Brazilian Journal of Medical and Biological Research (2001) 34: 851-859
ISSN 0100-879X

The DNA puff *BhB10-1* gene is differentially expressed in various tissues of *Bradysia hygida* late larvae and constitutively transcribed in transgenic *Drosophila*

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Abstract

We extended the characterization of the DNA puff *BhB10-1* gene of *Bradysia hygida* by showing that, although its mRNA is detected only at the end of the fourth larval instar, *BhB10-1* expression is not restricted to the salivary gland, the tissue in which this gene is amplified. Different amounts of *BhB10-1* mRNA were detected in other larval tissues such as gut, Malpighian tubules, fat body, brain and cuticle, suggesting that this gene is expressed differentially in the various tissues analyzed. Analysis of transgenic *Drosophila* carrying the *BhB10-1* transcription unit and flanking sequences revealed that the tested fragment promotes transcription in a constitutive manner. We suggest that either *cis*-regulatory elements are missing in the transgene or factors that temporally regulate the *BhB10-1* gene in *B. hygida* are not conserved in *Drosophila*.

Key words
- DNA puff
- Gene expression
- Development
- Ecdysone
- Transgenic *Drosophila*

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Research supported by FAPESP (No. 96/9378-9). J.F. Sousa was the recipient of a CAPES fellowship, N. Monesi was the recipient of a postdoctoral fellowship from FAPESP (No. 96/06418-0), and M.L. Paçó-Larson was the recipient of a CNPq fellowship (No. 520573/98-6).

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Received October 31, 2000
Accepted April 10, 2001

Introduction

DNA puffs are formed at discrete sites in the polytene chromosomes of sciarid salivary gland cells during the last larval instar. The molecular characterization of these genomic regions has demonstrated that they are sites of developmentally regulated gene amplification and transcription controlled by ecdysone (for a review, see Refs. 1-3).

Two groups of DNA puffs can be distinguished based on the time of puff expansion in *Bradysia hygida*. The first group expands around 26 h before the pupal molt (stage E7). Sixteen hours later, when the first group of puffs has regressed, the second group of DNA puffs reaches its maximum expansion. The only exception is the DNA puff B10 in which maximal expansion occurs during an intermediate time period between the first and second groups (4). A 2-kb *Eco*RI fragment from this puff, which contains the entire *BhB10-1* transcription unit, is amplified about 10-fold in the salivary gland during puff formation. The *BhB10-1* gene is complementary to two mRNA species of about 1.3 and 1.1 kb, whose expression accompanies the formation of DNA puff B10. The 1.3-kb mRNA species is initially detected at E7; however, 8 h later both mRNA species are present in the gland, and 16 h later only small amounts of the 1.1-kb mRNA species are

detected (5). Further characterization of the *BhB10-1* transcripts has demonstrated that the 1.3-kb mRNA species undergoes a poly A tail shortening process, giving rise to the 1.1-kb transcript, indicating that the *BhB10-1* gene is also regulated at the post-transcriptional level (6). In immunoblots, the BhB10-1 protein has been identified as a 23-kDa polypeptide present in the salivary gland and in the saliva of larvae at the time when the DNA puff B10 is formed (6).

The analysis of transgenic lineages of *Drosophila* carrying promoter sequences of the *II/9-1* gene of *Sciara coprophila* and the *BhC4-1* gene of *B. hygida* has indicated that the factors necessary for tissue-specific and developmentally regulated expression of sciarid DNA puff genes may be conserved in *Drosophila* (7,8). The transformed sequences so far tested are not amplified in the salivary gland of transgenic *Drosophila*, indicating that amplification and transcription of DNA puff genes are processes that can be uncoupled (7,8).

In the present study, we have extended the characterization of the DNA puff B10 gene of *B. hygida* by showing that the *BhB10-1* mRNA expression is induced at the end of the fourth larval instar in other larval tissues in addition to the salivary gland, the tissue in which the gene is amplified and the puff is formed. We have also analyzed the expression of the *BhB10-1* gene in six independent *Drosophila* transgenic lineages transformed with the 2-kb *Eco*RI genomic fragment from the B10 DNA puff, which contains the *BhB10-1* transcription unit and flanking sequences. We show that in these transgenic lineages the *BhB10-1* gene is ubiquitously expressed, suggesting that *BhB10-1* regulation is not maintained in *Drosophila*.

Material and Methods

Bradysia hygida

Larval tissues were obtained from female larvae of a laboratory culture kept at 20°C (9). Larval staging during the fourth larval instar, from stage E1 to E7, was based on eye spot pattern as described previously (4). Stage E1 occurs prior to DNA puff expansion and stage E7 corresponds to the time of the first DNA puff group formation. The DNA puff B10 is maximally expanded 8 h after E7. Whole animals were frozen in liquid nitrogen after staging, while larval tissues were dissected in insect saline and immediately frozen in dry ice/ethanol baths. Whole animals or tissues were kept at -80°C until processing.

Drosophila melanogaster

Drosophila lineages were kept at 25°C. Embryo collection and larval staging were as described by Ashburner (10). The time of puparium formation was used as the reference point for staging prepupae and pupae. White prepupae were removed from the food vials and maintained on Petri dishes on a moistened piece of filter paper. After the number of hours indicated for each experiment the animals were frozen in liquid nitrogen. For tissue collection, animals were dissected in insect saline solution and frozen in dry ice/ethanol.

Constructs and *P*-element transformation

The B1 construct consists of an ~2-kb *Eco*RI genomic fragment from DNA puff B10 of *B. hygida* (5), inserted into the *P*-element vector pCaSpeR 4 (11). The puff B10 fragment contains the *BhB10-1* transcription unit (876 bp) plus 816 bp of upstream flanking sequences and 241 bp of downstream flanking sequences. The B1 construct was injected into embryos of the *Drosophila melanogaster y*,*w* strain, together with the helper plasmid phsπ (12), at DNA concentrations of 0.5 and 0.1 µg/ml, respectively. *P*-element transformation was carried out following standard procedures (13,14).

Surviving G0 flies were mated individually with the parental strain and the resulting independent G1 transformants were used to establish homozygous lineages.

RNA extraction

Homogenization of frozen animals or tissues was performed in lysis buffer (10 mM Tris-HCl, pH 9.0, 2% SDS, 50 mM EDTA, 5% ethanol). After homogenization, total RNA was extracted by adding 10 volumes of Trizol, following the manufacturer's protocol (Gibco-BRL, Rockville, MD, USA). Total RNA concentrations were estimated by absorbance at 260 nm, assuming that 40 µg/ml RNA has an absorbance of 1.0.

Analysis of *BhB10-1* mRNA by Northern blot hybridization

Total RNA was fractionated on denaturing 1% agarose/formaldehyde gels (15) and blotted to a nylon membrane (Hybond N, Amersham Pharmacia Biotech, Uppsala, Sweden). Probes were labeled with [α^{32}P]-dCTP by random primer reactions. Hybridization was performed at 65°C in Church's buffer (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M $NaHPO_4$, pH 7.5). The final wash was 0.1 X SSC and 0.2% SDS at 65°C.

Ribonuclease protection assays

Ribonuclease protection assays (RPAs) were performed using the RPA II kit (Ambion, Austin, TX, USA), according to manufacturer instructions. The *BhB10-1* probe was a 316-bp fragment from the *BhB10-1* coding sequence (6). The actin probe consisted of a 650-bp fragment containing 5' noncoding and coding sequences of the *Drosophila* actin gene (16). The ribosomal probe was a 190-bp fragment from *B. hygida* 28S ribosomal DNA (NCBI accession number AF147103). After linearization of the template, *in vitro* transcription was performed in the presence of [α^{32}P]-UTP, using the MAXIscript T7/T3 kit (Ambion). The resulting *BhB10-1* transcript was 411 bp long (316 bp of *BhB10-1* sequences plus 95 bp from plasmid sequences), the resulting actin transcript was 701 bp long (650 bp of actin sequences plus 51 bp from plasmid sequences) and the resulting *B. hygida* ribosomal probe was 285 bp long (190 bp of rDNA sequences plus 95 bp from plasmid sequences). The *in vitro* transcribed probes were gel purified before use. The protected fragments were electrophoresed on 6% acrylamide/8 M urea gels.

Results

Developmental pattern of *BhB10-1* gene expression in *B. hygida*

In order to extend the characterization of *BhB10-1* gene expression in *B. hygida* we performed RPAs in whole animals at different developmental times and in salivary glands and carcasses of larvae at stage E7 + 8 h when the gene is abundantly transcribed in the salivary gland (5). The probe complementary to the *BhB10-1* mRNA is 411 bp long and results in the protection of a 316-bp fragment. As can be seen in Figure 1, the hybridization of total RNA from embryos, whole second- and third-instar larvae, whole fourth-instar larvae at stages E1, E7 and E7 + 8 h, whole pupae, and whole adults resulted in the protection of the 316-bp fragment only in samples obtained from late fourth-instar larvae (E7, E7 + 8 h) and early pupae. In agreement with previous data on the expression of *BhB10-1* mRNA in *B. hygida* salivary glands (5), the highest transcript levels were found at stage E7 + 8 h, i.e., coinciding with the formation of the DNA puff B10 in the salivary gland polytene chromosomes (Figure 1). Unexpectedly, we also detected high levels of *BhB10-1* mRNA in total RNA extracted from carcasses of larvae at stage E7 + 8 h (Figure 1), suggesting that this gene

is also expressed in larval tissues other than the salivary gland at the time when the corresponding DNA puff is formed.

In order to further investigate the expression of the *BhB10-1* gene, we analyzed its mRNA in Northern blots containing total RNA of different tissues of larvae at stages E1 and E7 + 8 h. As shown in Figure 2, the *BhB10-1* mRNA is detected only at stage E7 + 8 h and is present in different amounts in brain, gut, Malpighian tubules, fat body, integument, and salivary glands of larvae at stage E7 + 8 h. These results show for the first time that the transcript of a DNA puff gene is expressed in other tissues besides the salivary gland, tissue in which the gene is specifically amplified.

BhB10-1 gene expression in transgenic *Drosophila*

In order to obtain a system to investigate molecular mechanisms that regulate the *BhB10-1* gene, we obtained *Drosophila* transgenic lineages carrying a 2-kb genomic fragment from DNA puff B10. This fragment contains the entire *BhB10-1* transcription unit, 816 bp of upstream flanking sequences, and 241 bp of downstream flanking sequences. Six independent transgenic lineages, PB1-4, PB1-5, PB1-6, PB1-9, PB1-28 and PB1-33, were obtained. With the exception of lineage PB1-5, which harbors two copies of the transgene, all the other lineages contain a single copy of the transformed construct, as verified by Southern blot analysis (data not shown). The six transgene homozygous lineages are viable and we did not notice any morphological or behavioral changes.

BhB10-1 expression in the transgenic lineages was investigated by RPAs. The probe complementary to *BhB10-1* mRNA resulted in the protection of a 316-bp fragment which was detected in lineage PB1-33, but was absent in the *y*,*w* strain, indicating that there is no endogenous expression of the transgene in the parental strain (Figure 3, lanes B10). The antisense actin probe, employed as a loading control, is 650 bp long and results in the protection of a 260-bp fragment (Figure 3, lanes act).

The *BhB10-1* mRNA is ubiquitously expressed in lineage PB1-33. It is detected in embryos, first-, second- and third-instar larvae (L1/2, L3), during the prepupal (0, +3, +6, and +9 h), and pupal period (24 h pupa) and in adults of both sexes (Figure 3). This pattern of expression was demonstrated in the other five independently obtained lineages (data not shown), showing that the 2-kb fragment of *B. hygida* is able to drive transcription of the *BhB10-1* gene in *Drosophila.*

In order to determine whether the *BhB10-1* gene was expressed differentially in the salivary glands and carcasses of the transgenic *Drosophila*, steady-state expression levels were analyzed in salivary glands and carcasses from third-instar larvae, and prepupae at 0, +3, +6, and +9 h using RPAs (Figure 4). In the PB1-33 lineage, the *BhB10-1* mRNA was expressed at similar levels in the salivary gland and in carcasses throughout the developmental period analyzed (Figure 4). The stronger signal in carcasses was due to the different amounts of total RNA from carcasses employed in the assay (30 µg) as opposed to those employed for salivary gland samples (3 µg). Similar results were obtained for another independent transgenic lineage, PB1-5.

Taken together, the results obtained from the analysis of transgenic *Drosophila* lineages transformed with the 2-kb fragment from DNA puff B10 of *B. hygida* indicate that the transformed fragment contains elements which are able to promote *BhB10-1* transcription. However, in contrast to *B. hygida*, transgenic *Drosophila* express the *BhB10-1* mRNA in a constitutive manner throughout development, regardless of stage or tissue.

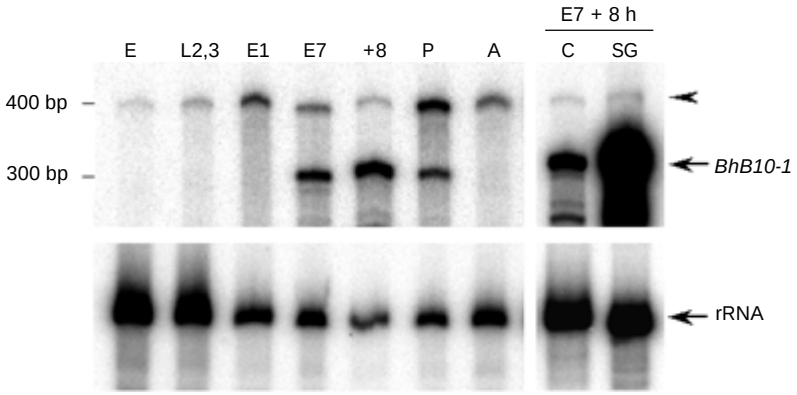

Figure 1. Developmental pattern of expression of the *BhB10-1* gene in *Bradysia hygida*. *Upper autoradiograms*, 50 µg of total RNA extracted from embryos (E), second- and third-instar larvae (L2,3), fourth-instar larvae at stage before DNA puff formation (E1) and adults (A); 2.5 µg of total RNA from whole fourth-instar larvae at the time when the first group of DNA puffs expands (E7), whole fourth-instar larvae (+8) or carcasses (C) from larvae 8 h after E7 when puff B10 is maximally expanded and young pupae (P), and 0.25 µg of total RNA extracted from salivary glands (SG) of larvae at stage E7 + 8 h. The RNAs were analyzed with a ribonuclease protection assay (RPA) with an antisense probe complementary to the *BhB10-1* gene. The protected fragment is 316 bp long (*BhB10-1,* arrow). The higher molecular weight fragment indicated by the arrowhead is the undigested *BhB10-1* probe. *Lower autoradiograms*, 10 ng of total RNA extracted from the same developmental stages and tissues as described above was analyzed by RPA with an antisense probe complementary to the 28S rRNA of *B. hygida*. Due to the abundance of the rRNA it was necessary to independently hybridize a dilution of the same RNA samples (10 ng) used in the above RPA with the rRNA probe employed as control for the amount of total RNA in the assay. The protected fragment is 190 bp long (rRNA, arrow). The migration of the RNA size markers is indicated on the left. The left side autoradiograms were exposed for 5 days, whereas the autoradiograms on the right side were exposed for 8 days, which accounts for the stronger signal in lanes (E7 + 8 h C/E7 + 8 h SG).

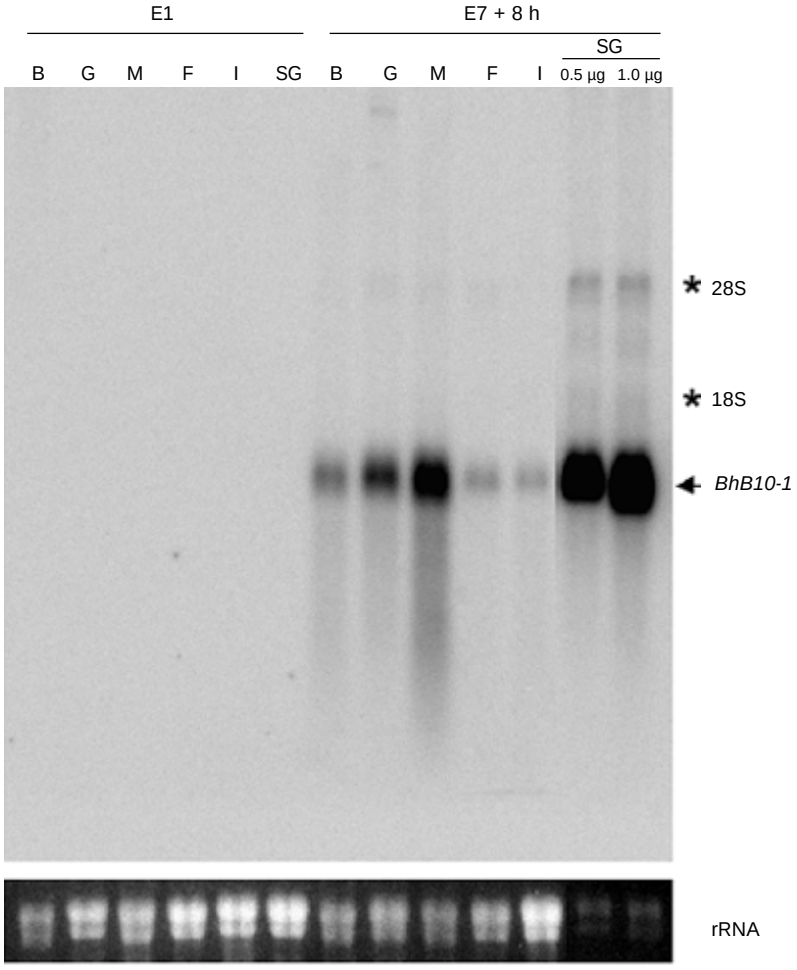

Figure 2. Northern blot characterization of *BhB10-1* mRNA expression in different tissues at the end of the fourth larval instar of *Bradysia hygida*. *Upper autoradiogram*, Northern blot after hybridization with the 2-kb genomic fragment from DNA puff B10, which contains the *BhB10-1* transcription unit (5). The lanes contain about 7 µg of total RNA extracted from brain (B), gut (G), Malpighian tubules (M), fat body (F) and integument (I) of larvae at a stage before puff formation (lanes E1) and larvae at the stage when puff B10 is maximally expanded (lanes E7 + 8 h), about 7 µg of total RNA extracted from salivary glands (SG) of larvae at E1, and 0.5 µg and 1.0 µg of total RNA extracted from salivary glands of larvae at E7 + 8 h (lanes SG). The arrow points to the *BhB10-1* mRNAs ranging from 1.3 to 1.1 kb. The extra bands of higher molecular weight are not reproducible. The lower picture is an image of the gel before blotting to show the loading of the lanes. The migration of the 28 and 18S mammalian ribosomal RNA, employed as molecular size markers, is indicated by asterisks on the right.

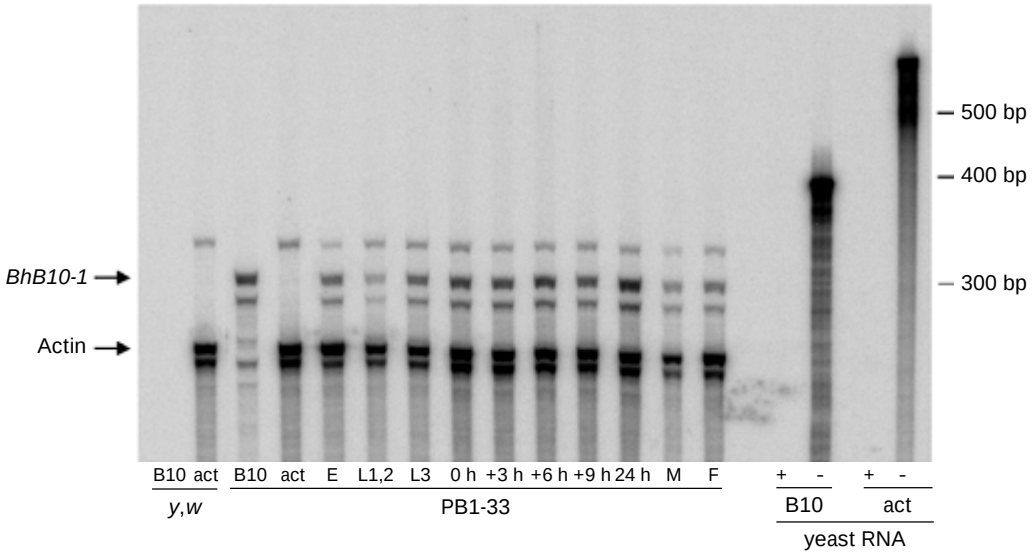

Figure 3. Developmental pattern of expression of *BhB10-1* mRNA in the transgenic lineage PB1-33. Forty micrograms of total RNA extracted from embryos (E), first- and second-instar larvae (L1,2), third-instar larvae (L3), prepupae at 0, 3, 6, and 9 h (0, +3, +6, and +9 h), pupae at 24 h (24 h), adult males (M) and adult females (F) from transgenic lineage PB1-33 was analyzed by RPA using two different radiolabeled probes. Probe (B10) is a 411-bp long RNA complementary to the *BhB10-1* gene which results in the protection of a 316-bp long fragment (*BhB10-1*, arrow). Probe (act) is a 700-bp long RNA complementary to the *Drosophila* actin gene, and the protected fragment is 260 bp long (actin, arrow). In lanes *y*,*w*, a mixture containing equal amounts of total RNA extracted from prepupae at 0, 3, 6 and 9 h from the parental strain *y*,*w* was separately hybridized with each of the probes (*y*,*w*/B10 and *y*,*w*/act). Note the absence of endogenous *BhB10-1* expression in the parental strain *y*,*w*. In lanes PB1-33/B10 and PB1-33/act, 40 µg of a mixture containing equal amounts of total RNA extracted from prepupae at 0, 3, 6 and 9 h from the transgenic lineage PB1-33 was hybridized with probes (B10) or (act) as indicated. In lanes yeast RNA, 10 µg of yeast RNA (negative control) was hybridized with the same amounts of probes (B10) and (act) that were employed in the assay and treated (B10/+, act/+) or not (B10/-, act/-) with ribonuclease. Lanes (-) show the size of the unprotected probes. The migration of the RNA size markers is indicated on the right.

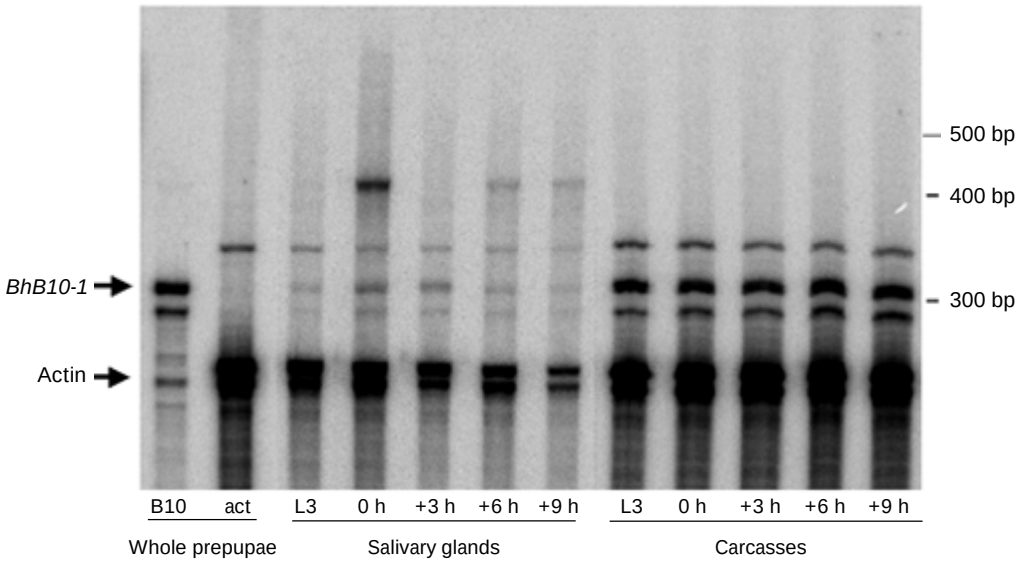

Figure 4. The *BhB10-1* mRNA is expressed in a constitutive manner in the salivary gland and in carcasses of late third-instar larvae and prepupae of the transgenic lineage PB1-33. Autoradiogram of an RPA in which 3 µg of total RNA extracted from salivary glands of third-instar larvae (L3) and prepupae at 0, 3, 6, and 9 h (0, +3, +6, and +9 h), or 30 µg of total RNA extracted from carcasses of third-instar larvae and prepupae at 0, 3, 6, and 9 h from the transgenic lineage PB1-33 was simultaneously hybridized with two radiolabeled probes. Probe (B10) is a 411-bp long RNA complementary to the *BhB10-1* gene and the protected fragment is 316 bp long (*BhB10-1*, arrow). Probe (act) is a 700-bp long RNA complementary to the *Drosophila* actin gene and the protected fragment is about 260 bp long (actin, arrow). In lanes (whole prepupae), 30 µg of a mixture containing equal amounts of total RNA extracted from whole prepupae at 0, 3, 6 and 9 h from transgenic lineage PB1-33 was hybridized with probes (B10) or (act) as indicated. The migration of the RNA size markers is indicated on the right.

Discussion

The developmentally regulated gene amplification that occurs in sciarid DNA puffs is generally interpreted as an evolutionarily selected mechanism which regulates the production of specific proteins required in large amounts during short periods of time (for a review, see Refs. 1-3). This view has been supported by the strict temporal correlation observed between amplification and activation of transcription of several DNA puff genes in the salivary glands of last-instar larvae (5,17-20), and implies a tissue-specific expression of the amplified genes. Our results showing that the transcription of an amplified DNA puff gene is not restricted to the larval salivary glands add a new perspective to this interpretation. The *BhB10-1* mRNA is detected by Northern blot hybridization in various other tissues of *B. hygida* late larvae, albeit in lower amounts than in the salivary gland in which the gene is amplified (5).

The expression of *BhB10-1* mRNA in larval tissues other than the salivary gland also has implications for the understanding of the biological role of the encoded protein. Although there are indications that DNA puff products constitute secretory polypeptides produced by the salivary gland (6,21-23), the function of these proteins is still unknown. Comparison of DNA puff polypeptide sequences with protein data bases results in no significant matches with other known protein sequences, which places DNA puff proteins in a distinct class of proteins (24). In previous work we have identified the *BhB10-1* gene product as a 23-kDa polypeptide in immunoblots of salivary glands from late larvae (6). However, using the same antibody we were unable to detect the 23-kDa polypeptide in tissues other than the salivary gland (data not shown), which could be attributed to the absence of BhB10-1 protein in these tissues. Alternatively, the failure in detecting BhB10-1 protein in other larval tissues could be due to the presence of lower amounts of the protein in these tissues. Favoring this last possibility is the observation that the BhB10-1 protein is detected in the salivary gland regions S1 and S3, but not in the S2 region (6). In the gland region S2, as in the other larval tissues analyzed in the present study, *BhB10-1* mRNA is present at much lower levels. Further investigation is needed to verify if the BhB10-1 protein is expressed in other larval tissues or if the regulation of the *BhB10-1* gene also includes mechanisms that restrict mRNA translation to the salivary gland.

The observation that the *BhB10-1* mRNA is detected at the same time in all tissues of late larvae analyzed suggests that the activation of the *BhB10-1* gene in these tissues is triggered by a common factor. A strong candidate for the activation of the *BhB10-1* gene at the end of the larval stage is ecdysone which is known to regulate DNA puffing in sciarid polytene chromosomes (25-28), and which induces DNA puff B10 formation when injected into early larvae (Fontes AM and Paçó-Larson ML, unpublished data). The observation of a peak in the ecdysteroid titer approximately 26 h before the pupal molt in *B. hygida* (Hartfelder K, Basso Jr LR and Paçó-Larson ML, unpublished data) also agrees with the idea that the induction of *BhB10-1* mRNA expression in all tissues of late larvae may be regulated by ecdysone. The higher levels of *BhB10-1* mRNA observed in the salivary gland are consistent with the presence of 10-fold more copies of the *BhB10-1* gene in this tissue (5). However, since amplification of the *BhB10-1* gene is detected only in the salivary gland (data not shown), the presence of different amounts of *BhB10-1* mRNA in the tissues analyzed could suggest that the regulation of *BhB10-1* may involve tissue-specific regulators. Another possibility is that the *BhB10-1* mRNA half-life differs between the analyzed tissues. In this context it is interesting to note that previous studies have shown that

the *BhB10-1* mRNA undergoes poly A tail shortening during development, suggesting that post-transcriptional controls as well as transcription activation are involved in the regulation of the *BhB10-1* gene (6). Taken together, the results presented here indicate that the control of the expression of amplified DNA puff genes is more complex than previously thought. A given gene can be expressed at different levels in various larval tissues at the time when the DNA puff is expanded in the salivary glands.

By employing *Drosophila* germ line transformation it has been shown that the factors involved in promoter regulation of the DNA puff gene *II/9-1* of *S. coprophila* (7), and the *BhC4-1* gene of *B. hygida* (8) are conserved between sciarids and *Drosophila*. In both cases, the promoter region needed for the temporal control of transcription in the salivary gland is not longer than 718 bp. In the present study, we show that a 2-kb fragment containing the *BhB10-1* transcription unit is capable of promoting constitutive transcription in *Drosophila*. However, the pattern of *BhB10-1* mRNA expression in the transgenic *Drosophila* lineages is not the same as that observed in *B. hygida*. In the transgenic lineages, *BhB10-1* mRNA was detected at all stages and in all tissues analyzed, suggesting that the factors involved in the regulation of the *BhB10-1* gene are not present in these animals. The absence of *cis*-regulatory elements in the transformed 2-kb fragment could explain these results. If this is the case, one might expect that the elements which activate *BhB10-1* transcription in *B. hygida* last-instar larvae would be located outside the tested fragment (positions -817 bp to +1116 bp, determined in relation to the *BhB10-1* gene transcription initiation site (6)). With this in mind, it is interesting to note that the *BhB10-1* 2-kb fragment contains an AT-rich sequence which is the only feature shared between the 5' flanking sequences of DNA puff genes (24), including the two DNA puff constructs which were expressed in a regulated manner in *Drosophila* (7,8). Alternatively, the absence of an increase in the steady-state levels of *BhB10-1* mRNA during the prepupal stage in transgenic *Drosophila* could be indicating that the factors involved in the induction of the *BhB10-1* gene in *B. hygida* late larvae are not conserved between *B. hygida* and *Drosophila*. The analyses of transgenic lineages transformed with fragments including larger portions of *BhB10-1* gene-flanking regions will help to clarify this question.

Acknowledgments

We thank Cirlei A.V. Saraiva and Benedita O. de Souza for dedicated technical assistance, Valdir Mazzucatto for the maintenance of the *Drosophila* room and Cláudia R.M. Faggion and Domingos S.S. Filho for maintenance of the *B. hygida* culture. We also thank Dr. K. Hartfelder and Dr. R.J. Ward, from FFCLRP/USP, and Dr. Ann Stocker, from IB/USP, for reviewing the manuscript.

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