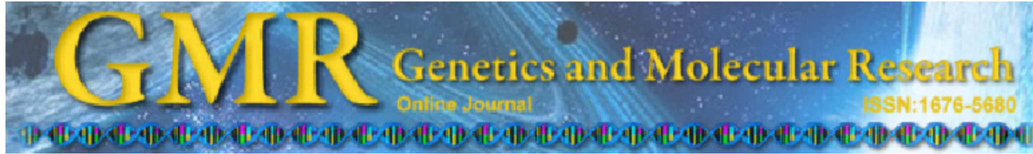

Molecular combing in the analysis of developmentally regulated amplified segments of *Bradysia hygida*

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ABSTRACT. Molecular combing technology is an important new tool for the functional and physical mapping of genome segments. It is designed to identify amplifications, microdeletions, and rearrangements in a DNA sequence and to study the process of DNA replication. This technique has recently been used to identify and analyze the dynamics of replication in amplified domains. In *Bradysia hygida*, multiple amplification initiation sites are predicted to exist upstream of the *BhC4-1* gene. However, it has been impossible to identify them using the available standard techniques. The aim of this study was to optimize molecular combing technology to obtain DNA fibers from the polytene nuclei of the salivary glands of *B. hygida* to study the dynamics of DNA replication in this organism. Our results suggest that combing this DNA without prior purification of the polytene nuclei is possible. The density, integrity, and linearity of the DNA fibers were analyzed, fibers 50 to 300 kb in length were detected, and a 9-kb fragment within the amplified region was visualized using

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biotin detected by Alexa Fluor 488-conjugated streptavidin technique. The feasibility of physically mapping these fibers demonstrated in this study suggests that molecular combing may be used to identify the replication origin of the *BhC4-1* amplicon.

Key words: Molecular combing; *Bradysia hygida*; Replication origin; Developmentally regulated amplified segment

INTRODUCTION

Experimental models widely used to study the nature and structure of replication origins in metazoa are systems that exhibit developmentally regulated gene amplification. Some examples of this phenomenon include DNA segments that form DNA puffs in Sciaridae and the chorionic gene-containing chromosome segment in *Drosophila melanogaster* (Claycomb and Orr-Weaver, 2005). Examples of DNA amplification induced *in vitro* in cultured mammalian cells include the dihydrofolate reductase gene amplification in ovarian cells (Dijkwell et al., 1991; Altman and Fanning, 2004) and the adenosine monophosphate deaminase 2 gene amplification in Chinese hamster lung cells (Toledo et al., 1998; Anglana et al., 2003; Courbet et al., 2008). Gene amplification has also been reported to occur during the course of tumorigenesis in distinct chromosomal regions (Myllykangas and Knuutila, 2006).

In all of these systems, the replication origins of these amplicons are activated more than once during the cell cycle, resulting in additional copies in the genome. These additional copies are structured differently when they originate in cell culture than when they occur during tumorigenesis (Debatisse et al., 1993; Omasa, 2002; Myllykangas and Knuutila, 2006) or in developmentally regulated amplified segments (Claycomb and Orr-Weaver, 2005).

Despite the use of sophisticated methodologies, the identification of amplification initiation sites during DNA replication in metazoan systems has been difficult (Mesner and Hamlin, 2009), and the development of alternative technologies such as molecular combing has yielded better physical and functional mapping of such genome segments (Lebofsky and Bensimon, 2003).

In the molecular combing method (Bensimon et al., 1994), DNA molecules with a random-coil configuration are attached to a hydrophobic silanized cover slip by their extremities. The cover slip is removed from the solution at a constant speed of 300 µM/s, resulting in the uniform extension and alignment of the DNA molecules owing to the force exerted by the receding air-water meniscus. Using this technique, the physical mapping of genomes and detection of target sequences can be performed (Bensimon et al., 1994; Lebofsky and Bensimon, 2003). The air-water meniscus exerts a constant perpendicular force on the attached DNA molecules, allowing for uniform orientation. The force exerted by the meniscus is sufficient to lengthen the DNA from its random-coil conformation but is insufficient to break the DNA extremity-cover slip surface interaction and the covalent bonds within the DNA molecules. Once in contact with air, the DNA molecules stick to the surface, preventing molecule retraction (Bensimon et al., 1994; Michalet et al., 1997; Lebofsky and Bensimon, 2003).

One of the major advantages of molecular combing is its capability for obtaining a high concentration of total genomic DNA without excessive breaking, which results in high-density fibers with the majority of DNA fragments measuring 200-700 kb (Michalet et al.,

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1997; Lebofsky and Bensimon, 2003). The high resolution of the combed DNA allows for the study of microdeletions (Michalet et al., 1997), amplifications (Herrick et al., 2000), and DNA rearrangements (Gad et al., 2001) via direct visualization of the rearranged genomic structures. Molecular combing can also be used to study the process of evolution (Koszul et al., 2004) and DNA replication (Lengronne et al., 2001; Norio and Schildkraut, 2001; Caburet et al., 2002; Lebosky and Bensimon, 2005). The feasibility of using molecular combing to identify and analyze the dynamics of replication during the developmentally regulated amplification of gene segments permits the use of a greater number of biological models in the study of DNA replication.

In *D. melanogaster*, the region comprising the chorion genes in the ovarian follicle cells has been shown to undergo gene amplification, and the DNA molecules obtained by this differential replication reportedly form an "onion skin" structure. *Rhynchosciara americana*, *Sciara coprophila*, and *Bradysia hygida* are members of the family Sciaridae in which specific regions of the genome are amplified in polytene cells of the salivary glands in the larvae (Claycomb and Orr-Weaver, 2005). Similar to chorionic gene amplification in *D. melanogaster*, gene amplification in *B. hygida* also forms an onion skin structure as a result of various levels of gene amplification in the DNA segments flanking the central region of amplification (Monesi et al., 1995).

Precise identification of the site of replication initiation for the amplified segments of the *BhC4-1* gene of *B. hygida* using neutral-neutral (N/N) and neutral-alkaline (N/A) two-dimensional gels was impossible, although the replication forks appeared to be upstream of the gene, suggesting the existence of a replication origin in this region (Coelho, 1997). Studies of this DNA segment have identified a region with numerous sites of bent DNA and a significant site localized to approximately 2000 bp upstream of the *BhC4-1* gene transcription start site (Fiorini et al., 2001). Sites of association with the nuclear matrix have also been identified in this segment (Mikami, 2000; Polinarski, 2005). The challenge of identifying the replication initiation site within this developmentally regulated amplification segment introduces difficulty into correlations with either the intrinsic bent DNA sites or the sites of association with the nuclear matrix in the *BhC4-1* gene amplicon with replication initiation. Therefore, new strategies such as molecular combing are necessary to identify replication initiation sites in the amplified segment of the *BhC4-1* gene.

MATERIAL AND METHODS

Biological material

B. hygida (Diptera: Sciaridae) was described by Sauaia and Alves (1968). This sciarid was reared in soil at 22°C and fed a fermented mate diet supplemented with yeast extract. Under these conditions, the length of the sciarid life is approximately 36 days, of which 6 days comprise embryogenesis and 22 days comprise the larval development period. The larval instar is characterized by the occurrence of 3 rounds of molting that delimit 4th larval stages (Laicine et al., 1984; Silva and Fernandez, 2000). The pupal stage lasts for 5 days and culminates in the emergence of the adult, which lasts approximately 3 days from mating to egg laying, thus closing the cycle life. This sciarid has a pair of salivary glands that contain the polytene chromosomes (Sauaia, 1971) and can be divided into 3 distinct morphological regions called S1, S2, and S3 (Rizzo, 1980).

The process of gene amplification in sciarids occurs during the last cycle of DNA replication in the salivary gland polytene chromosomes, and the DNA puffs occur exclusively at this location at the end of the fourth larval stage (Souza, 1999). *B. hygida* 4th-instar larvae develop 8 patterns of eyespots (E0 to E8) that provide markers for staging larval development and allow the distinction between the various puff patterns on the salivary gland polytene chromosomes (Sauaia, 1971).

Dissection of salivary glands

Salivary glands from 4th-stage larvae at ages E1, E3, and E7 were dissected using a stereomicroscope (Stemi DV4, Carl Zeiss). After gentle traction, the glands were separated from the fatty mass and stored at 4°C in 1X phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4).

Analysis of DNA integrity and high molecular weight

Extraction of high molecular weight DNA using agarose blocks was performed following a protocol described by Schurra and Bensimon (2009) with some modifications for our biological material. The glands were inserted into the plug mold (Bio-Rad) with a needle to avoid breaking and to maintain the integrity of the material. The molds were immediately filled with 1.5% low-melting-point agarose in 1X PBS at 42°C and incubated at room temperature for 15 min and then at 4°C for 30 min. The digestion of agarose blocks was carried out in ESP solution [0.45 M ethylenediaminetetraacetic acid (EDTA), 1% sarkosyl, and 2 mg/mL proteinase K] at 50°C overnight and stored in 0.5 M EDTA, pH 8.0. The blocks were visualized under a stereomicroscope before and after digestion with the ESP solution. The integrity and size of the DNA present in the agarose blocks were analyzed on 1% agarose gel in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) using pulsed-field gel electrophoresis (PFGE) according to manufacturer instructions.

Enzymatic digestion and staining

The agarose blocks containing the samples were incubated twice in excess (~100X the agarose block volume) TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with gentle agitation for 20 min and then digested with a rare restriction enzyme, *Not*I, in a final volume of 400 µL that included the volume of the block (1X buffer, 0.1 mg/mL bovine serum albumin, 125 U/mL *Not*I; Biolabs) for 3 h at 37°C. The blocks were then transferred to a tube containing 10 mL TE and incubated for 15 min at room temperature to wash the blocks. This process was repeated twice. After washing with TE, each block was transferred to a 2-mL tube containing 250 µL TE and 1 µL 1 mM YOYO®-1 iodide (Invitrogen) and incubated for 1 h in the dark (staining was also performed on some samples after the dissolution of the agarose blocks as described below).

Agarose block melting

Procedures that took place after staining with the fluorescent dye YOYO®-1 were carried out in the dark. Each block was transferred to a tube containing 1X β-agarase buffer (Bio-

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labs) in TE (final volume of 400 µL, including the volume of the agarose block), incubated for 15 min at 68°C, and transferred to 42°C for 15 min. For agarose digestion, 2.5 U β-agarase (Biolabs) was added to the sample and gently mixed. After incubating the solution for 1 h at 42°C, another 2.5 U β-agarase was added and incubated overnight at 42°C. The next day, 1.7 mL 0.25 M 2-(N-morpholino)ethanesulfonic acid solution (Sigma) was added and the sample was incubated for 30 min at 65°C. The tube was then cooled for 2 h at room temperature and stored at 4°C in the dark for periods ranging from a few hours to 4 weeks.

DNA processing on the molecular combing platform

Before using the DNA, we incubated the tubes at room temperature for 30 min and transferred them to the equipment tank. The DNA was combed onto silanized cover slips according to manufacturer instructions (Genomic Vision), placed on slides with cyanoacrylate superglue (Loctite), and incubated at 60°C for 2 h. The slides were then incubated at room temperature for 10 min and stored at -20°C before *in situ* hybridization.

Denaturation

DNA fibers from *B. hygida* salivary glands (isolated from glands of larvae at ages E1, E3, and E7) obtained by molecular combing technology were denatured by immersion in 1 N NaOH for 10 min. The reaction was stopped with rapid washing in 1X PBS at 4°C and subsequent washing in 1X PBS for 10 min with one exchange of buffer. The slides were then dehydrated with increasing ethanol concentrations (70, 85, and 100%) for 5 min in each bath. Slides prepared this way can be stored at 4°C until use.

Hybridization

For the hybridization, 1 µg from the clones 4-kb *Eco*RI and 5-kb *Hin*dIII, which contain the initial sequence of the *BhC4-1* gene and the downstream sequences, were linearized via digestion with *Eco*RI and *Hin*dIII (Biolabs), respectively, labeled with biotin using the BioNick Labeling System kit (Invitrogen), and purified on a G50 resin column (Roche). Hybridization was performed using 25 µL hybridization solution/slide, which contained 50 ng each labeled DNA, 18 µL hybridization buffer [10% dextran sulfate, 50% formamide, 2X saline-sodium-citrate solution (SSC 2X, 300 mM sodium chloride and 30 mM trisodium citrate)], 1% Tween-20, and 0.2 mg/mL competitor genomic DNA (human Cot-1 DNA, Invitrogen). The slides were incubated at room temperature for 15 min and the hybridization solution (pre-incubated with the probes for 10 min at 37°C) was placed on the slides, covered with a cover slip, and incubated for 5 min at 72°C, then sealed and incubated at 37°C overnight in a humid chamber. After hybridization, the slides were washed 3 times, 5 min each wash, at room temperature with 50% formamide in 2X SSC, pH 5.6, and then washed 3 additional times with 2X SSC, pH 7.0, and once with 1X PBS.

Immunodetection

Twenty-five microliters of blocking solution (1.5% blocking reagent [Roche] and 1X

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PBS with 0.05% Tween) was added to each slide, covered with a cover slip, and incubated for 30 min at 37°C in a humid chamber. After incubation, the cover slip was removed and the slide was washed 3 times with 1X PBS for 5 min each wash. This washing procedure was repeated between each incubation step with the fluorescent reagent or antibody. Samples were incubated for 30 min with 25 µL of the first detection solution containing streptavidin-Alexa Fluor 488 conjugate (Invitrogen) diluted 1:100 in blocking solution. After washing with PBS, as described above, the samples were incubated for 30 min with a 25-µL second layer detection solution containing biotin-conjugated rabbit anti-streptavidin antibody (Rockland/Tebu-bio) diluted 1:50 in blocking solution. The third and fifth detection layers were identical to the first layer described above, whereas the fourth was identical to the second layer. When the antibody incubations were complete, single-strand immunodetection was carried out using mouse anti-human single-stranded DNA antibody (Millipore) diluted 1:50 in blocking solution. Each sample was incubated with 25 µL solution, covered with a cover slip, and placed in a humid chamber at 37°C for 30 min. The second layer of detection was performed with goat anti-mouse-Cy5 (Abcam) diluted 1:100, and the third layer with donkey anti-goat-Cy5 (Abcam) diluted 1:100. After the last wash with 1X PBS, the slides were mounted with ProLong Gold antifade reagent (Molecular Probes).

RESULTS AND DISCUSSION

Systems that exhibit developmentally controlled gene amplification are potentially informative models for the study of mechanisms that regulate DNA replication and amplification. One such example is the formation of polytene chromosome DNA puffs in sciarid salivary glands. The molecular mechanisms involved in the control of DNA amplification are poorly understood, mostly owing to a lack of technologies for mapping functionally important elements in this process (Paçó-Larson et al., 2002). The combined use of N/A and N/N two-dimensional electrophoresis methods in the analysis of replication intermediates in DNA puffs permits the identification of regions with bidirectional replication origins, which are active during the amplification process in the DNA II/A puff of *S. coprophila* (Liang et al., 1993) and in the C3 puff of *R. americana* (Yokosawa et al., 1999).

The N/A electrophoresis method was used for the analysis of an 11-kb segment containing the transcription unit of the *BhC4-1* gene, which is amplified in the C4 puff of *B. hygida*. Using this technique, Coelho (1997) determined that the replication fork enters at the 5'-end of *BhC4-1* and moves unidirectionally through a 4-kb DNA fragment comprising the promoter region of this gene. However, the precise site of amplification initiation of this amplified segment cannot be determined using N/N and N/A two-dimensional electrophoresis (Coelho, 1997). Therefore, the development and application of a new method are needed for the identification of replication origins in this sciarid.

The purity and molecular size of the DNA sample are critical features of the molecular combing procedure (Michalet et al., 1997). In the protocol developed for this study, the salivary gland nuclei were not purified before DNA isolation to reduce the loss and degradation of starting material. Complete dissolution of the *B. hygida* larva salivary glands in buffer containing proteinase K is a method widely used when studying this sciarid and is routinely used in DNA preparations for quantitative and qualitative analyses (Monesi et al., 1995).

Our aim was to evaluate whether this strategy could be used for DNA sample prepara-

tion for molecular combing and thereafter to optimize the process to use less biological material and increase its efficiency. During salivary gland preparation from larvae of ages E1, E3, and E7, fat tissue was removed and the glands were incubated in 1X cold PBS. After dissection, the glands were immediately placed into PFGE molds and filled with low-melting-point agarose. The de-proteinization of glandular tissue was monitored using a stereomicroscope after incubation with ESP solution (Figure 1A and B).

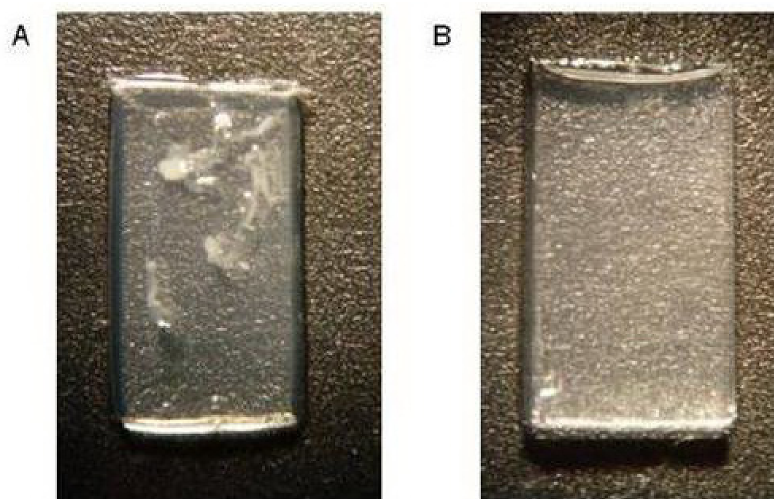

Figure 1. *Bradysia hygida* salivary gland agarose plugs. **A.** Agarose plug with salivary glands. **B.** Agarose plug with salivary glands digested with ESP solution.

Previous reports have stated that to prepare material for molecular combing of diploid cells, the optimal amount of DNA is obtained from 500,000 cells/50 μL low-melting-point agarose in the mold (Schurra and Bensimon, 2009). As each diploid mammalian cell has approximately 7 pg DNA (Gregory, 2005), this protocol results in blocks containing approximately 3500 ng DNA each. Based on this information, the number of salivary glands necessary for each block can be calculated. On average, because each *B. hygida* salivary gland cell has approximately 2.55 ng DNA and is composed of 100 cells, each pair of salivary glands has approximately 510 ng DNA (Paçó-Larson, 1976). Therefore, 6 pairs of glands yield 3060 ng DNA in an agarose block, which is approximately the amount required for the molecular combing technique. Our PFGE analysis confirmed that the direct preparation of DNA from *B. hygida* salivary glands yields high molecular size material suitable for use in the technique (data not shown).

After the digestion of the agarose blocks and their dissolution in appropriate buffer, the samples were used in the molecular combing process following a standard protocol (Michalet et al., 1997). The samples stored at 4°C were processed in separate batches based on the length of their storage (ranging from a few hours to 4 weeks). The appropriately combed DNA fibers were identified with fluorescence microscopy using YOYO®-1. No significant differences in fiber arrangement were found with respect to time of sample storage. By contrast, owing to the difference in the extent of amplification, the samples derived from the salivary gland at age E7 had a fiber density higher than that of samples from earlier larval ages (Figure 2).

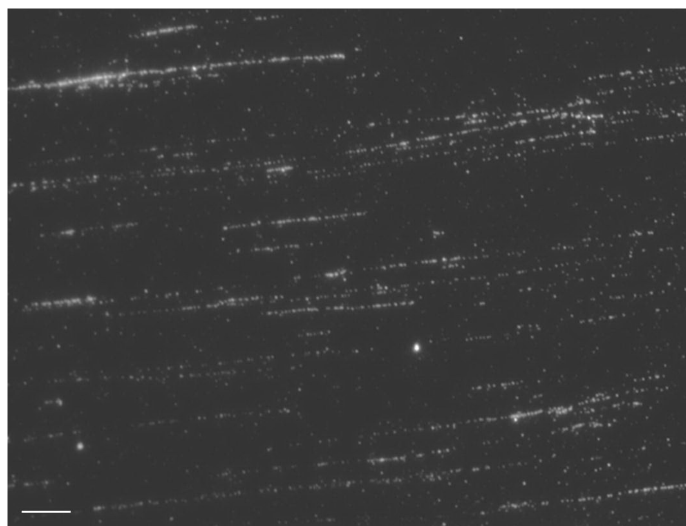

Figure 2. *Bradysia hygida* combed DNA array. Combed fibers from the salivary gland nuclei of E7 aged larvae were hybridized with mouse anti-ssDNA and detected with multiple layers of goat anti-mouse Cy5 and donkey anti-goat Cy5 antibodies. Bar = 5 µm (10 kb).

Our results indicate that the starting material must be doubled to obtain better results, even with the original DNA quantity of starting material we visualized DNA fibers ranging from 50 to 300 kb regardless of larval age (Figure 3). A series of slides representing samples of DNA fibers isolated from larvae of all ages was hybridized, and as expected, the *BhC4-1* domain was easily located on the E7 samples (Figure 4).

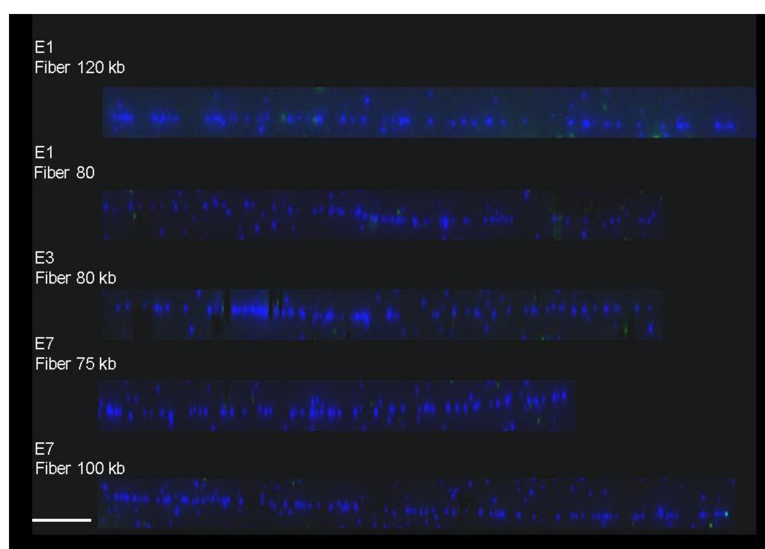

Figure 3. *Bradysia hygida* combed DNA fibers. The combed DNA fibers from the salivary gland polytene nuclei of E1, E3 and E7 larvae were hybridized with mouse anti-ssDNA and detected with layers of goat anti-mouse Cy5 and donkey anti-goat Cy5 antibodies. Bar = 5 µm (10 kb).

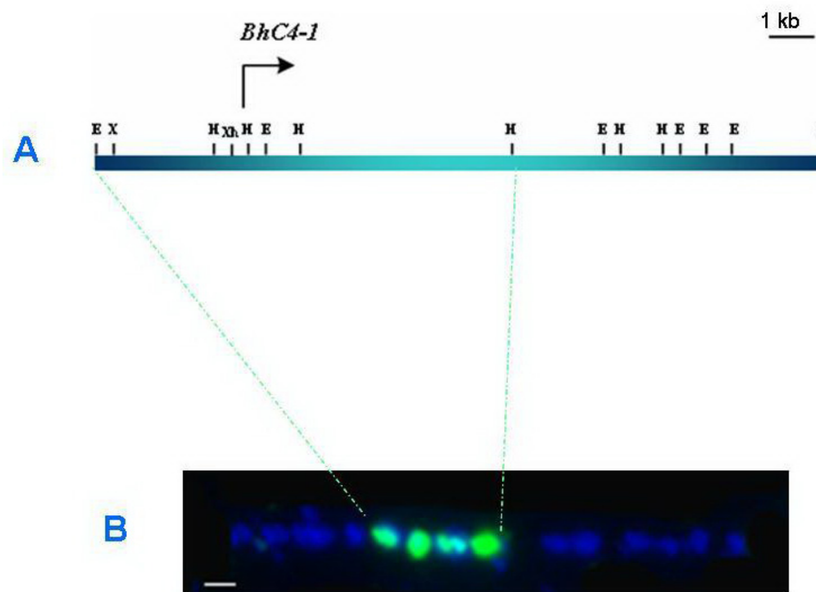

Figure 4. Combed *BhC4-1* amplicon. **A.** Physical map from the 4-kb *Eco*RI fragment, which contains the initial sequence of the *BhC4-1* gene, and the 5-kb *Hin*dIII segment, which includes the gene and downstream sequences. **B.** The combed fiber from salivary glands of E7 larvae was labeled with biotin and detected with fluorescein using routine methods. Bar = 1 µm (2 kb).

Our results also demonstrate the feasibility of preparing combed DNA samples without prior extraction of nuclei from *B. hygida* salivary glands. In samples prepared using this method, it will be possible to identify the sites of DNA replication initiation through fluorescent *in situ* hybridization using specific probes for the genomic region of interest combined with the detection of the incorporated halogenated nucleotides. Previous studies have reported the use of molecular combing technology for the analysis of DNA replication in *D. melanogaster* (Kolesnikova et al., 2009, 2010; Ivankin et al., 2011), but in contrast to the method implanted in this study, those procedures involved prior isolation of nuclei from the analyzed tissues.

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