

# cDNA of YP4, a Follicular Epithelium Yolk Protein Subunit, in the Moth, *Plodia interpunctella*

O.P. Perera and Paul D. Shirk\*

Center for Medical, Agricultural and Veterinary Entomology, Agricultural Research Service,  
U. S. Department of Agriculture, Gainesville, Florida

**YP4, a subunit of the follicular epithelium yolk protein in the moth, *Plodia interpunctella*, is produced in the follicle cells during vitellogenesis and after secretion is taken up into the oocyte and stored in the yolk spheres for utilization during embryogenesis. In order to identify the cDNA clones for YP4, a degenerate PCR primer was designed to six amino acid residues identified in the NH<sub>2</sub>-terminal sequence of mature YP4. The YP4 degenerate primer plus T7 reverse PCR primer produced a PCR product from a cDNA library for the majority of the YP4 coding sequence. Combined cDNA and 5' RACE sequencing showed the YP4 transcript to be 991 bp in length with a single open reading frame for a predicted polypeptide of 299 amino acids. Northern analysis showed a single YP4 transcript was present in ovarian RNA that was approximately 1 kb in length. The predicted amino acid sequence for YP4 from *P. interpunctella* was most closely related to the predicted YP4 protein from the moth, *Galleria mellonella*, and the spherulin 2a protein from the slime mold, *Physarum polycephalum*. Arch. Insect Biochem. Physiol. 40:157–164, 1999. Published 1999 Wiley-Liss, Inc.†**

**Key words:** oocyte; ovary; reproduction; PCR; 5'RACE

## INTRODUCTION

The production and accumulation of protein yolk in the eggs of many animals follows a similar paradigm; vitellogenin that is synthesized and secreted from extra-ovarian tissues is taken up by the oocyte and packaged in yolk spheres (bodies) to provide nutrients during embryogenesis. However, not all yolk proteins that are packaged in the oocytes of insects originate outside of the ovary. In the higher Diptera and in some Lepidoptera, the follicular epithelial cells that surround the oocyte also synthesize and secrete proteins that contribute a major component to the proteinaceous yolk (Bownes, 1982; Shirk et al., 1984). In moths, the yolk proteins produced by the follicular epithelium either have a homo- or hetero-subunit composition. The homo-subunit

type, such as paravitellogenin (70 kDa) from *Hyalophora cecropia* (Bast and Telfer, 1976; Telfer et al., 1981) and egg-specific protein (ESP at 72 kDa) from *Bombyx mori* (Ono et al., 1975; Irie and Yamashita, 1983; Zhu et al., 1986; Indrasith et al., 1988; Sato and Yamashita, 1991), consist

Abbreviations used: ESP = egg specific protein; FEYP = follicular epithelium yolk protein; Glx = glutamine and glutamic acid residues; MVg = microvitellogenin; YP2, yolk polypeptide 2; YP4 = yolk polypeptide 4; Vg = vitellogenin.

The sequence reported in this paper has been deposited in the GenBank database (accession no. AF092741).

\*Correspondence to: Paul D. Shirk, USDA ARS CMAVE, P.O. Box 14565, Gainesville, FL 32604. E-mail: pshirk@gainesville.usda.ufl.edu

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of a single subunit that associates as dimers or tetramers. The hetero-subunit type, such as follicular epithelium yolk protein (FEYP) with subunits YP2 (69 kDa) and YP4 (33 kDa) from *Plodia interpunctella* (Shirk et al., 1984; Bean et al., 1988) and other pyralid moths (Shirk, 1987), have two subunits that associate in dimeric and tetrameric forms. Typically, the FEYPs contribute approximately 25–40% of the proteinaceous material to the oocytes.

In the moth *P. interpunctella*, the FEYP is synthesized in the follicular epithelial cells of vitellogenic follicles present in pharate adult females (Shirk et al., 1984; Bean et al., 1988; Zimowska et al., 1994, 1995a,b). Although the two subunits are present in the yolk spheres of mature eggs in equal molar concentrations, the synthesis and secretion of YP2 by the follicular epithelial cells appear to precede the synthesis of YP4 as determined by immuno-gold labeling studies (Zimowska et al., 1994). YP2 was detected in the follicular epithelial cells and the early yolk spheres of oocytes in previtellogenic follicles prior to the onset of patency while YP4 was detected only after the onset of patency. Whether this temporal separation in appearance of the different subunits is the result of non-coordinate expression of the two genes or a result of a temporal difference in the apparent antigenicity of the two subunits could not be determined using the immuno-gold labeling technique. Previously, the cDNA for the YP2 subunit from *P. interpunctella* (Shirk and Perera, 1998) and the cDNA for YP4 from *Galleria mellonella* (Rajaratnam, 1996a) were cloned. To obtain primary sequence information for an analysis of YP4 structure and to provide tools for the spatial distribution of YP4 transcript present in these stages of follicles, we set about to clone the cDNA for YP4 from *P. interpunctella*.

## MATERIALS AND METHODS

The *P. interpunctella* (Hübner) colony was reared according to Silhacek and Miller (1972) in a 16 h light:8 h dark cycle at 30°C and 70% relative humidity. All molecular biological procedures were conducted as described in Sambrook et al. (1989) unless otherwise stated.

### cDNA Library Construction and Screening

Total RNA was extracted from the vitellogenic ovaries of late pharate adult females using an RNA extraction kit (Pharmacia Biotech, Piscataway, NJ). To construct the cDNA library,

poly(A)<sup>+</sup> RNA was isolated from total RNA by two cycles of chromatography through oligo(dT)-cellulose columns. The Library Construction Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida, prepared the cDNA library. The cDNA library was constructed from poly(A)<sup>+</sup> RNA of the ovaries. cDNA was synthesized with the Lambda ZAP II cDNA Synthesis Kit (Stratagene, La Jolla, CA) using an oligo(dT) primer with an *Xho*I linker. The cDNA was size-fractionated on a Sephacryl S-400 column with a minimum size cutoff of 0.5 kb. An *Eco*RI adaptor was added to the 5' end of the cDNA. The cDNA was cut with *Eco*RI and *Xho*I and then ligated into Lambda ZAP II arms that were restriction digested with *Eco*RI and *Xho*I. The ligation was packaged into lambda phage according to the manufacturer's protocols.

Affinity purified polyclonal antibodies for YP4 (Shirk et al., 1992) were prepared as described by Robinson et al. (1988). The cDNA library was screened with the affinity purified antibodies by the plaque method of Huynh et al. (1985).

### Amino Acid Sequence Analysis

The follicular epithelium yolk protein was purified as described previously (Bean et al., 1988) and resolved by SDS-PAGE. The protein was electroblotted to Trans-Blot membrane (BioRad, Hercules, CA) and the YP4 band was cut from the total membrane. The Protein Chemistry Core Facility of the Interdisciplinary Center for Biotechnology Research, University of Florida, sequenced NH<sub>2</sub>-terminal amino acids of YP4. The NH<sub>2</sub>-terminal amino acid sequence of the fragments was determined by automated dansyl-Edman degradation using an on-line sequencing analyzer (Model 470A, Applied Biosystems, Foster City, CA) as described by Walker (1984).

### PCR Analysis

Direct PCR of YP4 sequence from the pharate adult ovarian cDNA library utilized a degenerate forward primer (<sup>5</sup>GGNGARTTYAAYGAYGA<sup>3</sup>) corresponding to the six amino acid residues GEFNDD from the NH<sub>2</sub>-terminal sequence of YP4 in combination with the T7 reverse PCR primer specific for the pBluescript II SK phagemid.

The 3' RACE product for YP4 was produced using the 5' RACE kit (GIBCO/BRL, Gaithersburg, MD). The 3' RACE utilized the GEFNDD forward degenerate primer in conjunction with the 3' RACE adapter primer (<sup>5</sup>GGCCACGCGTC-

GACTAGTAC(T)<sub>17</sub><sup>3'</sup>) to amplify the sequence following the synthesis of the first cDNA strand.

The structure of the 5' end of the YP4 transcript was determined by 5' RACE essentially as directed in the kit (GIBCO/BRL). The 5' RACE utilized the YP4R1 reverse primer and the YP4R2 reverse primer as the primary and nested primers, respectively. The 5' RACE PCR products were TA cloned into *pCR2.1* (Invitrogen, Carlsbad, CA) and sequenced.

### DNA Sequencing

The DNA Sequencing Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida, sequenced the various cloned DNA. The sequencing of all cDNA and 5' RACE products was accomplished by the Taq DyeDeoxy Terminator (no. 401388) and Dye-Primer (no. 401386) Cycle Sequencing protocols developed by Applied Biosystems (a division of Perkin-Elmer Corp., Foster City, CA) using fluorescent-labeled dideoxynucleotides and primers, respectively. The labeled extension products were analyzed on a Model 373A DNA Sequencer (Applied Biosystems).

The DNA and the conceptual translation sequences were used to search peptide and nucleotide sequence databases using the BLAST (Altschul et al., 1990) network service. Alignments for the DNA sequences and the predicted amino acid sequences were produced using ClustalW within the MacVector 6.0.1 software (Oxford Molecular Group, Campbell, CA) as were the Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982).

### Northern Analysis

Total RNA from ovaries, testes, and somatic tissues of pharate adults was isolated using TRIZOL reagent (GIBCO/BRL). Northern analysis was carried out using NorthernMax (Ambion, Austin, TX) reagents. Five micrograms of total RNA of each sample was resolved in a 1% formaldehyde agarose gel. The gel was run for 4 h at 60 V with buffer recirculation. One lane of the 0.24–9.5kb RNA Ladder (GIBCO/BRL) was included in the gel to determine the size of the target mRNA. The RNA was then transferred onto a Nytran blotting membrane (Schleicher & Schuell, Keene, NH) by downward capillary transfer using the rapid transfer buffer of the NorthernMax reagent system. After 1 h, the transfer apparatus was disassembled, the membrane was washed in the transfer buffer briefly and UV cross-linked using a Stratalinker (Stratagene).

Prehybridization and hybridization of the Northern blot was carried out at 48°C in the NorthernMax hybridization buffer. The radiolabeled YP4 probe was synthesized by amplification of a 500-bp fragment of a YP4 clone isolated in the 5' RACE procedure. The amplification reaction contained 10 pg of template DNA, 7.5 mmol of alpha <sup>32</sup>PdCTP (10 µCi/mmol), 10 pmol each of PiYP4R2 and 5' RACE universal amplification primer (UAP), 100 nmol each of dATP, dGTP, and dTTP, and 2.5 mM MgCl<sub>2</sub> in a 15-µl volume. Cycling parameters were the same as for the amplification step in the 5' RACE procedure except that only 20 cycles of amplification were performed. Probe for the RNA Ladder was made by random prime labeling of λ DNA according to the kit manufacturer (Boehringer Mannheim, Indianapolis, IN). The probes were purified from unincorporated nucleotides and primers using a BioGel P60 (100-200 mesh) (BioRad) column. Heat denatured probe was added to the hybridization buffer and incubated overnight. At the end of hybridization, the probe was removed and the filter was washed twice with NorthernMax low stringency wash buffer at room temperature and then twice with high stringency wash buffer at 48°C for 15 min each. An X-ray film (Kodak, Rochester, NY, XOMAT AR) was exposed to the blot for 6 h to visualize the signal.

## RESULTS

### cDNA and Predicted Amino Acid Sequences of YP4

Approximately 10<sup>5</sup> plaques of the ovarian cDNA library were screened with antigen affinity purified antiserum to YP4 without any positive clones being identified. As an alternate strategy for the isolation of a YP4 cDNA clone, a degenerate PCR primer corresponding to the amino terminal amino acid sequence was identified and used to produce a PCR product.

The first 30 NH<sub>2</sub>-terminal amino acids of mature YP4 from 4-h-old eggs were determined. The sequence of the NH<sub>2</sub>-terminal amino acid residues were, RIDVQLSGEFNDDSHNNLKVYYSQASQASVI, which shared no strong similarity with any known sequences including YP4 from *G. mellonella*.

The degenerate forward PCR primer corresponding to the six amino acid residues GEFNDD from the NH<sub>2</sub>-terminal amino acid sequence of YP4 was made and used in combination with the T7 reverse PCR primer specific for the pBluescript

II SK phagemid. When utilizing these two primers with phage DNA from the pharate adult ovarian cDNA library, the PCR product was approximately 800 bp in size. By doing 3' RACE using mRNA from vitellogenic ovaries, the degenerate GEFNDD forward primer in combination with an oligo(dT) primer produced a product of 800 bp as well. The direct YP4 PCR product from the ovarian cDNA library was cloned into *pCR2.1* and sequenced. The sequence of the PCR product showed that it included a large open reading frame that corresponded in part to the NH<sub>2</sub>-terminal amino acid residues downstream from the GEEFNDD sequence used for the PCR primer. When aligned with the predicted amino acid sequence for YP4 from *G. mellonella* (Rajaratnam, 1996a), there was considerable identity shared between the partial sequence for YP4 from *P. interpunctella* and YP4 from *G. mellonella* (see

Fig. 3). In order to determine the 5' sequence of the YP4 transcript, ovarian mRNA was used to perform 5' RACE PCR as described to produce the sequence. The YP4 5' RACE products were cloned into *pCR2.1* and sequenced.

The complete cDNA sequence for YP4 was 989 bp in length with a single open reading frame that encoded a 299 amino acid polypeptide (Fig. 1). Northern analysis showed that a single transcript of approximately 1 kb was present in vitellogenic follicles from ovaries of pharate adult females (Fig. 2). The YP4 transcript was present in low amounts in pre-vitellogenic and post-vitellogenic follicles but not detected at all in other tissues of the female abdomen or in testes or pharate adult male abdomens. The 30 amino acid sequence derived from the NH<sub>2</sub>-terminal sequencing of YP4 was identical with amino acids 17 to 47 of the predicted amino acid sequence from

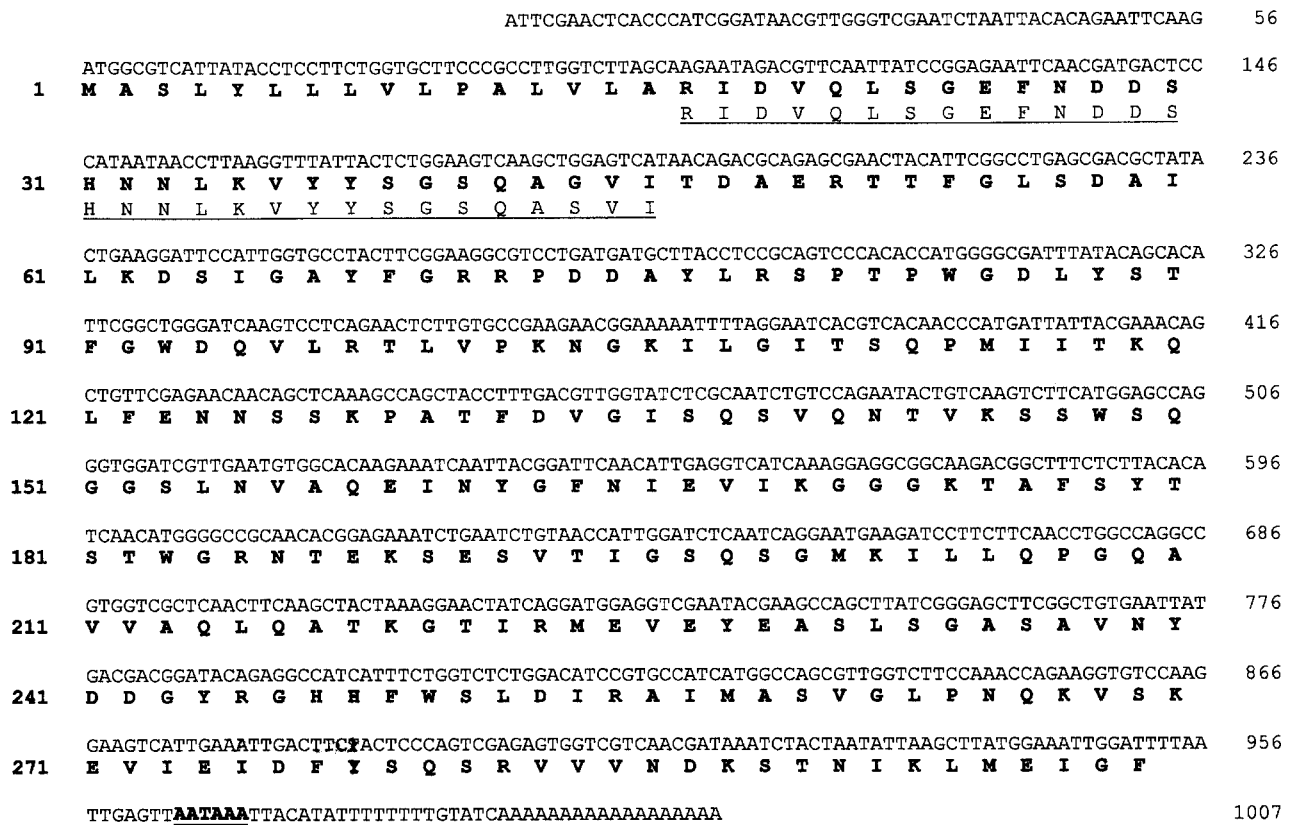


Fig. 1. The nucleotide sequence and predicted amino acid sequence for the consensus YP4 cDNA. Panel A. The YP4 sequence was completed by 5' RACE analysis. The 5' RACE utilized the YP4R1 (5'AGATTCAGATTTCTCCGTGTTG<sup>3</sup>) reverse primer and the YP4R2 (5'TGTGTAAGAGAAAGC-CGTCTT<sup>3</sup>) reverse primer as the primary and nested primers, respectively. The nucleotide position is indicated on the right, and the predicted amino acid position is indi-

cated on the left. The adenine of the first methionine codon is designated as +1 in the nucleotide sequence. The predicted amino acid sequence of the open reading frame is shown under the nucleotide sequence in the single-letter amino acid code. The single underlined sequence is the 30 amino acid sequence from the N-terminal of mature YP4. The bold-underlined sequence shows the poly(A)<sup>+</sup> addition signal beginning at base 1939.

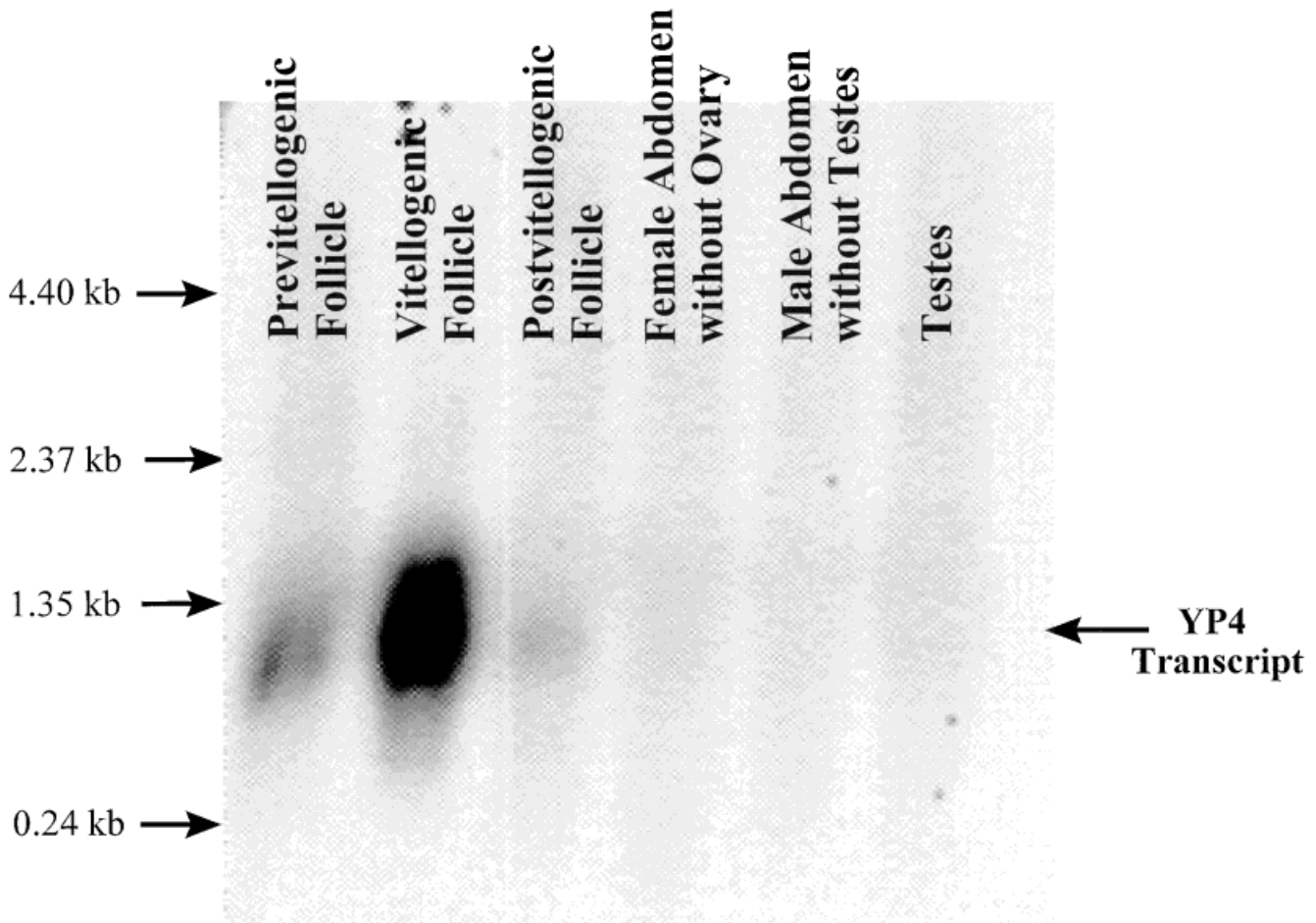


Fig. 2. Northern analysis of YP4 transcript in mRNA from late pharate adults. Total RNA (5  $\mu$ g per lane) from previtellogenic follicles, vitellogenic follicles, and post-vitellogenic follicles of late pharate adult female ovaries, abdomens without ovaries from late pharate adult females, testis, and abdomens without ovaries from late pharate adult males was

resolved in denaturing gel conditions and then blotted to a membrane. The blot was hybridized to labeled probe made from the 5' RACE clones as described. Labeled lambda DNA was included in the hybridization mix, and the position of the bracketing size markers is shown on the left.

the cDNA except for amino acid 45. This difference may be due to an error in the amino acid sequencing, but was not resolved by resequencing of the protein. The 3' noncoding region of the cDNA was 33 bp long and contained one AATAAA poly(A)<sup>+</sup> addition signal.

#### Sequence Similarity Between YP4 and Other Proteins

The cDNA sequence and the predicted YP4 amino acid sequence from *P. interpunctella* were BLAST searched against the available gene and protein banks. The greatest sequence similarity was shared with the YP4 gene from *G. mellonella* (smallest sum probability =  $9.3 \times 10^{-90}$ ; Rajaratnam, 1996a) (Fig. 3). The YP4 sequence also had similarity with the spherulin 2a gene from the

slime mold, *Physarum polycephalum* (smallest sum probability =  $1.7 \times 10^{-29}$ ; Bernier et al., 1987). The alignment of the *P. interpunctella* and *G. mellonella* YP4 gene products showed differences in the 5' and 3' regions. Of the first 30 NH<sub>2</sub>-terminal amino acids of the mature YP4 from eggs of *P. interpunctella*, the predicted amino acid sequence for YP4 from *G. mellonella* and YP4 from *P. interpunctella* had 4 identities and 8 conservative substitutions (40% similarity), while the last 50 amino acid residues of the C-terminal of the two proteins had 4 identities and 10 conservative substitutions (28% similarity). On the other hand, the central regions of the two proteins has 117 identities with 24 conservative substitutions without any insertions or deletions (70% similarity). Over the same central region, spherulin 2a

	1		100
PiYP4	MASLYLLLVL PALVL ARID VQLSGEFNDD SHNNLKVYYS GSQAGVITDA ERTTFGLSDA ILKDSIGAYF GRPDDAYLR SPTPWG . . . . . DLYSTFGWD		
GmYP4	-v-K--ail- FiPiiY-k-Q -nvVaSE-eA ETGEP . . . . G WKnvDi---N --Y--Q-t-N N--NAVQS-- -Q-----f-- ----- . . . . . ----QVY--P		
S2a	MAFQ -naHvG . . . N RTASSh . . . . -VVERiM--S d-RA---DGD N-FRAVERfR --W-TGawv-- --AIA-GVDL YQa-AHQ--R		
	101		200
PiYP4	QVLRTLVPKN GKILGITSQP MIITKQLFEN NSSKPATFDV GISQSVQNTV KSSWSQGGSL NVAQEINYGF NIEVIKGGGK TAFSYTSTIWG RNTEKSESVT		
GmYP4	--ars-SSSE S-Y-Qvs-K- S--LT-H-R- --tQ----Ka Q-Q-Q----- T-t-EK--E- T-g---E--- D-K-vSv--- -S-----R-- ESVS---t--		
S2a	--VTR-EPIS ST-HHPNDR TIVVTARLS- ---F-gE-Fa NL-nETT-sa Tt---STHGt E-g-SvS-. . S-G-vS-. -E -S-G-sYQ-- -GG-QtTASs		
	201		300
PiYP4	IGSQSGMKIL LQPGQAVVAQ LQATKGTIRM EVEYEASLSG ASAVNYDDGY RGHFWSLDI RAIMASVGLP NQKVSKEVIE IDFYSQSRV VNDKSTNIKL		
GmYP4	v--E--VE-T -E----ii-E -L--r--MEI Q-d---t--- -t---ANTF k---HfWaSg hQCcDD-WRL EPhR . . . . . 1PGSY-A-11 lqLSRCDQRc		
S2a	vSFvt-VTVH -----g-ivR -L-EQ-Wa-I TTR-R---t- HV-Q-fNPPH Q-----AHSv NS-LQAS--- T-IFIENIVd vG-fAn-h-D Mq-LV-GvIV		
	301	342	
PiYP4	MEIGF		
GmYP4			
S2a	PIgTDKIFRP LALKYDEIKE DDEKFADEKE QQKGEHKKET KH		

Fig. 3. Alignment of the predicted YP4 amino acid sequence of *P. interpunctella* with YP4 from *G. mellonella* and Spherulin 2a from the slime mold, *Physarum polycephalum*. . = insertions; - = identical amino acids between amino acid residues in YP4 and other amino acid sequences; lowercase

= conservative amino acid substitution; GmYP4 = YP4 from *G. mellonella* (smallest sum probability =  $9.3 \times 10^{-90}$ ); PiYP4 = YP4 from *P. interpunctella*; S2a = spherulin 2a from *P. polycephalum* (smallest sum probability =  $1.7 \times 10^{-29}$ ).

had 48% similarity with YP4 from *P. interpunctella*. Even with this level of similarity, comparison of the Kyte-Doolittle hydrophathy plots for these three proteins showed there was little correspondence of the hydrophilic/hydrophobic regions among them (data not presented).

## DISCUSSION

A complete cDNA sequence that coded for YP4 from *P. interpunctella* was derived from a combination of direct PCR and 5' RACE. The transcript for YP4 was abundant in vitellogenic follicles but barely detectable in pre- or post-vitellogenic follicles. These data are consistent with the observations using immunogold labeling with YP4 antiserum to detect the presence of the polypeptide. YP4 transcript was not detectable in other tissues of the vitellogenic female or males.

The YP4 cDNA from *P. interpunctella* encoded a single polypeptide of 299 amino acids. The predicted amino acid sequence has greatest similarity with YP4 from *G. mellonella* and to a lesser extent with spherulin 2a, a coat glycoprotein produced during encystment, from the slime mold, *P. polycephalum*. Over the entire sequence, the predicted amino acid sequences of the YP4 from *P. interpunctella* and *G. mellonella* had 133 identities (44%) with 59 additional conserved substitutions (64% total similarity). The overall similarity of YP4 with the predicted amino acid sequence for spherulin 2a was lower (50% for *P. interpunctella* and 47% for *G. mellonella*). Even though there was some

similarity between the YP4 sequences from the two moths and spherulin 2a, analysis of the sequences with the Kyte-Doolittle hydrophathy plots showed there was no apparent conservation of hydrophilic/hydrophobic regions between any of the three proteins (data not shown). The YP4 sequences from neither moth shows any similarity with the sequences for other yolk proteins produced by the follicular epithelium (i.e., YP2 from *P. interpunctella* and *G. mellonella*, ESP from *B. mori*, or YP1, YP2, and YP3 from *D. melanogaster* and other flies), or vitellogenin from any species.

Structural differences between the predicted amino acid sequences for YP4 from *P. interpunctella* and *G. mellonella* are also apparent in the amino acid composition ratios. The *G. mellonella* YP4 sequence has a high Glx (Glu + Gln) residue content (approximately 14%; Rajaratnam, 1996a) which is typical of many yolk proteins (Byrne et al., 1989). However, the *P. interpunctella* YP4 sequence has approximately 9.7% Glx residues which is more typical of other eukaryotic proteins (Table 1). Similar to *P. interpunctella* YP4, the predicted amino acid sequence for spherulin 2a is also in the typical eukaryotic range with 10.8% Glx content. When compared with other yolk proteins that have been cloned from Lepidoptera, YP4 from *P. interpunctella* and microvitellogenin from *Manduca sexta* (Wang et al., 1988) are the only two proteins that have a Glx content within the range of typical eukaryotic proteins (Table 1). What this means to protein function within the egg is not clear.

**TABLE 1. Glutamine/Glutamic Acid Content of Yolk Proteins From Lepidoptera\***

Species	Protein (accession no.)	%Glx
<i>M. sexta</i>	mVg <sup>1</sup> (M28820)	8.8
<i>P. interpunctella</i>	YP4 (AF092741)	9.7
<i>B. mori</i>	ESP <sup>2</sup> (D12521)	11.3
<i>P. interpunctella</i>	YP2 <sup>3</sup> (AF063014)	11.7
<i>G. mellonella</i>	YP2 <sup>4</sup> (U69881)	12.5
<i>B. mori</i>	Vg <sup>5</sup> (D13160)	12.8
<i>L. dispar</i>	Vg <sup>6</sup> (U90756)	12.9
<i>G. mellonella</i>	Yp4 <sup>7</sup> (U22425)	14

\*mVg = microvitellogenin from *M. sexta* (Wang et al., 1988); ESP = egg specific protein from *B. mori* (Inagaki and Yamashita, 1989); YP2 = YP2 from *P. interpunctella* (Shirk and Perera, 1998); YP2 = YP2 from *G. mellonella* (Rajaratnam, 1996b); Vg = vitellogenin from *B. mori* (Yano et al., 1994); Vg = vitellogenin from *Lymantria dispar* (Hiremath and Lehtoma, 1997); YP4 = YP4 from *G. mellonella* (Rajaratnam, 1996a).

Considering the close phylogenetic relationship between *P. interpunctella* and *G. mellonella* (Solis and Mitter, 1992), we find it interesting that both of the FEYP subunits, YP2 and YP4, have significantly divergent amino acid sequences between these two species without apparent impact on yolk formation or embryogenesis. A cladistic analysis of the family Pyralidae placed the Galleriinae subfamily, which includes *G. mellonella*, basal to the Phycitinae subfamily, which includes *P. interpunctella* (Solis and Mitter, 1992). For the YP2 subunit, the divergence appears in the amino terminus of YP2 from *P. interpunctella* that has a large sequence insert that is not present in YP2 of *G. mellonella* (Shirk and Perera, 1998). For the YP4 subunit, the divergence occurs in the amino and carboxyl terminal amino acid sequences. A BLAST search with the 60 amino acids from either the amino or carboxyl terminus of YP4 from *P. interpunctella* does not result in the selection of identity with YP4 from *G. mellonella*. This shows that over 120 amino acid residues (40% of the length) of these proteins share no identity. In addition, the Glx content is significantly different between YP4 from the two species. These data suggest that an analysis of the homologous genes from other members of the Pyralidae may provide a convenient marker for assessing the evolutionary divergence for this family.

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