Phosphorylation of ribosomal protein S6 is requisite for prothoracotropic hormone (PTTH)-stimulated specific protein synthesis and subsequent ecdysteroidogenesis in the prothoracic glands of the tobacco hornworm, Manduca sexta. To better understand the role of S6 in regulating ecdysteroidogenesis, S6 cDNA was isolated from a Manduca prothoracic gland cDNA library and sequenced. The deduced protein is comprised of 253 amino acids, has a molecular weight of 29,038, and contains four copies of a 10-amino acid motif defining potential DNA-binding sites. This Manduca S6 possesses a consensus recognition sequence for the p70S6k binding domain as well as six seryl residues at the carboxy-terminal sequence of 17 amino acids. Phosphoamino acid analysis revealed that the phosphorylation of Manduca prothoracic gland S6 is limited exclusively to serine residues. Although alterations in the quantity of S6 mRNA throughout the last larval instar and early pupal-adult development were not well correlated with the hemolymph ecdysteroid titer, developmental expression and phosphorylation of S6 were temporally correlated with PTTH release and the hemolymph ecdysteroid titer. These data provide additional evidence that S6 phosphorylation is a critical element in the transduction pathway leading to PTTH-stimulated ecdysteroidogenesis.

Insect molting and metamorphosis are elicited by a class of steroid hormones, ecdysteroids, originating in the prothoracic gland (1, 2). These glands are stimulated by a brain neuropeptide, prothoracicotropic hormone (PTTH),1 that acts via a cascade that includes a Ca2+/calmodulin-dependent increase in intracellular cAMP, activation of a CAMP-dependent protein kinase, and ultimate phosphorylation of S6, a protein of the ribosomal 40 S subunit (1–4).

Previous studies demonstrated that S6 in the prothoracic glands of the tobacco hornworm, Manduca sexta, was phosphorylated at up to five sites under PTTH stimulation (4, 5), a phenomenon typical of S6 phosphorylation in mammals (6). Temporal analysis of PTTH-stimulated S6 phosphorylation showed that phosphorylation and dephosphorylation of S6 closely paralleled the increase and decrease in PTTH-stimulated ecdysteroidogenesis (4). Most importantly, the multiple phosphorylation of S6 was inhibited completely by rapamycin, an inhibitor of S6 phosphorylation (7, 8), resulting in the inhibition of PTTH-stimulated specific protein synthesis and subsequent ecdysteroidogenesis (4). These data indicate that S6 phosphorylation is required for both specific protein synthesis and ecdysteroidogenesis in PTTH-stimulated glands.

Ribosomal protein S6 is the major substrate for several protein kinases in the eukaryotic ribosome, and it may have an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA (6). Although steroidogenic tissues such as the insect prothoracic glands do not respond to hormonal stimulation by cell proliferation, steroidogenesis in some mammalian tissues appears to require the rapid synthesis of relatively short-lived proteins (9), which in turn aid in the transit of cholesterol into the mitochondria for the synthesis of steroids (10, 11). In insects, RNA and protein synthesis are required for the full response of the prothoracic glands to PTTH (12–16). Although the rapid synthesis of 60-kDa (14), 50-kDa (β-tubulin), 70-kDa (Hsp70), and 100-kDa (15, 16) proteins in the prothoracic glands was observed following exposure to PTTH, calcium ionophore, or cAMP, the specific macromolecules necessary for the dynamic response of Manduca prothoracic glands to PTTH have not yet been identified. These observations suggest that S6 phosphorylation resulting from PTTH stimulation may be critical in regulating the synthesis of these proteins that are required for ecdysteroid biosynthesis, and this has led us to investigate further the relationships between S6 phosphorylation and ecdysteroidogenesis.

MATERIALS AND METHODS

Animals—M. sexta were reared on an artificial diet at 25 °C, >60% relative humidity under a photoperiod of 18 h light:6 h dark with 2400 h artificial Zeitgeber time (lights off) set at 2200 h Eastern Standard Time. A synchronous population of animals was selected by routinely staging on days zero of the third instar, fifth instar, and pupal stage (3, 4).

Chemicals—Standard and phosphate-free Grace’s insect tissue culture medium were obtained from Life Technologies, Inc. Carrier-free [32P]04 (10 mCi/ml) was from Amersham Corp., whereas rapamycin was a gift from Wyeth-Ayerst Research. Other reagents were from Sigma, Bio-Rad, Fisher, and U.S. Biochemical Corp.

cDNA Cloning and Sequencing—Prothoracic glands from day 7, fifth instar (V7) larvae were used to develop a cDNA library.2 First-strand cDNA was synthesized with an oligo(dT)12–15 primer using Moloney murine leukemia virus reverse transcriptase (TimeSaver cDNA synthesis kit; Pharmacia Biotech, Inc.). Second-strand cDNA was obtained by nick translation using DNA polymerase I, followed by the addition of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U64795.

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§ The abbreviations used are: PTTH, prothoracotropic hormone; PAGE, polyacrylamide gel electrophoresis.

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1 R. Rybczynski and L. I. Gilbert, unpublished data.
Cloning, Developmental Expression, and Phosphorylation of S6

EcoRI/NotI adapter to each end. cDNA in the large-insert library size range was ligated into the EcoRI-digested Zap II cloning vector. Phage packaged in vitro were plated (440,000 primary recombinants) and screened by standard plaque hybridization techniques using a full-length Drosophila S6 probe (graciously supplied by Dr. Kellie Watson). Prehybridization was performed in 3× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) containing 2× Denhardt’s medium, 0.5% SDS, and 50 μg/ml salmon sperm DNA. Hybridization used the same solution with the 32P-labeled Drosophila S6 probe, and washes in 0.1× SSC containing 1% SDS were performed at 50°C. Selected hybridizing plaques were taken through successive rounds of purification, and isolated recombinant phage were used for in vitro rescue of cDNA inserts in the pBluescript SK plasmid vector. Preliminary restriction mapping of cDNA inserts indicated that a single class of inserts was represented. Partial sequence analysis with T3 and T7 primers from both ends of nine selected inserts revealed that they were identical. Thus, a single clone, with 0.9 kilobase pair, was selected for sequencing.

DNA sequencing was performed on double-stranded plasmid DNA using the chain termination method with the Sequenase enzyme (U.S. Biochemical Corp.). Much of the sequencing was done using ordered sets across the cDNA insert (17). All regions of both strands were sequenced. Nucleotide sequences were analyzed with the Program Manual Software (Genetics Computer Group, Inc., Madison, WI).

Phosphoamino Acid Analysis—Prothoracic glands from V7 larvae were dissected as described previously (4), immediately frozen on dry ice, and kept at −80°C until extracted for RNA. Total RNA was prepared by the rapid RNA extraction procedure of Jowett (19). RNA was size fractionated on 1.0% agarose/formaldehyde gels and transferred to nylon membranes. The 0.9-kilobase pair cDNA insert of the Manduca S6 cDNA clone was excised from the pBluescript SK plasmid vector by SfiI + NotI digestion, gel purified, and labeled by random-primer synthesis with [32P]dCTP to a specific activity of >106 counts/min/μg. Nylon membranes were hybridized at 42°C with a S6 insert probe (2× 106 counts/min/ml) in a solution consisting of 5× SSC, 5× Denhardt’s solution, 50% formamide (w/v), 1% SDS, and 100 μg/ml sonicated salmon testis DNA. Following hybridization, blots were washed in 2× SSC plus 0.5% SDS at 50°C, dried briefly before two short washes with 0.1× SSC, then autoradiographed.

Antibody Preparation—A synthetic peptide corresponding to the C-terminal 21 amino acids of deduced Manduca prothoracic gland S6 protein was generated and conjugated to BSA. Two female New Zealand White rabbits were initially injected with 200 μg of bovine serum albumin-conjugated synthetic peptide mixed with RIBI adjuvant system (RIBI ImmunoChem Research, Inc., Hamilton, MT) at a ratio of 1:1 and boosted once with the same amount of immunogen 4 weeks later. Antiserum was titred by enzyme-linked immunosorbent assay, precipitated with ammonium sulfate, and affinity-purified with a protein A column (Bio-Rad). Antibody specificity was examined by Western blot analysis (20) using both gland lysate and purified 80 S ribosomes from prothoracic glands separated by one-dimensional SDS-PAGE or two-dimensional PAGE (8).

Western Blot Analysis of S6 Expression—Prothoracic glands were dissected from the last larval instar (V7–V8) and animals during early pupal-adult development (P0–P4) as described previously (4), homogenized by sonication (10 s) in 10 ml Tris buffer, pH 7.5, containing 0.15 M NaCl. The homogenate was centrifuged for 10 min at 10,000 × g. Proteins in the supernatant were quantified (21), subjected to SDS-PAGE (10% gel) separation, and transferred onto nitrocellulose membrane. The membranes were then immunostained with the Manduca S6 antibody as described previously (3) and analyzed densitometrically (Molecular Dynamics model 300A densitometer).

S6 Phosphorylation—To investigate whether in vivo the S6 phosphorylation state was correlated with the hemolymph ecdysteroid titers, prothoracic glands were dissected from different developmental stages as indicated and immediately placed on dry ice. 80 S ribosomes were purified, separated by two-dimensional PAGE, and silver stained (4).

RESULTS

cDNA Cloning and Sequence Analysis—The S6 cDNA from Manduca prothoracic glands includes 33 nucleotides of the 5′ noncoding sequence, an open reading frame of 759 bases corresponding to a protein of 253 residues with a calculated molecular mass of 26,014 daltons. The supernatant was lyophilized and resuspended in pH 1.9 electrophoresis buffer containing cold phosphoamid acid standards (0.2 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine). The phosphoamid acid standards were separated by electrophoresis using 10× 10-cm thin-layer cellulose plates at pH 1.9 for 30 min in the first dimension and 3.5 for 20 min in the second dimension (18) (HTLE-FF apparatus; CBS Scientific, Del Mar, CA). The plates were stained with ninhydrin following electrophoresis so that the positions of these phosphoamid acid standards could be monitored and subjected to autoradiography.

RNA Preparation and Northern Blot Analysis—Prothoracic glands from V7 larvae were dissected and subjected to autoradiography (4). The labeled band corresponding to S6 was cut out and subjected to SDS-PAGE (12.5%) electrophoresis and autoradiography. The RNA was size fractionated on 1.0% agarose/formaldehyde gels and transferred to nylon membranes. The 0.9-kilobase pair cDNA insert of the Manduca S6 cDNA clone was excised from the pBluescript SK plasmid vector by SfiI + NotI digestion, gel purified, and labeled by random-primer synthesis with [32P]dCTP to a specific activity of >106 counts/min/μg. Nylon membranes were hybridized at 42°C with a S6 insert probe (2× 106 counts/min/ml) in a solution consisting of 5× SSC, 5× Denhardt’s solution, 50% formamide (w/v), 1% SDS, and 100 μg/ml sonicated salmon testis DNA. Following hybridization, blots were washed in 2× SSC plus 0.5% SDS at 50°C, dried briefly before two short washes with 0.1× SSC, then autoradiographed.
Species—Sequence comparison of Manduca S6 to the S6 of four other species, ranging from yeast to Drosophila, rat, and human (Fig. 2), revealed that this ribosomal protein is highly conserved at the amino-terminal region but much less so at the carboxyl terminus. The deduced Manduca prothoracic gland S6 polypeptide shares 79% identity (89% similarity) with the Drosophila ribosomal protein S6 (23–25), 75% identity (84% similarity) with the rat (26) and human (27, 28) homolog of S6, and 60% identity (77% similarity) with the yeast S10 homolog of S6 (29). The deduced Manduca S6 protein is 4 amino acids longer than the S6 of Drosophila, rat, and human and contains a total of 16 seryl residues, the same number as in Drosophila S6, whereas there are only 15 seryl residues in rat and human and 11 in yeast. It should be noted that the positions of all six potentially phosphorylatable seryl residues located in the carboxyl-terminal region of the Manduca S6 are not identical to that in other species (Fig. 2). The Manduca S6 sequence reveals four copies of a 10-amino acid motif that is also common to the other four species whose consensus sequence includes an initial proline residue and four to six basic amino acids (Fig. 2). This motif is postulated to be a nuclear localization signal (26). The S6 sequences of all five species also reveal a consensus recognition sequence for the mitogen-activated p70s6k (30, 31). The prothoracic gland cytosol (PG) was labeled with 32P, treated with rapamycin, and challenged with PTTH as described previously (4). Ribosomal 80 S proteins were purified and subjected to SDS-PAGE (12.5%) electrophoresis. The Coomassie Blue-stained ribosomal 80 S proteins in the absence (Fig. 3a, lane 1) or presence (Fig. 3a, lane 2) of rapamycin were subjected to autoradiography. The labeled band corresponding to S6 in the absence of rapamycin (Fig. 3a, lane 3) was excised from the gel and subjected to partial acid hydrolysis and two-dimensional thin layer electrophoresis. Rapamycin treatment inhibited PTTH-stimulated phosphorylation limited to seryl residues at the carboxyl-terminal region (32, 33), no analogous information existed for the Manduca S6 or for the S6 of any other insect. Therefore, a phosphoamino acid analysis was carried out. Prothoracic gland S6 or for the S6 of any other insect. Therefore, a phosphoamino acid analysis was carried out. Prothoracic gland S6 were isolated from PTTH-stimulated glands in the presence (a, lanes 2 and 4) or absence (a, lanes 1 and 3) of 10 nM rapamycin and subjected to SDS-PAGE separation (12.5% gel). Lanes 1 and 2, Coomassie Blue-stained; lanes 3 and 4, autoradiographs. The phosphorylated ribosomal protein S6 band (a, lane 1) was cut from the gel, hydrolyzed under acid conditions, and processed for phosphoamino acid analysis (b). The directions of electrophoresis are indicated by arrows, P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Primary Structure of the Manduca S6 Protein—The deduced primary structure of the Manduca ribosomal protein S6 consists of 253 amino acids (Fig. 1), and the molecular weight is 29,038 for the unmodified protein. This is close to the 31 kDa estimated from SDS-PAGE gel analysis (3, 4). This S6 has an excess of basic residues (33 arginyl, 33 lysyl, and 3 histidyl) when compared to the acidic residues (11 aspartyl and 16 glutamyl). The basic amino acids comprise 27.3% of the total, while the acid residues make up 10.7% of the total. The estimated isoelectric point is 11.48. There are three cysteiny l residues at position 12, 83, and 100, but it is not known if they form disulfide bridges. There are a total of 16 seryl residues in the Manduca S6, 6 of which are located within the carboxy- terminal sequence of 17 amino acids. This includes the serines at positions 237, 239, 243, 245, 246, and 249.

Comparison of the Manduca S6 Sequence to That of Other

Species—Sequence comparison of Manduca S6 to the S6 of four other species, ranging from yeast to Drosophila, rat, and human (Fig. 2), revealed that this ribosomal protein is highly conserved at the amino-terminal region but much less so at the carboxyl terminus. The deduced Manduca prothoracic gland S6 polypeptide shares 79% identity (89% similarity) with the Drosophila ribosomal protein S6 (23–25), 75% identity (84% similarity) with the rat (26) and human (27, 28) homolog of S6, and 60% identity (77% similarity) with the yeast S10 homolog of S6 (29). The deduced Manduca S6 protein is 4 amino acids longer than the S6 of Drosophila, rat, and human and contains a total of 16 seryl residues, the same number as in Drosophila S6, whereas there are only 15 seryl residues in rat and human and 11 in yeast. It should be noted that the positions of all six potentially phosphorylatable seryl residues located in the carboxyl-terminal region of the Manduca S6 are not identical to that in other species (Fig. 2). The Manduca S6 sequence reveals four copies of a 10-amino acid motif that is also common to the other four species whose consensus sequence includes an initial proline residue and four to six basic amino acids (Fig. 2). This motif is postulated to be a nuclear localization signal (26). The S6 sequences of all five species also reveal a consensus recognition sequence for the mitogen-activated p70s6k (30, 31). The prothoracic gland cytosol (PG) was labeled with 32P, treated with rapamycin, and challenged with PTTH as described previously (4). Ribosomal 80 S proteins were purified and subjected to SDS-PAGE (12.5%) electrophoresis. The Coomassie Blue-stained ribosomal 80 S proteins in the absence (Fig. 3a, lane 1) or presence (Fig. 3a, lane 2) of rapamycin were subjected to autoradiography. The labeled band corresponding to S6 in the absence of rapamycin (Fig. 3a, lane 3) was excised from the gel and subjected to partial acid hydrolysis and two-dimensional thin layer electrophoresis. Rapamycin treatment inhibited PTTH-stimulated phosphorylation limited to seryl residues at the carboxyl-terminal region (32, 33), no analogous information existed for the Manduca S6 or for the S6 of any other insect. Therefore, a phosphoamino acid analysis was carried out. Prothoracic gland S6 or for the S6 of any other insect. Therefore, a phosphoamino acid analysis was carried out. Prothoracic gland S6 were isolated from PTTH-stimulated glands in the presence (a, lanes 2 and 4) or absence (a, lanes 1 and 3) of 10 nM rapamycin and subjected to SDS-PAGE separation (12.5% gel). Lanes 1 and 2, Coomassie Blue-stained; lanes 3 and 4, autoradiographs. The phosphorylated ribosomal protein S6 band (a, lane 1) was cut from the gel, hydrolyzed under acid conditions, and processed for phosphoamino acid analysis (b). The directions of electrophoresis are indicated by arrows, P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

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Comparison of the Manduca S6 Sequence to That of Other
S6 phosphorylation completely (Fig. 3a, lane 4; see also Refs. 3 and 4), and this was used as a control to confirm that the excised band represents S6. Phosphoamino acid analysis revealed that S6 was phosphorylated exclusively at the serine residues (Fig. 3b), a result consistent with all past reports on the subject (34).

S6 Antibody Specificity—The high specificity of the S6 antiserum against the carboxyl-terminal 21 amino acids of the deduced Manduca S6 was demonstrated by Western blot analysis (Fig. 4). The antibody detected only a single band of 31 kDa in both prothoracic gland cytosol (Fig. 4b, lanes 1 and 2) and 80 S ribosomes purified from prothoracic glands (Fig. 4b, lanes 3 and 4). The blot containing equivalent amounts of prothoracic gland cytosol (Fig. 4a, lanes 1 and 2) and 80 S ribosomes (Fig. 4a, lanes 3 and 4) was stained with Amido Black and used as a reference. Preimmune serum displayed no immunoreactivity to S6 (data not shown).

To determine if the immunostained band corresponding to S6 in one-dimensional SDS-PAGE is indeed S6 and whether the antibody recognizes all forms of the phosphorylated or unphosphorylated S6, two-dimensional PAGE was performed. The purified 80 S ribosomal proteins from PTTH-stimulated or control glands were separated by two-dimensional PAGE (4), transferred onto nitrocellulose membranes, and immunostained with the purified antibody (Fig. 5). S6 was phosphorylated at all five sites in PTTH-stimulated glands as shown by the Ponceau S-stained membrane (Fig. 5b), and only one site was phosphorylated in control glands (Fig. 5a), a result consistent with previous studies (4, 5). These same blots were then destained in water to remove the Ponceau S stain and immunostained with the antibody. The antibody recognized all forms of the prothoracic gland S6 protein in both PTTH-stimulated (Fig. 5d) and control glands (Fig. 5c) with virtually equal affinity to all forms of S6 when compared to the corresponding Ponceau S-stained blots (Fig. 5, a and b).

S6 Gene Expression—To investigate the functional relationship between prothoracic gland S6 expression at both the transcriptional and translational levels and ecdysteroid biosynthesis, both Northern and Western blot analyses were performed to monitor the expression of S6 through the last larval instar and during early pupal-adult development. The developmental Northern blot analysis revealed a 0.9-kilobase transcript expressed throughout the fifth larval instar and early pupal-adult development (Fig. 6b). The S6 mRNA is abundant in the prothoracic glands since total RNA (Fig. 6a) was probed. The densitometric analysis of S6 mRNA expression (Fig. 6c) revealed that the level of S6-specific mRNA was relatively high at stages V1–V3, started to decrease at V4, reached its lowest level at V7–V9, recovered slightly after pupation, and declined again at P1–P4. These results reveal that the peak of S6 mRNA was
transcripts for the S6 gene in the prothoracic glands of fifth instar larvae (V1–V9) and animals during early pupal-adult development (P0–P4). The filters containing 20 µg of total RNA per lane were hybridized with the Manduca S6 cDNA probe. a shows the total RNA loaded per lane on the gel before transfer as a reference. b indicates the developmental profile of S6 mRNA expression in the prothoracic glands. c reveals the densitometrical analysis of S6 mRNA expression of b after calibration with the densitometrical data of total RNA of a. Hemolymph ecdysteroid titers were redrawn from the data of Grieneisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

S6 Protein Expression—Using the highly specific antibody generated against the Manduca S6 protein, developmental changes in the amount of S6 protein in the prothoracic glands were analyzed by Western blot through the last larval instar (V1–V9) and early pupal-adult development (P0–P4). Proteins (20 µg/lane) were subjected to SDS-PAGE (10% gel) before immunostaining. The immunostained blots were analyzed densitometrically. The data show the developmental profile of S6 in the prothoracic glands (a) and fat body (b). The blot inserts above a and b show the typical immunoreactivity of S6. The data are expressed as the means of five separate experiments; bars, S.E. Hemolymph ecdysteroid titers were redrawn from the data of Grieneisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

FIG. 6. Northern blot analysis of S6 mRNA in the prothoracic glands of fifth instar larvae (V1–V9) and animals during early pupal-adult development (P0–P4). The filters containing 20 µg of total RNA per lane were hybridized with the Manduca S6 cDNA probe. a shows the total RNA loaded per lane on the gels before transfer as a reference. b indicates the developmental profile of S6 mRNA expression in the prothoracic glands. c reveals the densitometrical analysis of S6 mRNA expression of b after calibration with the densitometrical data of total RNA of a. Hemolymph ecdysteroid titers were redrawn from the data of Grieneisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

FIG. 7. Temporal relationship between the level of S6 protein and the hemolymph ecdysteroid titer during the fifth larval instar (V1–V9) and early pupal-adult development (P0–P4). Proteins (20 µg/lane) were subjected to SDS-PAGE (10% gel) before immunostaining. The immunostained blots were analyzed densitometrically. The data show the developmental profile of S6 in the prothoracic glands (a) and fat body (b). The blot inserts above a and b show the typical immunoreactivity of S6. The data are expressed as the means of five separate experiments; bars, S.E. Hemolymph ecdysteroid titers were redrawn from the data of Grieneisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

(S7a). S6 immunoreactivity was relatively constant until V5, increased rapidly at V6, was almost double in V7 glands relative to V5, and decreased rapidly by V8. S6 immunoreactivity remained at a low level in P0–P2 prothoracic glands but increased dramatically at P3–P4 in concert with the ecdysteroid titer. Both of the peaks of S6 immunoreactivity (Fig. 7) paralleled the large peaks in the hemolymph ecdysteroid concentration (35, 36). To demonstrate that the fluctuation in S6 immunoreactivity in parallel with the hemolymph ecdysteroid titer was not simply coincidental, Western blot analysis was conducted for the fat body and midgut S6 during the same developmental period. The results from both fat body (Fig. 7b) and midgut (data not shown) revealed that the pattern of S6 immunoreactivity differed dramatically from that of the prothoracic gland S6 and was not directly correlated with the ecdysteroid titer.

In Vitro S6 Phosphorylation—Our previous in vitro studies showed that S6 phosphorylation is a prerequisite for PTTH-stimulated protein synthesis and subsequent ecdysteroidogenesis. However, the in vivo relationships between PTTH release, S6 phosphorylation and the hemolymph ecdysteroid titer remain conjectural. Fig. 8 reveals that S6 phosphorylation in vivo was closely correlated with the hemolymph ecdysteroid titer; S6 remained in a dephosphorylated state in prothoracic glands from stages V1, V5, V7, V9, and P1, but was phosphorylated at
sequence differences between *Manduca* and yeast are more evenly distributed, 56.2% of the substitutions are located within the carboxyl-terminal half of the protein. In addition, *Manduca* S6 contains only six serine residues at the carboxyl terminus portion rather than the seven occurring in more evolutionarily advanced species such as the fruit fly, rat, and human S6, but there are four more serines than was found in the S6 of more primitive species, e.g. yeast.

With the cloned S6 cDNA probe (Fig. 1) and specific S6 antibody (Figs. 4 and 5) in hand, we investigated the possible correlation between S6 expression and ecdysteroid biosynthesis, the latter requiring specific protein synthesis (3, 4, 15). The small increase in the larval hemolymph ecdysteroid titer at V₆ is responsible for cellular reprogramming, whereas the major surge in the larval hemolymph ecdysteroid titer takes place at about stage V₇ and is responsible for initiating the larval-pupal molt (38). The peak at P₃ elicits pupal-adult development (36). Thus, the latter two dramatic increases in the hemolymph ecdysteroid titer are responsible for the two metamorphic molts of this insect. Northern blot analysis revealed that transcriptional expression of S6 through larval and early pupal-adult development was not correlated with the ecdysteroid titer, i.e. the S6 mRNA signal was at its lowest level at stages V₇–V₈ and P₃–P₄ (Fig. 6). However, Western blot analysis demonstrated that the quantity of S6 gene product peaked at stages V₇–V₈ and P₃–P₄ in concert with the peaks in the hemolymph ecdysteroid titer (Fig. 7). The rapid decline in S6 mRNA (Fig. 6) accompanied the rapid increase in the level of S6 protein at the times that the hemolymph ecdysteroid titer peaked, suggesting that the increase in S6 results from increased translation, and that more ribosomal S6 protein is required at these stages, perhaps to facilitate the synthesis of specific proteins (enzymes?) required for ecdysteroidogenesis. Although direct evidence for a specific role of S6 in protein synthesis and ecdysteroidogenesis is not yet available for the *Manduca* prothoracic gland, in mammalian cells, following activation of the cells by mitogens, mRNAs encoding ribosomal proteins (including S6) and elongation factors are shifted from nonactive ribosomes to the active polysome fraction within 30 min and synthesis of specific proteins begins prior to the general increase in protein synthesis (39).

Our previous in vitro studies showed that high levels of S6

In Fig. 8. Temporal relationship between the level of S6 phosphorylation *in vivo* and the hemolymph ecdysteroid titer during the fifth larval instar (V₁–V₉) and early pupal-adult development (P₀–P₉). Prothoracic glands from indicated stages of larval and pupal-adult development were dissected and immediately placed on dry ice. 80 S ribosomes were purified, subjected to two-dimensional PAGE, and silver-stained. Phosphorylation sites are designated as 1–5. 0 represents the unphosphorylated form of S6. Hemolymph ecdysteroid titers were redrawn from the data of Grieiisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

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**DISCUSSION**

Phosphorylation of the S6 protein in the 40 S ribosomal subunit is a critical event in the initiation of cell growth and proliferation and is well conserved among eukaryotic organisms (6). A full-length cDNA encoding the *Manduca* prothoracic gland S6 has been cloned and sequenced. Sequence comparisons between *Manduca* and other species, ranging from yeast to invertebrate to vertebrate, revealed that the key elements required for S6 function are well conserved (Fig. 2). All four copies of the 10-amino acid motif postulated to be a nuclear localization signal (26), a consensus recognition sequence for copies of the 10-amino acid motif postulated to be a nuclear localization signal (26), and immediately placed on dry ice. 80 S ribosomes were purified, subjected to two-dimensional PAGE, and silver-stained. Phosphorylation sites are designated as 1–5. 0 represents the unphosphorylated form of S6. Hemolymph ecdysteroid titers were redrawn from the data of Grieiisen et al. (35) and Warren and Gilbert (36) for comparison purposes.

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V₃, V₆, and P₃. All five sites were phosphorylated at V₆, whereas only one or two sites were phosphorylated at stages V₃ and P₃. Phosphorylation of S6 at a single site is sufficient for the initiation of ecdysteroidogenesis (4). The phosphorylation of S6 at stages V₅, V₆, and P₃ occurred in concert with the three peaks of hemolymph ecdysteroids.

**DISCUSSION**

Phosphorylation of the S6 protein in the 40 S ribosomal subunit is a critical event in the initiation of cell growth and proliferation and is well conserved among eukaryotic organisms (6). A full-length cDNA encoding the *Manduca* prothoracic gland S6 has been cloned and sequenced. Sequence comparisons between *Manduca* and other species, ranging from yeast to invertebrate to vertebrate, revealed that the key elements required for S6 function are well conserved (Fig. 2). All four copies of the 10-amino acid motif postulated to be a nuclear localization signal (26), a consensus recognition sequence for the mitogen-activated p70S6K, and five phosphorylatable localization signal (26), and immediately placed on dry ice. 80 S ribosomes were purified, subjected to two-dimensional PAGE, and silver-stained. Phosphorylation sites are designated as 1–5. 0 represents the unphosphorylated form of S6. Hemolymph ecdysteroid titers were redrawn from the data of Grieiisen et al. (35) and Warren and Gilbert (36) for comparison purposes.
phosphorylation are closely associated with increased rates of protein and ecdysteroid synthesis and that inhibition of S6 phosphorylation by rapamycin resulted in the inhibition of specific protein synthesis and subsequent ecdysteroid synthesis (3, 4). The present data (Fig. 8) revealed that the multiple phosphorylation of S6 occurred in vivo as well in concert with the increase in hemolymph ecdysteroidogenesis at all three critical periods of development, i.e. Vp, V6, and P3, and therefore, with the release of endogenous PTTH. The data therefore indicate that the release of PTTH in vivo initiates S6 phosphorylation, which in turn elicits specific protein synthesis and subsequent ecdysteroidogenesis. Studies in other biological systems have also shown a correlation between a high level of S6 phosphorylation and an increase in protein synthesis, one of the earliest events required for cell growth and proliferation (6). S6 phosphorylation is an event that is believed to facilitate the movement of inactive 80 S ribosomes into actively translating polysomes (39), increase translational efficiency (40), selectively translate protein (41), and regulate a specific class of messages with a polypyrimidine tract at their 5′ cap site (42).

Recent studies also suggest that S6 may play a role in the up-regulation of ribosome biogenesis (6) and in controlling tumor production in Drosophila (23, 25). It is possible that the increased levels of S6 protein expression that correlate with the peaks of hemolymph ecdysteroids may not only facilitate specific protein synthesis required for ecdysteroidogenesis but may also be involved in the feedback regulation of ecdysteroid synthesis. Thus, S6 not only participates in “housekeeping” roles but may be critically involved in the control of insect growth, molting, and metamorphosis.

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REFERENCES
1. Gilbert, L. I., Combret, W. L., Smith, W. A., and Rountree, D. B. (1988) BioEssays 8, 153–157.
2. Gilbert, L. I., Rybczynski, R., and Tober, S. S. (1996) in Metamorphosis (Gilbert, L. I., Tata, J. R., and Atkinson, B. G., eds) pp. 60–98, Academic Press, Inc., San Diego.
3. Song, Q., and Gilbert, L. I. (1994) Dev. Genet. 15, 332–338.
4. Song, Q., and Gilbert, L. I. (1995) Insect Biochem. Mol. Biol. 25, 591–602.
5. Song, Q., and Gilbert, L. I. (1996) Arch. Insect Biochem. Physiol. 31, 465–480.
6. Stewart, M. J., and Thomas, G. (1994) BioEssays 16, 809–815.
7. Price, D. J., Grove, J. R., Calvo, V., Avruj, D., and Bierer, B. E. (1992) Science 257, 973–977.
8. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992) Nature 358, 79–73.
9. Garren, L. D., Nye, R. L., and Davis, W. W. (1965) Proc. Natl Acad. Sci. U. S. A. 53, 1443–1450.
10. Orme-Johnson, N. R. (1990) Biochim. Biophys. Acta 1020, 213–231.
11. Whitehouse, B. J. (1992) J. Endocrinol. 134, 1–3.
12. Smith, W. A., Rountree, D. B., Bollenbacher, W. E., and Gilbert, L. I. (1987) in Progress in Insect Neurochemistry and Neurophysiology (Borkovec, A., and Gelman, D., eds) pp. 319–322, Humana Press, Clifton, NJ.
13. Smith, W. A., and Gilbert, L. I. (1989) J. Exp. Zool. 252, 202–270.
14. Klieghley, D. A., Lou, K. J., and Smith, W. A. (1990) Mol. Cell. Endocrinol. 74, 239–237.
15. Rybczynski, R., and Gilbert, L. I. (1994) Insect Biochem. Mol. Biol. 24, 175–189.
16. Rybczynski, R., and Gilbert, L. I. (1995) Mol. Cell. Endocrinol. 115, 73–85.
17. Henikoff, S. (1984) Gene (Amst.) 28, 351–359.
18. Hunter, T., and Selton, B. M. (1979) Proc. Natl Acad. Sci. U. S. A. 77, 1311–1315.
19. Jowett, T. (1986) in Drosophila: A Practical Approach (Roberts, D. D., ed) pp. 275–286, IRL Press, Oxford.
20. Towein, H. T., Stachelin, T., and Gordon, J. (1979) Proc. Natl Acad. Sci. U. S. A. 76, 4350–4354.
21. Bradford, M. (1976) Anal. Biochem. 72, 248–254.
22. Proudfoot, N. J., and Brownlee, G. G. (1976) Nature 263, 211–214.
23. Stewart, M. J., and Denell, R. (1993) Mol. Biol. Evol. 10, 1041–1047.
24. Spencer, T. A., and Mackie, G. A. (1993) Biochim. Biophys. Acta 1172, 332–334.
25. Watson, K. L., Konrad, K. D., Woods, D. F., and Bryant, P. J. (1992) Proc. Natl Acad. Sci. U. S. A. 89, 11302–11306.
26. Chan, Y.-L., and Wool, I. G. (1988) J. Biol. Chem. 263, 2891–2896.
27. Lott, J. B., and Mackie, G. A. (1988) Gene (Amst.) 65, 31–39.
28. Heinze, H., Arnold, H. H., Fischer, D., and Krupa, J. (1988) J. Biol. Chem. 263, 4139–4144.
29. Leer, R. J., van Raamsdonk-Duin, M. M. C., Molenaar, C. M. T., Cohen, L. H., Mager, W. H., and Plantza, R. J. (1992) Nucleic Acids Res. 10, 5869–5878.
30. Ferrari, S., Band, H. R., Hofstenge, J., Rusman, R. B., and Thomas, G. (1991) J. Biol. Chem. 266, 22770–22775.
31. Flotow, H., and Thomas, G. (1992) J. Biol. Chem. 267, 3074–3078.
32. Krieg, J., Hofstenge, J., and Thomas, G. (1988) J. Biol. Chem. 263, 11473–11477.
33. Bandi, H. R., Ferrari, S., Krieg, J., Meyer, H. E., and Thomas, G. (1993) J. Biol. Chem. 268, 4530–4533.
34. Lawen, A., Burger, M., and Martini, O. H. W. (1989) Eur. J. Biochem. 183, 245–253.
35. Grieneisen, M. L., Warren, J. T., and Gilbert, L. I. (1983) Insect Biochem. Mol. Biol. 23, 13–23.
36. Warren, J. T., and Gilbert, L. I. (1986) Insect Biochem. 16, 65–82.
37. Wettenthaler, R. E. H., and Morgan, F. J. (1984) J. Biol. Chem. 259, 2084–2091.
38. Bollenbacher, W. E., Vedelviks, W. V., Gilbert, L. I., and O’Connor, D. J. (1975) Anal. Biochem. 64, 46–53.
39. Nielsen, P. J., Duncan, R., and McConkey, E. H. (1965) Proc. Natl Acad. Sci. U. S. A. 52, 1400–14008.
40. Thomas, G., Thomas, G., and Luther, H. (1981) Proc. Natl Acad. Sci. U. S. A. 78, 5712–5716.
41. Jeffersies, H. B. J., Thomas, G., and Thomas, G. (1994) J. Biol. Chem. 269, 4367–4372.