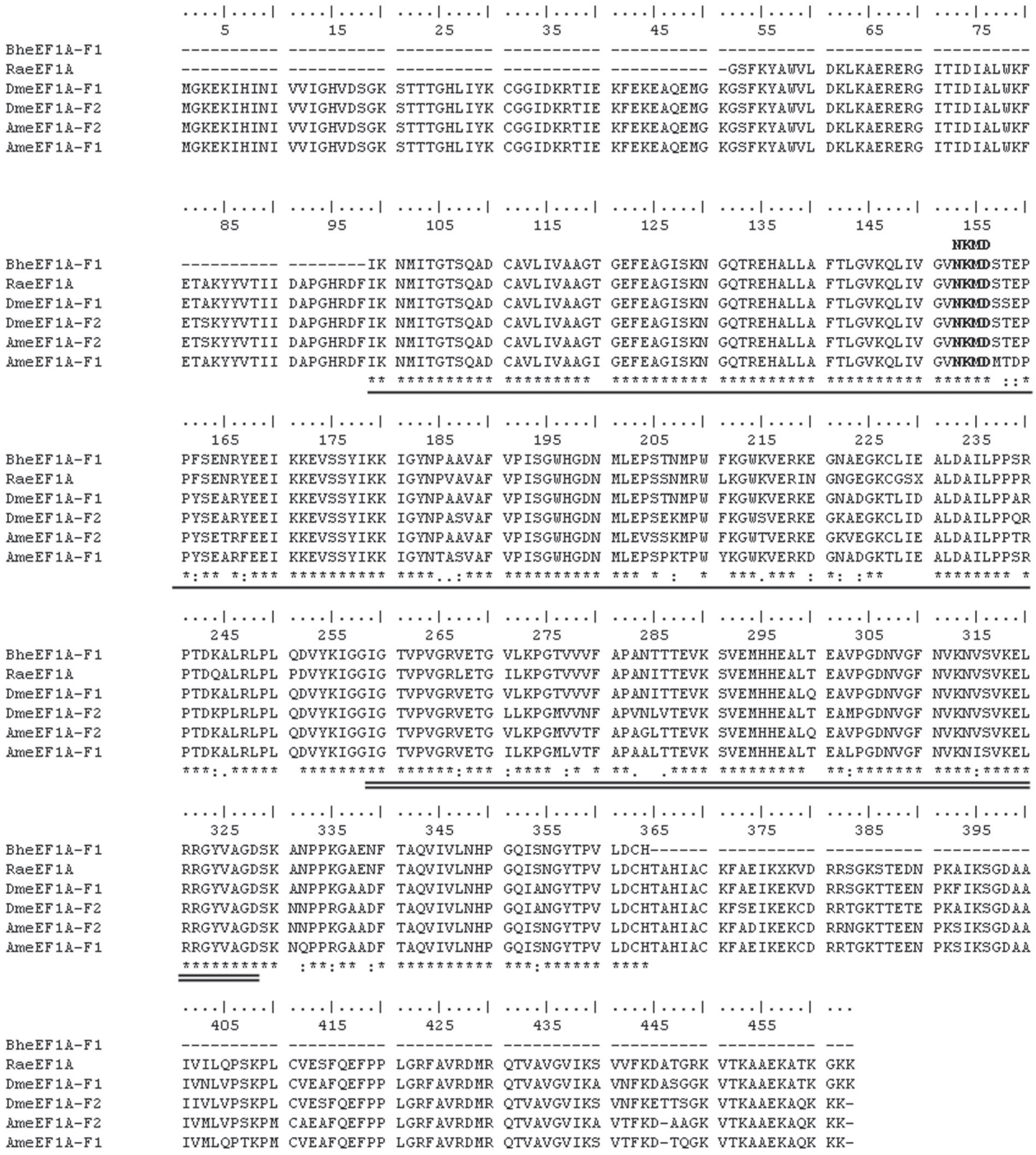

Figure 1. Multiple alignment of eEF1A deduced amino acid sequences. The BheEF1A-F1 deduced amino acid sequence (GenBank accession number GQ119627) was aligned with the deduced eEF1A amino acid sequences of *Rhynchosciara americana* (RaeEF1A, CAA46922.1), *Drosophila melanogaster* (DmeEF1A-F1, NP_477375.1), *D. melanogaster* (DmeEF1A-F2, NP_524611.1), *Apis mellifera* (AmeEF1A-F2, NP_001014993.1), and *A. mellifera* (AmeEF1A-F1, NP_001011628.1) using the CLUSTALW program (25). The consensus GTP-binding motif (NKMD) is indicated in bold. The domain I or G domain is underlined. The domain II is double-underlined.

salivary gland polytene chromosomes of fourth-instar larvae. As shown in Figure 2, the *BheEF1A-F1* probe hybridizes in two different chromosomal regions: the 3d region of the A chromosome (Figure 2A) and the 6a region of the X chromosome (Figure 2B). Labeling in the A3d region was always detected, whereas labeling in the X6a region was detected in the majority of the chromosomes analyzed. Extensive examination of four independent experiments did not reveal any labeling in the B or C chromosome (data not shown). Since the hybridization signal in the A3d region was more intense, we propose that this region corresponds to location of the *BheEF1A-F1* gene. The weaker hybridization signal detected in the X6a region suggests that the *B. hygida* genome might contain a second gene homologous to the *eEF1A* gene.

In order to extend the characterization of the *BheEF1A-F1* gene, a Southern blot containing 10 µg salivary gland genomic DNA of larvae at late E7 (E7+), digested with seven different restriction enzymes, was hybridized to the *BheEF1A-F1* cDNA. As can be observed in Figure 2D, the *BheEF1A-F1* probe detected four *Nco*I fragments, three genomic fragments in both *Hin*dIII and *Xho*I digestions, and two genomic fragments in the *Eco*RI, *Bam*HI, *Sal*I, and *Xba*I digestions. We consistently detected genomic fragments of different labeling intensities regardless of the restriction enzyme employed (Figure 2D). The *BheEF1A-F1* cDNA fragment contains both an *Hin*dIII and an *Nco*I restriction site (Figure 2C) and at least three genomic fragments were detected when either restriction enzyme was used to digest the DNA. In the case of the remaining five enzymes tested, for which there are no restriction sites in the probe, we detected at least two genomic fragments. We suggest that the more intensely labeled fragments in the Southern blot correspond to genomic fragments containing the *BheEF1A-F1* gene and we attribute the weaker signals to the detection of a second gene that is similar to the *BheEF1A-F1* gene.

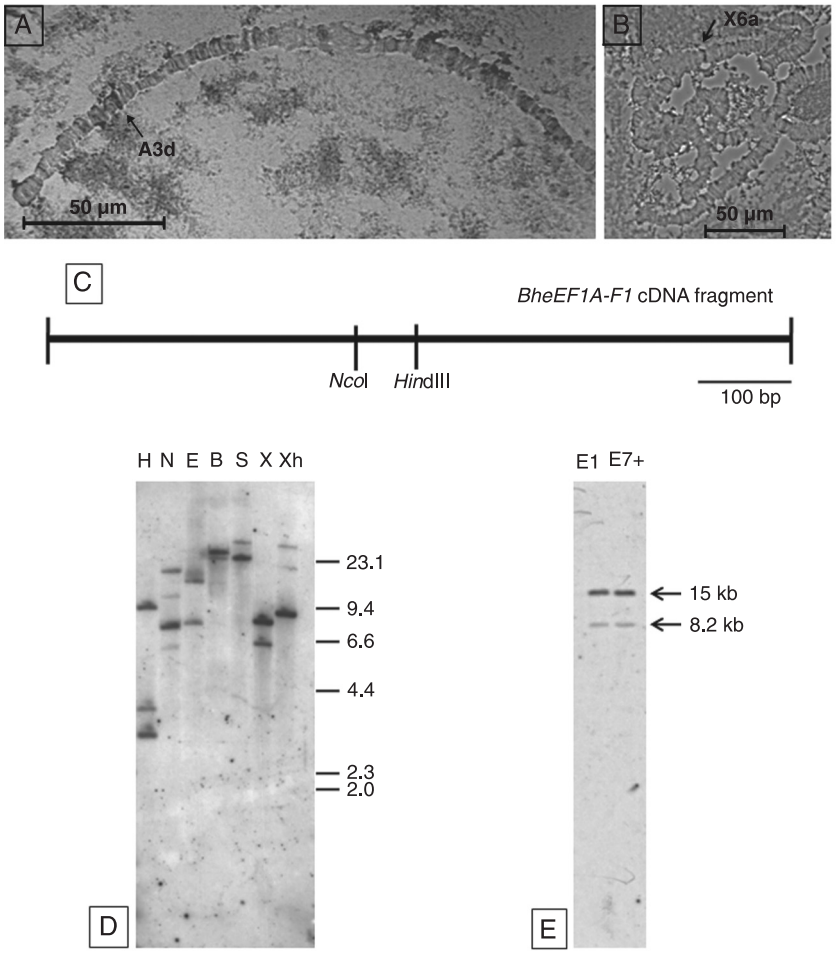

Figure 2. Cytogenetic mapping and Southern blot characterization of *BheEF1A*. *A*,*B*, *In situ* hybridization. Salivary gland polytene chromosomes were hybridized with a digoxigenin-labeled *BheEF1A-F1* cDNA fragment and counterstained with Toluidine blue. The arrows point to the A3d (A) and the X6a (B) *loci*, in which the product of the peroxidase reaction was detected. *C*, Restriction map of the *BheEF1A-F1* cDNA fragment. Both *Hin*dIII (nucleotide 397) and *Nco*I (nucleotide 333) sites are shown. *D*, Southern blot containing identical amounts of genomic DNA extracted from salivary glands at late E7 and digested with different enzymes, was hybridized with the *BheEF1A-F1* cDNA fragment, followed by detection with the CDPstar reagent. The restriction enzymes employed are indicated on top of the blot: *Hin*dIII (H), *Nco*I (N), *Eco*RI (E), *Bam*HI (B), *Sal*I (S), *Xba*I (X), and *Xho*I (Xh). *E*, Southern blot containing identical amounts of *Eco*RI-digested DNA extracted from salivary glands at ages E1 and late E7 (E7+), after hybridization with the *BheEF1A-F1* cDNA fragment, followed by detection with the CDPstar reagent.

Two copies of the *eEF1A* gene are also found in different insect orders. *D. melanogaster* and *A. mellifera* both contain two copies of the *eEF1A* gene encoding highly similar proteins that differ both in sequence and exon and in intron organization. The *D. melanogaster eEF1A-F1* gene consists of two exons separated by an intron. In contrast, the *D. melanogaster eEF1A-F2* gene is organized into five exons separated by four introns (22,23). In *A. mellifera* the *eEF1A-F1* gene is closely related to the *D. melanogaster eEF1A-F2* gene (31) and is organized into three exons

separated by two introns (24). The *A. mellifera eEF1A-F2* gene shows high similarity to the *D. melanogaster eEF1A-F1* gene and presents four exons separated by three introns (24). The existence of two copies of *eEF1A* genes in both *D. melanogaster* (Diptera) and *A. mellifera* (Hymenoptera), together with the recently found *eEF1A* gene duplication in *Xyleborini* (Coleoptera) (32), suggests that *eEF1A* duplication might be more widespread in the holometabolous insect orders. Previous studies have suggested that the *eEF1A* gene was duplicated in parallel in the Diptera and in the Hymenoptera (24). Taken together, these data further support the existence of two paralogous copies of the *eEF1A* gene in the nematoceran Dipteran *B. hygida*.

Sciarid DNA puffs have been previously characterized as genomic regions in which developmentally regulated gene amplification occurs (33-38). The only DNA puff-forming regions found on chromosomes A and X constitute the A14 and X4 regions, respectively (6). Therefore, the identification of both the A3d and X6a regions in the *in situ* hybridization experiments revealed that the *BheEF1A* gene is not located in DNA puff-forming regions. In order to investigate whether the *BheEF1A-F1* gene is amplified during the end of the fourth larval instar, a Southern blot containing identical amounts (10 µg) of *Eco*RI-digested salivary gland genomic DNA of larvae at age E1 (before gene amplification) and late E7 (after gene amplification) was hybridized to the *BheEF1A-F1* probe (Figure 2E). In both cases two genomic fragments were identified. Regardless of the age analyzed, both samples showed that the hybridization signal of the 15-kb fragment was stronger than that of the 8.2-kb fragment. In addition, the intensity of the hybridization signals for each fragment was closely similar when both samples, E1 and E7+, were compared (Figure 2E). These results reveal that at E7+, a developmental time when the amplification process has already occurred in the DNA puff-forming regions, the number of copies of the *eEF1A* gene is the same as before the gene amplification process, age (E1), and therefore constitutes a non-amplified gene. These results agree with previous experiments using the *R. americana eEF1A* gene as a non-amplified control gene to estimate the amplification levels in both the *B. hygida* DNA puff C4 amplified domain and the *R. americana* C3 amplified domain (39,40).

Our results contribute to the characterization of the *eEF1A* of *B. hygida*, which constitutes the second sciarid *eEF1A* sequence to be deposited in the public databases. The availability of this cDNA sequence coupled to the data suggesting the existence of two paralogs in *B. hygida*, a nematoceran Dipteran, will contribute to future insect phylogeny studies.

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