Grand Valley State University

ScholarWorks@GVSU

2009

Construction of a transgene to analyze the function of the 3'UTR of the Hdc gene on spatial expression of histidine decarboxylase in Drosophila melanogaster

Embriette Hyde
Grand Valley State University, hydee@mail.gvsu.edu

Martin G. Burg
Grand Valley State University, burgm@gvsu.edu

Construction of a transgene to analyze the function of the 3'UTR of the *Hdc* gene on spatial expression of histidine decarboxylase in *Drosophila melanogaster*

Embriette R. Hyde and Martin G. Burg,
Dept. of Biomedical Science, Grand Valley State University

ABSTRACT: Histamine has been shown to be an important neurotransmitter used in the nervous system of *Drosophila melanogaster.* Histidine decarboxylase (HDC) is the enzyme that catalyzes the synthesis of histamine. All genetic information necessary for *Hdc* gene expression has been shown to be present in a 9.4kb genomic DNA fragment. A previous study fused the 5'-UTR of *Hdc* to the gene encoding eGFP. Microscopic analysis of flies transformed with the pHdc-eGFP transgene showed a weak pattern of eGFP expression in the nervous system as compared to histamine localization, indicating that another genomic region may be necessary for robust *Hdc* expression. Studies in other systems have suggested that the 3'UTR of a gene may play an important role in its expression. Current efforts are focused on the construction of a transgene that fuses the 3' UTR of *Hdc* to the existing pHdc-eGFP transgene. Examination of GFP expression in flies bearing this new transgene (pHdc-eGFP-3'UTR) may demonstrate the role that the 3' UTR has in *Hdc* expression.

INTRODUCTION: When a mutation disrupts a gene, the function of the gene may be revealed through changes in how the organism functions. By studying both the mutant and its genetically normal counterpart, the identification of the protein and the process that is disrupted may be revealed at the molecular level. The mutation may be found to target the gene itself or could affect the regulatory region of the gene that controls its expression. These regulatory regions, known as promoters and enhancers, are critical to the regulation of the gene's expression pattern, both spatially and temporally. It is through the identification and characterization of these regulatory elements that the regulation of gene expression can be better understood.

There are many mutations, representing about 30 genes, which have been identified in *Drosophila melanogaster* that are thought to disrupt synaptic transmission between photoreceptors and their target interneurons. These mutations were identified by alterations in the electrical response to photoreceptor activity in the visual system of Drosophila (1). These mutations disrupt the on-/off- transient components of the electroretinogram (ERG), which is an extracellular recording of the light-evoked mass response of the retina. From among this group of mutations, the gene encoding histidine decarboxylase (*Hdc*) was identified as being functionally important for neuronal communication (2, 3). Histidine decarboxylase catalyzes the synthesis of histamine from histidine. The identification of this mutant provided definitive evidence that histamine is the neurotransmitter used by photoreceptors and other peripheral sensory receptor cells, as the mutants are rendered functionally blind (4). Using rabbit polyclonal histamine antibodies (5), examination of wild type flies

indicated that histamine was present in both the brain and visual centers but was not detected in some of the *Hdc* mutants (3-7). Using the germ-line transformation technique of *Drosophila*, introduction of a 9.4 kb genomic DNA fragment thought to contain the wild type *Hdc* gene into the mutants achieved "mutant rescue" (8-10). This result demonstrated that all of the regulatory information needed for normal *Hdc* gene expression was confined to this 9.4 kb fragment (8-10). Recently, we were able to construct a novel gene, pHdc-eGFP, which consists of the 5' promoter region of *Hdc* fused to the marker protein eGFP, and used it to transform flies (11). Initial fluorescent microscopy examination of eGFP expression in the transgene bearing flies indicated fluorescent cells appeared in locations where histaminergic cells were normally located; however, not all cells were visualized that were identified with histamine staining (Fig. 1; see also 12). This result, along with recent studies in other species (13), suggests the importance of the 3'UTR in gene expression, indicating that the 3'UTR may contain regulatory regions necessary for full expression of eGFP in histaminergic cells.

MATERIALS AND METHODS:

Restriction endonuclease digests: All restriction endonuclease digest reactions were performed using restriction endonucleases and buffers supplied by Promega Corporation (Madison, WI). Single digests were performed by adding 14μl of double de-ionized water, 2μl of buffer, 2μl of DNA template, and 2μl of restriction endonuclease (always added last) to a 0.6 ml microtube. The reactions were then incubated for one hour in a 37^0C water bath.

PCR: Polymerase Chain Reaction (PCR) was performed utilizing Roche Applied Sciences FastStart High Fidelity PCR system. 5μL each of 10X HF buffer, forward primer, and reverse primer (0.4μM each) were added to the reaction tube along with the DNA template (volumes varied by reaction) and PCR grade water up to a total reaction volume of 50μL The Eppendorf MasterCycler was used and programmed as follows: the initial denaturation at 95^oC for 2 minutes was followed by 35 cycles of: 30s denaturation at 95^oC, 30s of annealing at specified primer melting temperature, and 30s-3min of elongation at 72^oC (time of elongation varied with DNA fragment length). A final elongation at 72^oC for 4 minutes completed the reaction.

PCR Purification: PCR purification was performed on fragments generated by restriction endonuclease digests as well as PCR reactions. Qiagen's QIAquick PCR Purification Kit was used in the following manner: 5 volumes of PB1 Buffer was added to 1 volume of digest or PCR product in a 1.7 ml tube and mixed. This mixture was added to a QIAquick Spin column in a 2 ml collecting tube and centrifuged at 14,000 rpm for one minute. The flow-through was discarded and 0.75 ml Buffer PE was added to the column and centrifuged at the same speed for one minute. The flow-through was discarded and centrifugation repeated. The column was then placed in a clean 1.7 ml tube and 50 μl double deionized water was added to the column followed by one minute of centrifugation at the same speed. Resulting DNA was stored at -20^oC.

TA Cloning: TA cloning was used to isolate the 5' promoter region of *Hdc*, the coding region of GFP, and the 3' UTR of *Hdc*. Each respective fragment was TA cloned using Promega's pGEM-T Easy Vector System. For ligations, DNA to vector concentration ratios varied from 3:1, 1:1, and 1:3. Promega's 2X Rapid Ligation buffer was used at a volume of 5 µl per reaction. 1 µl of pGEM-T Easy Vector was added to the reaction, and the volume of DNA insert varied with each reaction (in the range of 0.3-1.0 µl). Double de-ionized water was added to bring the total reaction volume to 10 µl. Ligation reactions were allowed to incubate at 4°C overnight (at least 8 hours). For cell transformations, Promega's JM 109 *E. coli* High Efficiency Competent Cells were used. 2 µl of each ligation reaction was added to a 1.7 ml Microtube on ice followed by the addition of 50 µl of Competent Cells. The mixture was chilled on ice for 20 minutes and then the cells were heat shocked for 45-50 seconds at 42°C. The cells then were placed on ice for another 2 minutes. At the end of this incubation period, 950 µl room temperature SOC media was added to each tube and the cells were incubated for one and one half hours at 37°C with shaking at approximately 150 rpm. After incubation, 50 µl, 100 µl, and 200 µl volumes of each tube were plated onto two LB-Ampicillin plates and incubated at 37°C for 12-16 hours.

Subcloning: Once the 5' promoter of *hdc,* the coding region of GFP, and the 3'UTR of *Hdc* were each TA cloned into the pGEM-T Easy Vector, a series of subcloning reactions was performed in an attempt the ligate the three fragments together. The insert fragment was isolated from the pGEM-T Easy vector by a double digest with the same restriction endonucleases used to open the vector that the fragment was to be ligated into. A typical ligation reaction used 1 µl of NEB's 10X T4 DNA Ligase Buffer, 1 µl each of vector and insert DNA, 1 µl of NEB's T4 DNA Ligase, and 7 µl double deionized water for a total reaction volume of 10 µl. Ligation reactions were incubated for 10 minutes at room temperature according to New England Biolabs' protocol for sticky end ligations.
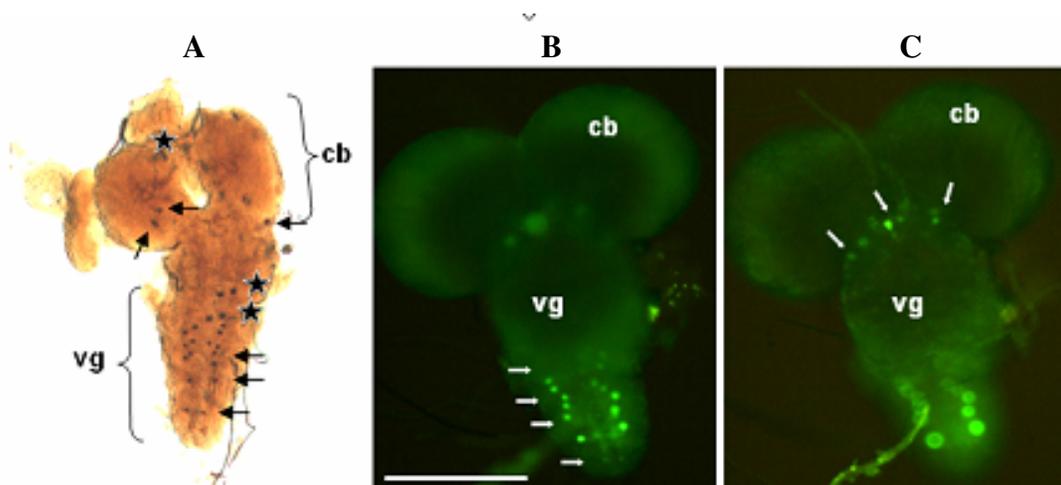
Figure 1: Images comparing histamine staining in a wild type *Drosophila* strain (A) to GFP expression in flies transformed with the pHdc-eGFP transgene. Histamine staining (A) is indicated by a dark brown color. Arrows in (A) indicate cells that also express GFP in the transformants (B and C). Stars in (A) indicate cells that do not express GFP in the transformants and are absent in (B) and (C). Bar in (B) indicates 50 µm.

Figure 2. Structure of the *Hdc* gene and location for the eGFP protein within the *Hdc* gene structure in the two pHdc-eGFP gene fusions. A) *Hdc* gene structure, indicating the protein coding region (shaded) and the 5'UTR and 3'UTR regulatory regions. B) Structure of the pHdc-eGFP transgene that has been transformed into flies which demonstrates partial but not complete Hdc expression. (C) Structure of the proposed pHdc-eGFP-3'UTR transgene to be generated through this work and used to transform flies.

Plasmid Mini-Preps: DNA was isolated from the plasmids resulting from TA Cloning reactions by utilizing Qiagen's QuickLyse Miniprep Kit and associated protocol. An isolated colony was incubated for 12-16 hours in 2 ml LB-Ampicillin broth at 37°C with shaking at 250 rpm. 1.5 ml of this culture was then added to a 2 ml tube and centrifuged at 14,000 rpm (this speed was used for all subsequent centrifugations) for one minute to pellet bacterial cells. The medium was removed from the pelleted cells and 400 μl ice cold lysis solution was added to the cells and mixed thoroughly by vortexing at high speed for 30 sec. This lysate was then transferred to a QuickSpin column and centrifuged one minute. 400 μl diluted Buffer QLW was then added to the column followed by another minute of centrifugation. The flow through was discarded at this point and another one minute centrifugation was performed to dry the column. The column was then transferred to a clean 1.7 ml tube and 50 μl water were added to the column. The tube was centrifuged for one minute and the resulting DNA stored at -20°C.

Gel electrophoresis: DNA products were identified by running the sample on a 1% agarose gel in a TAE buffer containing EtBr followed by visualization with a UV camera and Kodak ID software. The DNA samples were loaded into the wells of the gel with 6X loading dye. The gel was run at 80V for 2-3 hours before visualization.

RESULTS:

Isolation of the promoter region of *Hdc* plus the open reading frame of eGFP: A restriction endonuclease digest followed by PCR was utilized to excise the promoter region of *Hdc* plus the open reading frame of eGFP from the pGreenPelican + p*Hdc* plasmid created previously. Initial efforts to excise this fragment involved a double digest reaction using NcoI and XbaI, which are located at the 5' and 3' ends of the ORF, respectively (sequence analysis verified). Due to probable methylation of the XbaI site; however, the expected 750bp NcoI-XbaI fragment was not isolated. Therefore, a second digest reaction was attempted utilizing

NheI and SpeI, including the promoter region of *Hdc* upstream of the eGFP ORF and 3' to the termination codon of GFP, respectively (Figure 3, Step 1). Agarose gel electrophoresis indicated that the 1,132 bp NheI-SpeI fragment had been successfully isolated. Band extraction techniques were then utilized to purify the open plasmid fragment (approximately 13 kb) for later use. Primers pHdceGFPNheI-1 and pHdceGFPSpeI-1 were utilized in a PCR reaction with the pGreenPelican + p*Hdc* plasmid as the DNA template in order to isolate a pure NheI-SpeI fragment. Agarose gel electrophoresis indicated that the resulting PCR product was the anticipated NheI-SpeI fragment.

Amplification and Sequencing of the NheI-SpeI fragment: The 1,132bp NheI-SpeI fragment was TA cloned into the pGEM-T Easy Vector for rapid amplification (Figure 3, Step 1). Plasmid DNA was isolated from bacterial cells as described in Materials and Methods. Samples from different colonies were then prepared for sequencing, which was performed by Retrogen, Inc. (San Diego, CA). Sequencing results of this fragment were compared to same regions of the genomic DNA (the promoter of *Hdc* and the entire genomic region of GFP) to ensure that the PCR reactions did not introduce errors in the DNA sequence.

Division of the NheI-SpeI fragment into two fragments and the addition of restriction endonuclease sites: Two primers, the previously utilized pHdceGFPNhe1-1 and the primer ReverseLinear1 were utilized to PCR amplify a 917 bp fragment from the pGEM-T Easy plasmid containing the NheI-SpeI fragment. The resulting fragment contained the promoter of *Hdc*

and the ORF of GFP up to the first termination codon. The region of GFP 3' to this termination codon and including the second termination codon was also PCR amplified from the same DNA template utilizing the primers ForwardLinear1 and pHdceGFPSpeI-1. Agarose gel electrophoresis indicated that both fragments (917 bp and 286 bp, respectively) had been successfully isolated from their plasmids. A BglII restriction endonuclease site was then engineered into the 917 bp fragment utilizing the primers pHdceGFPNheI-2 and HindBgl2rev in a PCR reaction. The resulting fragment was characterized by the presence of an NheI site at its 5'end and a BglII site at its 3'end. The fragment was then TA cloned into the pGEM-T Easy Vector. An XbaI restriction endonuclease site was engineered into the 286 bp fragment utilizing the primers HindXbaIfor and pHdceGFPSpeI-2 in a PCR reaction. The resulting fragment was characterized by the presence of an XbaI site at its 5'end and a SpeI site at its 3'end. The fragment was TA cloned into the pGEM-T Easy vector.

Isolation of the 3'UTR of *Hdc*: PCR was utilized to isolate the 3'UTR of *Hdc* from the plasmid g*Hdc* + pCasper3, which contains the entire genomic region of *Hdc* (Figure 3, Step 2). Primers 5' and 3' to the 3'UTR (Hdc3'for1 and Hdc3'rev1) were designed and utilized in this PCR reaction. Polyacrylamide gel electrophoresis indicated that the approximately 750 bp fragment had been successfully isolated from the g*Hdc* + pCasper3 vector. The fragment was PCR purified and used as the DNA template for a second PCR reaction in which two different primers (Hdc3'for2 and Hdc3'rev2) were utilized to engineer

Step One:

pGreenPelican + pHdc → 1.13kb NheI-SpeI GFP fragment (NheI ... SpeI) + pGem-T Easy Vector (3,015bp) → pGem-T Easy Vector + NheI-SpeI GFP fragment

Step Two:

pCasper3-gHdc → Hdc 3'UTR (XbaI) → Hdc 3'UTR + BglII site (BglII ... XbaI) → pGEM-T Easy + Hdc-3'UTR

Steps Three and Four:

pGem-T Easy + NheI-TAA (NheI, TAA) + pGem-T Easy + TAG-SpeI (TAG, SpeI) + pGem-T Easy + 3'UTR → Plasmid with eGFP and Hdc 3'UTR (NheI, SpeI)

Step Five:

Plasmid with eGFP and 3'UTR (NheI, SpeI) → NheI-SpeI-GFP-3'UTR fragment (NheI ... SpeI) → Final transgene: pHdc-eGFP-3'UTR (NheI, SpeI)

Figure 3: Experimental design for the construction of the transgene pHdc-eGFP-3'UTR. Step One describes the isolation of the NheI-SpeI fragment containing the promoter of *hdc* and the ORF of GFP from the pGreenPelican + pHdc plasmid and the subsequent TA cloning of the fragment into the pGEM-T Easy Vector. Step Two describes the isolation of the 3'UTR of *hdc* from the pCasper3 +gHdc plasmid and the subsequent TA cloning of the fragment into the pGEM-T Easy Vector. The isolation in both steps one and two was achieved utilizing PCR. Steps Three and Four describe the sub-cloning reactions that were attempted in order to ligate together the promoter of *hdc*, the ORF of GFP, and the 3'UTR of *hdc* in a 5'-3' direction, forming one fragment approximately 2kb in size. Step Five illustrates the final step that was to be completed in the construction of the transgene. The original pGreenPelican + pHdc plasmid from Step One is opened by a double digest with NheI and SpeI. The fragment created in Steps Three and Four is isolated from the pGEM-T Easy Vector by a double digest with NheI and SpeI. The resultant "sticky ends" allow ligation of this fragment back into the original vector. The resulting transgene can then be used to transform *Drosophila* embryos and transformant flies analyzed for GFP expression.

restriction endonuclease sites into the fragment. A BglII site was engineered into the 5' end of the fragment and an restriction endonuclease sites into the fragment. A BglII site was engineered into the 5' end of the fragment and an XbaI site was engineered into the 3' end. These restriction endonuclease sites were engineered into the fragment to enable its ligation to the *Hdc* promoter + the ORF of GFP on the 5' end and the region of GFP past the termination codon on the 3' end. The resulting fragment was then TA cloned into the pGEM-T Easy vector.

Amplification and Sequencing of the 3'UTR: The 750 bp 3'UTR was TA cloned into the pGEM-T Easy Vector for rapid amplification (Figure 3, Step 2). Plasmid DNA was isolated from bacterial cells as described above and samples from four different colonies were prepared for sequencing, which was performed by Retrogen, Inc. Sequencing results of this fragment were compared to same region of the genomic DNA to ensure that the PCR reactions did not introduce errors into the sequence of the fragment. All four samples had the same sequence, which was identical to the genomic region on file.

Attempted sub-cloning reactions: Sub-cloning reactions were utilized in an attempt to ligate the three fragments (the promoter of *Hdc* + the ORF of GFP, the 3'UTR of *Hdc* and the 3'region of GFP) together (Figure 3, Steps 3 and 4). The resulting fragment would then be sub-cloned back into the original pGreenPelican plasmid utilizing the NheI and SpeI sites existing on both the fragment and the open plasmid (Figure 3, Step 5). The first sub-cloning attempt involved a double digest of the pGEM-T

Easy + 3'UTR vector with SacII and XbaI to open the plasmid. Then, the 3'region of GFP was isolated from its pGEM-T Easy vector by a double digest with the same enzymes; the resulting fragment was band purified. The open vector and the DNA fragment were then incubated in a sub-cloning reaction mixture, plasmid DNA was isolated and subsequently digested with various enzymes including BglII, XbaI, and SpeI to ensure successful ligation. Polyacrylamide gel electrophoresis showed that ligation had been unsuccessful as insert DNA fragment seemed to "fall out" of the plasmid when it had not been digested with the appropriate enzymes. The second sub-cloning attempt involved opening the pGEM-T Easy + 3'UTR plasmid via a double digest reaction utilizing SacI and Bgl2. The NheI fragment containing the promoter of *Hdc* and the ORF of GFP was then isolated from its pGEM-T Easy vector via a double digest reaction utilizing the same enzymes; the resulting fragment was band purified. The same protocol for the first sub-cloning attempt was followed. Gel electrophoresis of plasmid DNA that had been digested with various endonucleases including NheI, BglII, and XbaI indicated that this ligation was also unsuccessful.

DISCUSSION: For the initial stages of the project, both restriction endonuclease digest reactions and PCR proved to be highly accurate and successful methods for isolating specific DNA fragments. Sub-cloning reactions were not successful. This may be due to methylation of restriction endonuclease sites by the competent cell line utilized or other changes in the DNA such as super coiling or degradation. A different competent cell line, such as XL-1 Blue may be used in a future sub-cloning

attempt and may yield more successful results. Additionally, it is possible that the 5'promoter of *Hdc,* the ORF of GFP, and the 3'UTR of *Hdc* may be ligated together utilizing PCR ligation methods. Once a successful method of sub-cloning is identified, the final product can be re-ligated into the 13 kb pGreenPelican vector that the original fragment was digested out of, utilizing the NheI and SpeI restriction endonuclease sites. *Drosophila* embryos may then be transformed with the new transgene and the resulting transformant flies analyzed for GFP expression to determine the effect of the 3'UTR on gene expression. If the histaminergic cells that did not express GFP in the flies with the pHdc-eGFP transgene express GFP in flies transformed with the pHdc-eGFP-3'UTR transgene, it can be concluded that the 3'UTR plays an important role in the regulation of *Hdc* expression.

References:

1. Pak, W.L. (1995) Drosophila in vision research: The Friedenwald Lecture. *Invest. Ophthal. & Visual Sci.* **36(12):** 2340-2357.

2. Burg, M.G., Sarthy, V., Koliantz, G., and W.L. Pak (1993) Genetic and molecular identification of a *Drosophila* histidine decarboxylase gene required in photoreceptor transmitter synthesis. *EMBO J.* **12(3):** 911-919.

3. Melzig, J., Buchner, S., Wiebell, F., Wolf, R., Burg, M., Pak, W.L., and E. Buchner (1996) Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. *J. Comp. Physiol. A.* **179:** 763-773.

4. Pollack, I. and Hofbauer, A. (1991) Histamine-like immunoreactivity in the visual system and brain of Drosophila melanogaster. *Cell Tissue Research* **266:** 391-398.

5. Buchner, E., Buchner, S., Burg, M.G., Hofbauer, A., Pak, W.L., and I. Pollack (1993) Histamine is a major mechanosensory neurotransmitter candidate in *Drosophila melanogaster. Cell and Tissue Res.* **273:** 119-125.

6. Kroodsma, D. and M.G. Burg (2004) Identification of histidine decarboxylase expression using a histamine antibody staining technique. *GVSU McNAir Scholars Journal* **8:** 29-33.

7. Romero-Caldero´, R., G. Uhlenbrock., J. Borycz, A.F. Simon, A. Grygoruk1, S.K. Yee, A. Shyer, L. C. Ackerson, N.T. Maidment, I.A. Meinertzhagen, B.T Hovemann, D. E. Krantz1 (2008) A Glial Variant of the Vesicular Monoamine Transporter Is Required To Store Histamine in the Drosophila Visual System. PLoS Genetics 4(11): e1000245. doi:10.1371/journal.pgen.1000245

8. Burg, M.G. and W.L. Pak (1994) Cis-acting regulatory region required for brain-specific expression of the histidine decarboxylase gene of *Drosophila. Abstr. Soc. Neurosci.* **20:** 915.

9. Burg, M.G. and W.L. Pak (1995) Identification of cis-acting regulatory regions required for photoreceptor and brain-specific expression of the histidine decarboxylase gene of *Drosophila.* Neurobiology of *Drosophila* Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (pg.34).

10. Burg, M.G. and W.L. Pak (1995) Separation of cis-acting regulatory regions required for photoreceptor and brain-specific expression of the histidine decarboxylase gene of *Drosophila. Invest. Ophthal. and Vis. Sci.* **36(4):** 1979.

11. Anderson, E. and M.G. Burg (2008) Synthesis of a HDC-GFP Promoter-Gene Fusion as a Tool to Identify Histaminergic Cells and Examine HDC Regulation, GVSU McNair Scholars Journal, Vol. 11.

12. Duffy, J. (2002) GAL4 System in *Drosophila*: A Fly Geneticist's Swiss Army Knife Genesis **34:**1–15.

13. Miller, E., Burg, M.G. and E. Anderson (2008) Control of *Hdc*expression: initial results from reporter-gene fusion studies, Midwest *Drosophila* meeting, Allerton Park, IL.

14. Shen, C. et al.. (2001) Triplex forming oligonucleotide targeted to 3'UTR downregulates the expression of the bcl-2 proto-oncogene in HeLa cells. *Nucleic Acids Research.* **29.23**: 622-28.