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A *Drosophila* RNA helicase gene, *pitchoune*, is required for cell growth and proliferation and is a potential target of d-Myc

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SUMMARY

This article describes the characterization of a new *Drosophila* gene that we have called *pitchoune* (*pit*) (meaning small in Provence) because mutations in this gene produce larvae that cannot grow beyond the first instar larval stage although they can live as long as 7-10 days. All the tissues are equally affected and the perfectly shaped larvae are indistinguishable from first instar wild-type animals. Analysis of mutant somatic clones suggests a function in cell growth and proliferation, which is supported by the fact that cell proliferation is promoted by *pit* overexpression. Tagged-Pit, when transfected in S2 cells, localizes mainly to the nucleolus, pointing towards a possible role in ribosome biogenesis and, consequently, in protein biosynthesis. *pit* encodes a DEAD-box RNA helicase, a family of proteins involved in the control of RNA structure in many cellular processes and its closest homologue is a human DEAD-box RNA helicase, MrDb, whose corresponding gene transcription is directly activated by Myc-Max heterodimers (Grandori, C., Mac, J., Siëbelt, F., Ayer, D. E. and Eisenman, R. N. (1996) *EMBO J.* 15, 4344-4357). The patterns of expression of *d-myc* and *pit* are superimposable. Ectopic expression of *myc* in the nervous system drives an ectopic expression of *pit* in this tissue indicating that in *Drosophila* as well, *pit* is a potential target of d-Myc. These results suggest that *myc* might promote cell proliferation by activating genes that are required in protein biosynthesis, thus linking cell growth and cell proliferation.

Key words: DEAD-box, RNA helicase, *Drosophila*, Cell growth, Proliferation, *myc*

INTRODUCTION

Cell proliferation and growth of tissues are extremely intricate processes and very accurate controls must operate to ensure their coordination in the whole organism.

During early *Drosophila* embryogenesis, cells divide rapidly and their number increases exponentially; during this process, the embryo utilizes maternal nutrients and maintains its volume constant. By contrast, in larval stages, growth of larval tissues (two orders of magnitude) can be attributed mainly to an enlargement of the larval cells by endoreplication of their DNA without cell division. However, the diploid imaginal cells (and histoblasts as well), which became quiescent at the end of embryogenesis and are the precursors of adult structures resume their divisions and increase in number up to a thousand fold while cell size remains unchanged. These two different types of growth of the larval tissues are submitted to an absolute requirement for external feeding with appropriate molecules such as, for example, growth factors (Smith and Orr-Weaver, 1991; Edgar and Lehner, 1996; Serrano and O'Farrell, 1997).

Cell proliferation is primarily regulated during the G_1 phase of the cell cycle when cells sense their environment before replicating their DNA (Pardee, 1989; Norbury and Nurse, 1992). The G_1/S transition is particularly dependent upon protein synthesis and, in *Drosophila* for example, several mutations affecting growth rate and cell proliferation are located in partners of the protein synthesis machinery. *Minute* mutations are characterized by a haplo-insufficient phenotype associated with a delay in development, a general reduction in body size and short bristles (For a review, see Kay and Jacobs-Lorena, 1987). Homozygous *Minute* mutant embryos are able to pursue their development up to hatching but they die as early first instar larvae (Morata and Ripoll, 1975). The *Minute* genes that have already been molecularly characterized encode ribosomal proteins suggesting their involvement in protein biosynthesis. Similarly, reduction of the number of rRNA gene copies in *bobbed* mutations or of 5S RNA in *mini* mutants leads to comparable phenotypes.

Loss of function of the single (as opposed to several in other vertebrates) *Drosophila* gene coding for the receptor for

inositol triphosphate (InsP3) produces severe growth defects, the larvae being unable to pass over the second instar larval stage (Acharya et al., 1997). Release of InsP3 is eventually triggered by growth factor signalling molecules such as Wint1 in vertebrates (Slusarski et al., 1997) and, additionally, InsP3 contributes as a second messenger to the signal transduction pathway that regulates the p70S6 kinase (p70S 6k) activity, which is sensitive to inhibition by rapamycin (For a review, see Chou and Blenis, 1995). The cognate substrate for p70S 6k is the ribosomal protein S6 to which is attributed a pivotal role in the translational activity of the ribosome (Stewart and Thomas, 1994). In this same line, it has recently been postulated that, in yeast, a translational control of the G_1 cyclin Cln3p could couple cell growth to cell division (Polymenis and Schmidt, 1997).

Drosophila d-Myc is encoded by the *diminutive* locus and hypomorphic mutants in the gene display extraordinary growth defects (Gallant et al., 1996; Schreiber-Argus et al., 1997). The proto-oncogene *c-myc* codes for a basic helix-loop-helix leucine zipper (HLH-LZ) transcription factor whose ability to activate transcription and to participate in cell proliferation is dependent upon dimerization with an other HLH-LZ protein, the Max protein (Evan and Littlewood, 1993; for a review, see Amati and Land, 1994). c-Myc expression is activated by a variety of mitogenic stimuli that appear very early during the transition converting quiescent cells into proliferating cells and is maintained constant in these latter cells thus excluding a direct control of the cell cycle by the oncogene. While c-Myc's role in the activation of transcription is well documented, little is known about the nature of its targets. Some recent observations, however, are consistent with the idea that Myc could trigger cell proliferation by modulating protein synthesis. One of the identified Myc target genes is the eukaryotic initiation factor of translation eIF4E (Jones et al., 1996). In addition, Grandori et al. (1996) have cloned a human RNA helicase gene that encodes a Myc regulated DEAD box (MrDb) protein and which is a direct target of Myc-Max heterodimers. Finally, very recent results strongly support the participation of several yeast nucleolar DEAD-box-containing RNA helicases in rRNA processing and ribosome biogenesis and it is important to note that mutations in their genes all lead to growth arrest (Sachs and Davis, 1990; Ripmaster et al., 1992; O'Day et al., 1996; Liang et al., 1997; Kressler et al., 1997; Venema et al., 1997).

In this paper, we report the identification and characterization of the *Drosophila* gene *pitchoune* (*pit*) that encodes a novel DEAD-box protein containing all the signature sequences of this family of proteins with the exception of the DEAD-box itself in which a valine has replaced the consensus alanine. Moreover, Pitchoune shares a large number of similarities with the human MrDb helicase. As only sparse information is available on the pathways that Myc could control in association with its partners, this has led us to investigate whether *pit* could represent an in vivo target for Myc and to unravel the cellular processes in which such a target could be involved. We show that *pit* is a gene essential for cell growth that appears to be activated by Myc. The Pit protein is localized mainly to the nucleolus suggesting that it could exert its function by participating in some step of ribosome biogenesis and/or of rRNA processing and maturation.

MATERIALS AND METHODS

Genetic stocks

Deficiency stocks containing Df(3R)e-B52/TM3(93C-F) and Df(3R)e-N19/TM2(93B-94A) (Lindsley and Zimm, 1992), In(1)w^{m4}, as well as stocks to generate germline clones and somatic clones, were provided by the Bloomington *Drosophila* Stock Center. The *pit* mutations pit^4, pit^5 and pit^{10} were produced by imprecise excision of the B1-93F line (Ruohola et al., 1991). The balancer chromosome in the deficiency and in pit^{10} stocks was replaced by the TM3, DlacZ, which expresses β-galactosidase in a pattern identical to that of Deformed. Analysis of the small larvae phenotypes was performed on the progeny from a cross between pit^{10}/+ flies that had been obtained by back crossing pit^{10}/TM6B flies with Canton-S wild-type flies.

Isolation and analysis of genomic and cDNA clones

Standard molecular biology methods were used (Sambrook et al., 1989). Genomic DNA flanking the 1A122 P-element insertion (Perrimon et al., 1991; Zaffran et al., 1997) was isolated by using the plasmid rescue technique. The largest clone obtained, a region of 5 kb flanking the 1A122 P-element (p1A122 Pst1), was used to screen a λ-EMBL3 Canton-S genomic library (Clontech Laboratories, Inc.), a Canton-S 4-8 hour embryos cDNA library (Brown and Kafatos, 1988) and a λ-gt11 embryonic cDNA library (Zinn et al., 1988). Approximately 5×10^5 plaques were screened from each cDNA library. Fragments of the genomic and cDNA clones were subcloned into pBlueScript (Stratagene). Both strands of the longest cDNA clone pZN5 were sequenced by Genome Express (Grenoble, France). The GenBank accession number for the *pit* cDNA sequence is U84552.

P-element-mediated transformation

pUAS-*d-myc* and pUAS-*pit* were constructed by ligating the corresponding cDNAs (Gallant et al., 1996; this work) into pUAST (Brand and Perrimon, 1993). P(UAS-*d-myc*) and P(UAS-*pit)* were introduced into the fly germline by standard P-element transformation methods (Rubin and Spradling, 1982). Several independent transformants were obtained and lines of interest were then either made homozygous if viable and fertile, or balanced over *CyO* or *TM3*.

Construction of a *pit* minigene and rescue experiments

The minigene P(w^+-MN *mod/pit*) was constructed by ligating the pZN5 *pit* cDNA to a 4 kb fragment issued from the *modulo* gene (Garzino et al., 1992). A hsp terminator from CaspeR hs was inserted into the *Bam*HI site of pBS-SK$^-$ containing the pZN5 *pit* cDNA and a *Eco*RV fragment from the *modulo* gene control region (generous gift from Dr L. Perrin) was inserted upstream of the *pit* cDNA. The construct was digested by *Kpn*I-*Not*I to produce two fragments (1.7 and 5 kb long) which were sequentially inserted into *Kpn*I-*Not*I cut CaspeR4. The construct corresponding to the correct orientation of the minigene was injected in *yw* embryos and several independent lines were obtained.

For rescue experiments, flies bearing both the pit^{10} mutation and the P(w^+-MN *mod/pit*) element on the second chromosome were constructed (*w*; P(w^+-MN*mod/pit); pit*^{10}/TM6B,*Tb*) and crossed. Rescued pit^{10}/pit^{10} animals were recognized by their *Tb* minus phenotype. Three different transgenic lines on the second chromosome were used.

In situ hybridization on whole-mount embryos and on ovaries

Digoxigenin (DIG)-labelled antisense or sense RNA probes were generated from DNA with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Boehringer) and were used for whole-mount in situ hybridization of fixed staged embryos and ovaries as described in François et al. (1994). The DIG-labelled RNA probes were detected with the aid of a preadsorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer) and NBT/BCIP as substrate. The embryos

and ovaries were mounted in Geltol medium (Immunotech, France) for further observation.

Generation of mutant *pit* germline and somatic clones

Homozygous mutant *pit* clones were generated in the germline by using the autosomal FLP recombinase-dominant female sterile technique (Chou and Perrimon, 1992) and in the somatic line as described by Xu and Rubin (1993). The chromosome bearing the pit^{10} mutation was recombined with a P(ry^+, neo-FRT^{101})82B chromosome to obtain P(ry^+, neo-FRT^{101})82B, pit^{10} chromosomes, which were selected both for G418 resistance and for the presence of the pit^{10} mutation. 15 individual chromosomes were selected and balanced over TM3 or TM6B.

For the germline clones, the larvae (48-72 hours after egg laying) derived from a cross between P(ry^+, neo-FRT^{101})82B,pit^{10}/TM3 females and *yw*,P(ry^+, hsFLP); P(ry^+, neo-FRT^{101})82B, P(w^+, ovo^{D1})3R1, P(w^+, ovo^{D2})3R2/TM3 males were heat shocked at 37°C for 1 hour. The adult females of *yw*,P(ry^+, hsFLP); P(ry^+, neo-FRT^{101})82B, pit^{10}/P(ry^+, neo-FRT^{101})82B, P(w^+, ovo^{D1})3R1, P(w^+, ovo^{D2})3R2 genotype were crossed with wild-type or pit^{10}/TM3 males. These females, however, did not lay eggs. A control was performed with females carrying the P(ry^+, neo-FRT^{101})82B and P(ovo^D)FRT82B chromosomes. Their ovaries (from 5- to 6-day-old females) were dissected and stained with Hoechst dye.

Somatic clones were produced from the offspring of a cross between *yw*,P(ry^+, hsFLP); P(ry^+, neo-FRT^{101})82B, P(w^+, *c-myc*)87E, Sb^{63}, P(y^+,ry^+)96E/Dp males and yw; P(ry^+, neo-FRT^{101})82B, pit^{10}/TM6B females. A chromosome bearing a *Minute* mutation, P(ry^+, neo-FRT^{101})82B, P(w^+, *c-myc*)87E, Sb^{63}, M3(96C), was constructed by recombination with the *Minute* mutation M3(96C). Larvae at different stages were heat shocked at 37°C for 1 hour. Homozygous mutant clones for pit^{10} were *y* and not Sb^{63} and were observed in *yw*,P(ry^+, hsFLP); P(ry^+, neo-FRT^{101})82B, P(w^+, *c-myc*)87E, Sb^{63}, P(y^+,ry^+)96E/P(ry^+, neo-FRT^{101})82B, pit^{10} females. A control was performed by crossing males carrying the same FRT-labelled chromosome and the flipase with females carrying the P(ry^+, neo-FRT^{101})82B chromosome, which has been used to recombine the pit^{10} mutation with a FRT chromosome.

Transient transfections

The pCaSpeR hs-*pit* construct was achieved as follows. A 675 bp fragment corresponding to the 5′ end of the *pit* cDNA was prepared using PCR and the following oligonucleotides: (a) 5′ TGCTGTCGACGAAGATCGTTAAGC 3′, which was complementary to the 5′ end and introduced a *Sal*I site, and (b) 5′ ACCCACGATATCCCGTCCCTTTAGTAGG 3′, which was complementary to the 3′ end of the fragment and introduced a *Eco*RV site. After purification, the fragment was cleaved with *Sal*I and *Eco*RV and inserted into the *Sal*I and *Eco*RV sites of the modified vector pBS-*c-myc* constructed as described in Cerini et al. (1997). This plasmid (pBS-*c-myc-pit*) was sequenced to verify that the correct open reading frame for Pit had been established in frame with the ATG and the coding sequence for c-Myc. A 1.5 kb fragment that corresponds to the 3′ end of the *pit* cDNA was excised from pBS-KS^+ (Stratagene) using *Eco*NI and *Sma*I and was inserted in pBS-*c-myc-pit* cleaved using the *Eco*NI site in the 675 bp *pit* fragment and the *Sma*I site in the polycloning site of the vector. Finally, the reconstituted full-length *pit* cDNA bearing the *c-myc* epitope tag was cloned in the *Not*I site of the pCaSper hs polylinker (Thummel and Pirrotta, 1991).

Schneider line 2 cells were transfected as described previously using the calcium phosphate coprecipitation method (Courey and Tjian, 1988). The cells were plated in 2-chamber Lab Tek chamber slide system (Nalge Inc., USA) the day before transfection and each well received 1 μg CaSper hs-*pit* DNA. 44-48 hours post-transfection, the cells were heat shocked for 15 minutes at 37°C and allowed to recover for 90 minutes at room temperature. They were then fixed, for 15 minutes, in 4% formaldehyde in PBS and washed three times with PBS containing 0.3% Triton X-100. After blocking, for 1 hour, with 10% fetal calf serum in PBS, 0.3% Triton X-100, the cells were incubated overnight at 4°C with AJ1, a mouse monoclonal antibody that is specific for the nucleolus (Frasch et al., 1986; 1/2 dilution) and anti-Myc (rabbit IgG that recognizes the same epitope as mAb 9E10 (Evan et al., 1985)) (Santa Cruz biotechnology, 1/100 dilution). After several washings, the reactions were revealed by incubation for 1 hour at room temperature with affinity-purified secondary antibodies conjugated either with TRITC or with FITC (Jackson Immuno Research Laboratories; 1/100 dilution). Finally, the slides were mounted in Permafluor (Immunotech) and were observed under an Axiophot Zeiss microscope or a Zeiss Confocal microscope.

Antibody staining and histology of larval tissues

Staining of imaginal discs to detect somatic clones was performed as described in Xu and Rubin (1993). They were incubated overnight at 4°C with the same anti-Myc antibody as above, anti-Fasciclin III or anti-Invected (Developmental Studies Hybridoma Bank, Baltimore; 1/2 and 1/5 dilution, respectively), anti-Histone H3P (Euromedex, 1/200 dilution) and they were revealed with the same secondary antibodies as in the case of S2 transfected cells. Salivary glands and the midgut were dissected from wild-type and mutant larvae and processed for basic fuschin staining as described by Wieschaus and Nüsslein-Volhard (1986). Dissected tissues were incubated in vitro for 30 minutes at room temperature with BrdU (Sigma; 60 μg/ml in PBS) and further treated as described in Truman and Bate (1989) with an anti-BrdU antibody (DAKO; 1/50 dilution).

RESULTS

Isolation of *pit* genomic and cDNA clones and chromosomal location

Plasmid rescue of the original enhancer trap line 1A122 whose reporter gene is expressed in all myogenic cells (Perrimon et al., 1991; Zaffran et al., 1997) and screening of a genomic library led to the isolation of a λ phage clone (λ-9.2) containing a 15 kb insert (Fig. 1) which was used to screen cDNA libraries. Two different classes of cDNAs were isolated. One of them corresponds to the *how* gene (Zaffran et al., 1997). The other transcription unit will be referred to as *pitchoune* (*pit*), a word from the Provençal language illustrating the small mutant phenotype. Northern blot analysis showed that the longest cDNA clone (pZN5, 2205-bp) issued from that latter transcription unit hybridized to a single 2.4 kb transcript present both in preblastoderm (0-2 hour) and 8-12 hour embryos, indicating a maternal as well as a zygotic expression of this transcript. Different probes distributed along the total length of the pZN5 cDNA gave no evidence that the two transcripts could result from differential splicing.

A genomic walk around the *pit* unit allowed the establishment of a partial genomic structure of the *pit* gene (Fig. 1B) and showed that the transcription unit is divided into at least 3 exons (Fig. 1C). By in situ hybridization experiments to larval salivary glands polytene chromosomes, the gene was mapped to band 93 F on the right arm of chromosome III (data not shown), distal to the 93E region in which a cluster of homeobox genes has been previously located (Kim and Nirenberg, 1989) and in agreement with the initial location of the P-element insertion 1A122 (Zaffran et al., 1997).

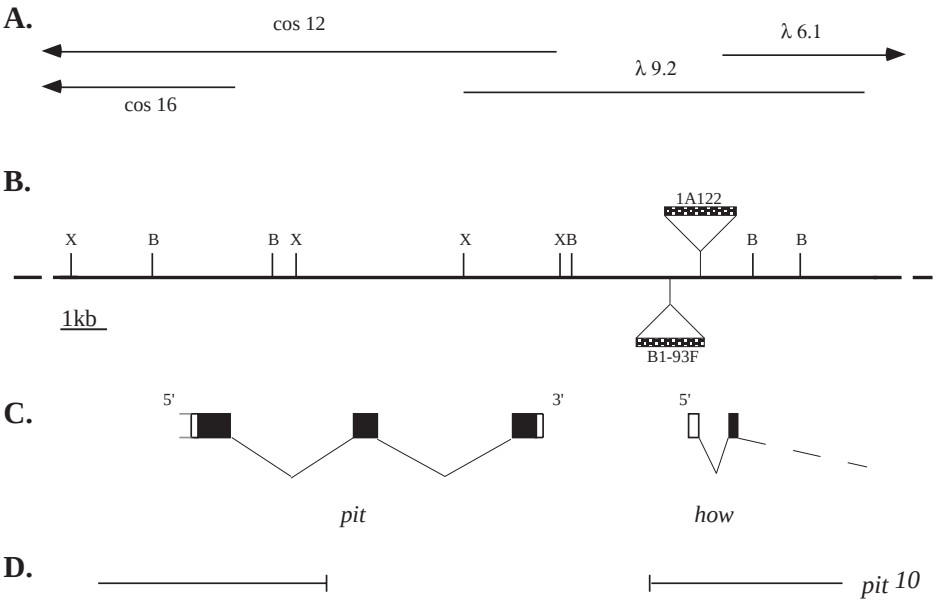

Fig. 1. The *pit* locus. (A) Physical map showing the overlapping genomic λ phages, which covered the 93F region. (B) The location of two enhancer trap P-elements 1A122 (Perrimon et al., 1991) and B1-93F (Ruohola et al., 1991) is indicated by triangles and some restriction enzymes sites are shown. X, *Xho*I; B, *Bam*HI. This map has not been oriented with respect to the centromere. (C) Structure of the cDNA in the genomic region. The left transcription unit corresponds to *pit* whereas the right transcription unit corresponds to *how* (Zaffran et al., 1997). The open boxes represent untranslated sequences of the cDNA and filled boxes indicate the coding sequences. A partial intron-exon structure of *pit* is also shown. (D) Deletion produced in the lethal mutation pit^{10} generated by imprecise excision of the B1-93F insertion. The 3′ of *pit* mRNA has been deleted. The breakpoint has not accurately been determined in the intron I. The GenBank accession number for the *pit* cDNA sequence is U84552.

The *pit* gene encodes a RNA helicase with an unusual DEAD-box (DEVD), homologous to human MrDb, whose gene is a direct target of Myc

The pZN5 cDNA clone contained an open reading frame of 1900 nucleotides (nucleotides 64-2079), encoding a protein constituted of 672 amino acids (about 75 kDa) (Fig. 2A). Given the length of the single mRNA detected by northern blot and taking into account a ~100 nt poly(A) stretch, the 5′-untranslated region of the gene could extend further upstream by approximately hundred nucleotides. A putative consensus poly(A) addition signal was found at position 2168 in the cDNA clone (underlined in Fig. 2A).

Searches in sequences database libraries revealed that the deduced Pit protein sequence displayed a significant score of homology with members of the DEAD-box family of ATPase RNA-dependent helicases that have been implicated in diverse cellular functions such as RNA splicing, ribosome assembly, initiation of translation and stabilization of RNA (Fuller-Pace, 1994). Particularly, the Pit-encoded protein contained, in its core region, the eight motifs with strong sequence conservation that are hallmarks of the family including the ATPase A and B motifs, the SAT motif and the HRIGR region (Linder et al., 1989; Schmid and Linder, 1992). In Pit, however, the alanine in DEAD was replaced by a valine (DEVD). This change was confirmed by sequencing two different cDNAs isolated from two unrelated libraries (see Materials and Methods). Furthermore, the genomic 5 kb *Pst*I fragment obtained by plasmid rescue from the 1A122 line was sequenced around the DEAD box and was shown to also have the DEVD sequence. It can therefore be concluded that this replacement was not due to polymorphism or to a mutation occurring haphazardly in the cDNA analyzed. Another ATPase RNA-dependent helicase from *Drosophila melanogaster*, unambiguously belonging to the DEAD-box family, has been reported to contain a DECD sequence (Eberl et al., 1997).

Fig. 2. Sequence of Pit and comparison with related DEAD-box helicases. (A) Sequences of the pZN5 *pit* cDNA and of the deduced protein. The pZN5 cDNA contains an ORF of 672 amino acids. The 8 highly conserved motifs of the DEAD-box family are boxed. The bipartite nuclear targeting sequence (Dingwall and Laskey, 1991) and the D/E stretch recovered in nucleolin (Ghisolfi et al., 1992) are underlined (short and long dashed lines, respectively). The consensus polyadenylation signal is over and underlined. (B): Alignment of the helicase domains of Pit and MrDb (Grandori et al., 1996). Amino acids found identical in the two sequences are boxed in grey squares. The eight motifs of the DEAD-box-containing helicases are underlined. (C) A dendrogram illustrating the similarities among members of a subfamily of DEAD-box-containing helicases. This result is based on an analysis using, in the Gene Works program, the UPGMA Tree Window. The length of the longitudinal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. Pit and MrDb are evolutionary very close and are probably orthologs. Two other sequences in yeast with unknown functions are also very closely related. Pit is a close relative to human DDX10 and to DBP4, which is a yeast nucleolar protein involved in ribosome biogenesis. a, Grandori et al., 1996; b, accession number Q09916; c, accession number Q03532; d, Savitsky et al., 1996; e, Liang et al., 1997; f,: Gururajan et al., 1991; g, Jamieson and Beggs, 1991; h, Lasko and Ashburner, 1988; i, Hirling et al., 1989; j, Weaver et al., 1997; k, Metz et al., 1992; l, O'Day et al., 1996; m, Schwer and Guthrie, 1991; n, Chen and Lin, 1990; o, Burgess et al., 1990; p, Company et al., 1991.

A.

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AACCATTAAAGTCGATTAAAAAGCATACATAGTCAGCAATATGTCTATCGGGAGAAGCTGCTGATGAAGAAGATC  75
                                                          M  K  K  K  I    4

GTTAAGCGGGAGAAGATGAAGAAGGAGCTCTCGCAGAAGAAGGGAAACAAGAAGGCCCAAAAGCAGGAGCCACCC 150
V  K  R  E  K  M  K  K  E  L  S  Q  K  K  G  N  K  K  A  Q  K  Q  E  P  P   29

AAACAAAATGGCAATAAACCCAGTAAAAAACCGGAGAAACTCAGTAAAAAGCATGTGGCCAAGGATGAGGATGAC 225
K  Q  N  G  N  K  P  S  K  K  P  E  K  L  S  K  K  H  V  A  K  D  E  D  D    54

GACTTAGAGGAGGATTTCCAGGAGGCGCCGCTGCCCAAGAAGAAGCAACAAAAACAGCCTCCAAAAAAGCAACAA 300
D  L  E  E  D  F  Q  E  A  P  L  P  K  K  K  Q  Q  K  Q  P  P  K  K  Q  Q    79

ATTCAGGTGGCCAACTCGGATTCGGAATCTGATGACGATGAGCAGGAGGATGAGGCAGATGAGGATAGCGATTTG 375
I  Q  V  A  N  S  D  S  E  S  D  D  D  E  Q  E  D  E  A  D  E  D  S  D  L   104

GACGAAGCAGCCGAAGTTGATGAGGAGGATGTTGACAGCGGGAGTGAAGATGATGACCAGCAGGAAGATGAAGAT 450
D  E  A  A  E  V  D  E  E  D  V  D  S  G  S  E  D  D  D  Q  Q  E  D  E  D    129

GAAGAAGAACCTGTGCCAGCCAAGAAAACGAAGTTGCTTCCCAACAAGTCCAAGGCACAGAATGGCAAACCAGCT 525
E  E  E  P  V  P  A  K  K  T  K  L  L  P  N  K  S  K  A  Q  N  G  K  P  A   154

AAAGACGACGAGCCCTTTACCGTGGAATCCTCACTGGCTGCACTGGATTACCGAGATTCGGATGATCGCAGCTTT 600
K  D  D  E  P  F  T  V  E  S  S  L  A  A  L  D  Y  R  D  S  D  D  R  S  F   179

GCCTCTTTAAAGGGCGCCGTGTCCGAGGCCACATTGCGAGCCATTAAGGAGATGGGTTTTACCGAGATGACTGAA 675
A  S  L  K  G  A  V  S  E  A  T  L  R  A  I  K  E  M  G  F  T  E  M  T  E   204

ATTCAGTCGAAATCGCTGACACCCCTACTAAAGGGACGGGATCTGGTGGGTGCTGCCCAGACGGGTTCCGGCAAA 750
I  Q  S  K  S  L  T  P  L  L  K  G  R  D  L  V  G  A  A  Q  T  G  S  G  K   229

ACCCTGGCCTTCCTGATACCCGCCGTCGAGCTGATAAACAAGCTGAGGTTTATGCCACGCAATGGTACCGGCGTG 825
T  L  A  F  L  I  P  A  V  E  L  I  N  K  L  R  F  M  P  R  N  G  T  G  V   254

ATCATCATCTCACCTACCCGAGAGTTGTCCATGCAAACTTTCGGTGTGCTCAAAGAGCTGATGGCACACCACCAT 900
I  I  I  S  P  T  R  E  L  S  M  Q  T  F  G  V  L  K  E  L  M  A  H  H  H   279

CACACTTATGGCTTGGTAATGGGCGGCTCCAATCGGCAGGTGGAGAGCGAGAAGTTGGGCAAGGGCATCAACATT 975
H  T  Y  G  L  V  M  G  G  S  N  R  Q  V  E  S  E  K  L  G  K  G  I  N  I   304

CTGGTGGCCACACCGGGTCGTCTGCTGGATCATTTACAAAACTCGCCCGACTTTCTGTACAAGAACCTTCAGTGC 1050
L  V  A  T  P  G  R  L  L  D  H  L  Q  N  S  P  D  F  L  Y  K  N  L  Q  C   329

CTGATTATCGATGAAGTGGATCGAATTCTGGAGATCGGTTTTGAGGAGGAGCTTAAGCAAATAATTAATCTGCTG 1125
L  I  I  D  E  V  D  R  I  L  E  I  G  F  E  E  E  L  K  Q  I  I  N  L  L   354

CCAAAACGCCGCCAGACAATGCTCTTCTCGGCCACACAGACGGCTCGCATCGAAGCGCTTTCCAAGCTGGCCCTT 1200
P  K  R  R  Q  T  M  L  F  S  A  T  Q  T  A  R  I  E  A  L  S  K  L  A  L   379

AAATCGGAACCAATTTATGTAGGGGTTCACGATAACCAGGACACGGCGACCGTGGATGGACTCGAGCAGGGGTAC 1275
K  S  E  P  I  Y  V  G  V  H  D  N  Q  D  T  A  T  V  D  G  L  E  Q  G  Y   404

ATTGTTTGCCCCTCGGAGAAGCGACTGCTCGTGCTCTTCACGTTCCTCAAAAAGAATCGTAAGAAGAAGGTGATG 1350
I  V  C  P  S  E  K  R  L  L  V  L  F  T  F  L  K  K  N  R  K  K  K  V  M   429

GTGTTCTTCTCGTCCTGCATGTCCGTCAAATACCACCACGAGCTCTTCAACTACATTGATCTGCCAGTGACCTCC 1425
V  F  F  S  S  C  M  S  V  K  Y  H  H  E  L  F  N  Y  I  D  L  P  V  T  S   454

ATCCACGGTAAACAAAAGCAAACGAAACGAACGACCACCTTCTTCCAGTTCTGCAACGCGGAATCTGGGATTCTC 1500
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TAAATGTTTCCAATTCAAAAAAAAA                                                   2200
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B.

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MrDb   EDTSFASLCNLVNENTLKAIKEMGFTNMTEIQHKSIRPLLEGRDLLAAAK 164

PIT    TGSGKTLAFLIPAVELINKLRFMPRNGTGVIIISPTRELSMQTFGVLKEL 274
MrDb   TGSGKTLAFLIPAVELIVKLRFMPRNGTGVLILSPTRELAMQTFGVLKEL 214

PIT    MAHHHHTYGLVMGGSNRQVESEKLGKGINILVATPGRLLDHLQNSPDFLY 324
MrDb   MTHHVHTYGLIMGGSNRSAEAQKLGNGINIIVATPGRLLDHMQNTPGFMY 264

PIT    KNLQCLIIDEVDRILEIGFEEELKQIINLLPKRRQTMLFSATQTARIEAL 374
MrDb   KNLQCLVIDEADRILDVGFEEELKQIIKLLPTRRQTMLFSATQTRKVEDL 314

PIT    SKLALKSEPIYVGVHDNQDTATVDGLEQGYIVCPSEKRLLVLFTFLKKNR 424
MrDb   ARISLKKEPLYVGVDDDKANATVDGLEQGYVVCPSEKRFLLLFTFLKKNR 364

PIT    KKKVMVFFSSCMSVKYHHELFNYIDLPVTSIHGKQKQTKRTTTFFQFCNA 474
MrDb   KKKLMVFFSSCMSVKYHYELLNYIDLPVLAIHGKQKQNKRTTTFFQFCNA 414

PIT    ESGILLCTDVAARGLDIPQVDWIVQYDPPGDQASIIHRVGRTARGSGTSG 524
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PIT    HALLLMRPEELGFLRYLKAAKVPLNEFEFSWQKIADIQLQLEKLIAKNYF 574
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PIT    LNQSAKEAFKSYVRAYDSHQLKQIFNVNTLDLQAVAKSFGFLVPPVVDLK 624
MrDb   LHKSAQEAYKSYIRAYDSHSLKQIFNVNNLNLPQVALSFGFKVPPFVDLN 564

PIT    VARPSGSDRKSDVGGGGFGFYKKMNEGSASKQRHFK               651
MrDb   VNSNEGKQKKRGGGGGFGYQKTKKVEKSKIFKHISK               599
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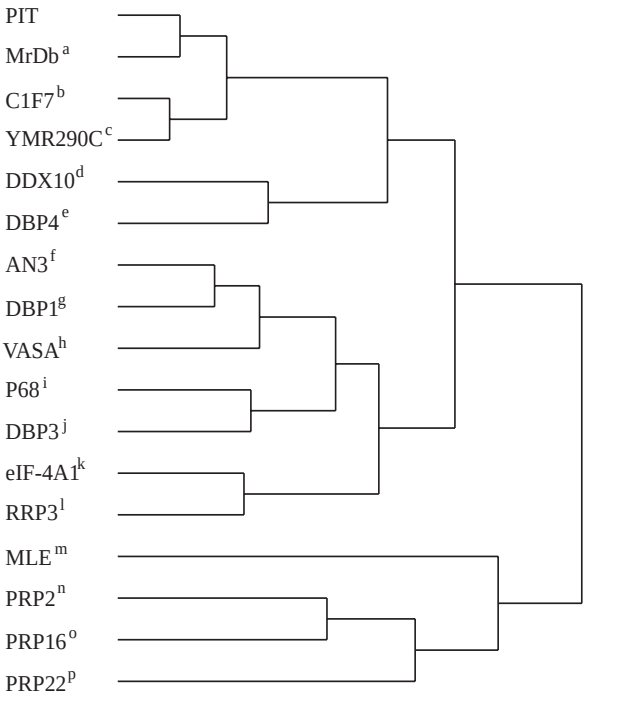
C.



Finally, the N-terminal domain (1 to 175 aa) of Pit had a very polar structure rich in lysine and Asp-Glu residues, which suggested a relationship, although only distantly, to the nucleolin family (Ghisolfi et al., 1992). The general organization of this N-terminal domain shared as well important overall similarities with the nucleolar P68 protein (Iggo et al., 1991). The bipartite nuclear localization signal (Dingwall and Laskey, 1991) was also found in the N-terminal part of Pit (underlined in Fig. 2A), suggesting that this protein exerts its function in the nucleus.

Interestingly, Pit shared very important similarities with human MrDb that extended over the entire length of the two proteins (Grandori et al., 1996; 59% identities) (Fig. 2B) and with two predicted yeast RNA helicases of unknown function (Fig. 2C). The human MrDb RNA helicase was isolated as being a direct Myc-Max heterodimers target gene (Grandori et al., 1996). An even higher level of homology was observed when only the helicase domain (77% identity over 500 amino acids) was considered. Moreover, the conservation between these two proteins extended outside the helicase region since the N-terminal domain of both proteins shared a poly(K) stretch and a D/E-rich region. This analysis suggests that Pit, MrDb and the yeast putative RNA helicases could define a new subgroup within the DEAD-box family that is clearly apparent from the evolutionary tree in Fig. 2C.

Expression of the *pit* transcript

The *pit* transcript was uniformly distributed in preblastoderm embryos, thus suggesting a maternal expression (Fig. 3A). This was confirmed by in situ hybridization on ovaries in which *pit* was ubiquitously expressed in late egg chambers (Fig. 3F). The transcript reproducibly displayed only very low, if any, expression in stage 2 egg chambers (Fig. 3F). At gastrulation, *pit* was expressed in the invaginating mesoderm. At early stage 11, *pit* expression was essentially visible in the mesoderm, from which it rapidly disappeared during germband extension, in the anterior and posterior precursors of the midgut and in the precursors of the salivary glands in parasegment 2 (Fig. 3B). At this stage, all these territories are mitotically active (Foe, 1989) and are programmed to enter into endoreplicative cycles (Smith and Orr-Weaver, 1991). A strong expression was also observed in the anal plate. In late stages, expression became restricted to the differentiating midgut (Fig. 3E), the Malpighian tubules and the mesodermal sheath of gonads (not shown). This late expression was, however, at a much lower level than that observed in the anterior and posterior midgut from stage 10-13 embryos. Also, weak expression was detectable in the epidermis at the end of embryogenesis (not shown). Finally, *pit* was ubiquitously expressed in the imaginal discs (not shown).

Overall during development, but especially in the early stages following gastrulation, *pit* expression closely paralleled that of *d-myc* (Gallant et al., 1996; Schreiber-Agus et al., 1997). This was also true in oogenesis (Fig. 3F and Gallant et al., 1996) during which *d-myc* is present in large amounts except in stage 2 egg chambers.

Ectopic expression of *d-myc* drives ectopic expression of *pit*

The almost completely superimposable *pit* and *d-myc* expression patterns as well as the similarities existing between the Pit sequence and that of MrDb, strongly support the hypothesis that *Drosophila pit* might also be a target for the transcriptional factor d-Myc. d-Myc is encoded by the *diminutive* locus (Gallant et al., 1996; Schreiber-Agus et al., 1997). The expression of *pit* was not, however, noticeably affected in ovaries of females homozygous for the hypomorphic allele of *diminutive*, dm^1. This result might indicate that *d-myc* is not required for the expression of *pit*. However, the low level of *d-myc* in the mutants might be sufficient to promote a high enough amount of *pit* leading to an apparently normal expression. In the same line, we were not able to see any difference in the embryonic expression of *pit* in dm^1 homozygous mutants. As is the case for *pit*, *d-myc* is maternally expressed and it is not known whether the maternal protein is stable throughout embryogenesis. As yet, no complete loss-of-function allele of *diminutive* is known.

Our attempts to demonstrate a possible interaction between *pit* and *d-myc* were then turned towards an ectopic *d-myc* expression by using an UAS-*d-myc* cDNA driven by a variety of tissue-specific GAL4-expressing lines. Since *d-myc* RNA was present neither in the nervous system nor in differentiating muscles, the 1407 and 24B lines, which respectively express GAL4 in the central and peripheral nervous system (Luo et al., 1994) and in all the muscles (Brand and Perrimon, 1993; Zaffran et al., 1997), were used. As shown in Fig. 4, *pit* was expressed in the central nervous system in embryos derived

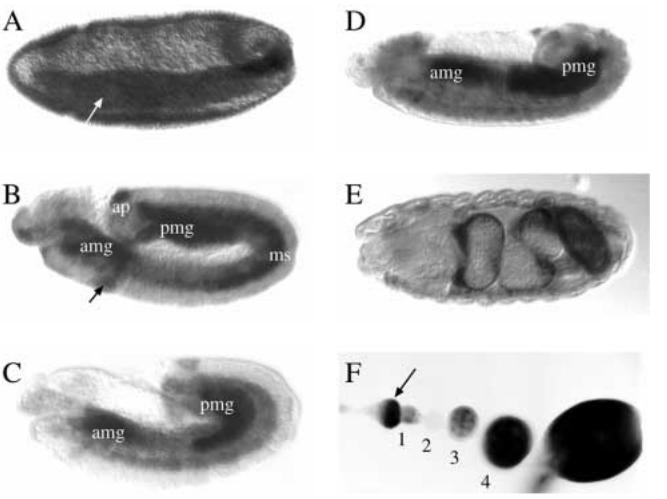


Fig. 3. Expression of the *pit* mRNA during development. In situ hybridization with a DIG-labelled riboprobe from the pZN5 cDNA. (A) In an early gastrula embryo, *pit* is expressed in the invaginating mesoderm (arrow). Strong maternal expression is also visible in the entire blastodermal cells. (B) Early stage 11 embryo. The mesoderm (ms), the anterior midgut precursors (amg) and the posterior midgut precursors (pmg) are labelled. Strong expression is also observed in the precursors of the salivary glands (in PS2, arrow) and of the anal plate (ap). (C) Stage 12 embryo. Strong expression is maintained in the amg and pmg but also persists in the other territories described in B. (D) Late stage 12 embryo. Only the amg and pmg are strongly labelled. A weak segmental expression (not in focus) is observed on the ectoderm. (E) At stage 16, the entire epithelial sheet of the intestine is labelled. (F) The ovariole is stained strongly in the germarium (arrow) and in egg chambers from stage 4 onward. A weak or null expression is reproducibly observed in stage 1 and 2 egg chambers.

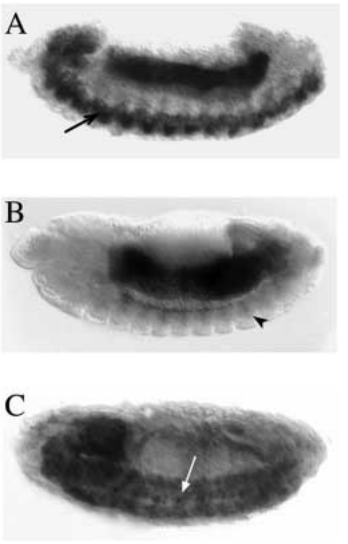


Fig. 4. *pit* expression in embryos ectopically expressing *d-myc*. Stage 13 embryos in A and B were processed for in situ hybridization by using a DIG-labelled *pit* riboprobe. (A) Embryos from a cross between flies bearing the UAS-*d-myc* cDNA and 1407-GAL4 flies. In addition to its normal endogeneous expression in the midgut, *pit* mRNA is strongly expressed in the central nervous system (arrow). (B) Control embryo issued from UAS-*d-myc* cDNA transgenic flies. *pit* expression is restricted to the midgut. (C) Embryo from the same cross as in A but hybridized with a DIG-labelled *d-myc* cDNA probe. Ectopic expression in the central nervous system is observed (white arrow).

from the 1407 GAL4 line, suggesting that d-Myc could behave, at least in that tissue, as a transcriptional activator of *pit*. In contrast, no evident ectopic expression of *pit* could be demonstrated in muscle precursors when the *d-myc* driver was 24B. There are several likely reasons for a lack of induction of *pit* in muscle. For example, the Myc protein is known to dimerize with Max to make a heterodimer that activates transcription (Blackwood and Eisenman, 1991). d-Max expression in muscle has not been clearly established (Gallant et al., 1996) and a too low concentration in this tissue might impair the transcriptional activation of the Myc targets. In conclusion, these results strongly support the hypothesis of *pit* being a target for Myc transcriptional activation and, as such, of playing a particular role. Of course, it is not possible to anticipate from these experiments whether or not *pit* is a direct target of d-Myc.

pit is an essential gene required for cell growth and proliferation

The B1-93F line (also called B1-3-12) in the 93F region has been located in close proximity to *pit* (Fig. 1). B1-93F is homozygous viable and no differences were observed when the pattern of expression of *pit* was compared in wild-type embryos and in homozygous B1-93F embryos.

A P-element mobilization screen with B1-93F led to two complementation groups of lethal mutations. A mutation in one of these complementation groups, pit^{10}, resulted from a small deletion of a 3.5 kb long genomic region starting from the initial site of insertion and extending towards the *pit* transcription unit. A part of the transposon, including the *lacZ* gene still resided at the site of insertion. The proximal breakpoint fell within the second exon of *pit* leading to an almost complete deletion of the helicase domain (Fig. 1). In contrast, no gross molecular alterations could be observed in the *how* gene which is in close proximity (Zaffran et al., 1997). The mutant alleles that specifically modified the *how* function were able to complement the pit^{10} mutation. Because this latter mutation removed most of the helicase domain, it was considered to be a loss-of-function allele and probably even a null mutation. Genetically, the phenotype of pit^{10} in *trans* to the deficiency Df(3R)e-BS2 was indistinguishable from that of homozygous pit^{10} animals. The pattern of the cDNA expression was not altered in pit^{10} homozygous embryos nor in embryos bearing pit^{10} in *trans* to the deficiency Df(3R)e-BS2 (not shown), suggesting the presence of a truncated mRNA unable to produce a functional protein. Two other mutations in the *pit* gene were obtained by mobilization of the B1-93F P-transposon. pit^{4} in *trans* to pit^{10} produced the same phenotype as homozygous pit^{10} animals (see below). Contrary to pit^{10}, this mutation did not complement loss-of-function mutations of *how* (Zaffran et al., 1997). Finally, homozygous pit^{5} animals were completely viable but were lethal in *trans* to Df(3R)e-BS2 or to pit^{10}. In this latter case, however, larvae developed normally but with a delay and, although they eventually were able to pupariate, they never gave adults.

Homozygous pit^{10} animals displayed important growth defects. Larvae hatched normally and initially showed a healthy behaviour, although with a constant delay in their timetable when compared to wild-type larvae. In a pit^{10}/+ cross, 75% of embryos hatched 24±2 hours after egg laying while 25% of the embryos that corresponded to homozygous pit^{10} embryos hatched later with a delay amounting to 7±4 hours.

Furthermore, the pit^{10} mutants failed to grow beyond the first instar larval stage and they never accomplished their metamorphosis (Fig. 5A). Heterozygous larvae as well as wild-type controls continued their growth throughout each of the larval stages and developed normally. In contrast, the mutants were arrested at the first larval stage, although they could survive longer than 7 days. The mutant larvae appeared normal and all of the tissues that could be examined had a wild-type morphology indistinguishable from that of a first instar wild-type larva (Fig. 5B,C).

The first attempts to rescue the homozygous mutant pit^{10} phenotype by expressing UAS-*pit* cDNA under the control of various GAL4 driver lines failed. This result was interpreted as reflecting the fact that none of these drivers including e22cGAL4, daGAL4, 24B or hsGAL4 perfectly mimicked the embryonic expression pattern of *pit*. A chimeric *pit* minigene was therefore constructed by fusing the *pit* pZN5 cDNA to a 4 kb genomic fragment (generous gift from Dr L. Perrin) belonging to the *modulo* gene (Graba et al., 1994) and capable of directing the expression of a reporter gene in a pattern identical to the embryonic spatiotemporal expression pattern of *mod* or of *pit* (Dr L. Perrin, personal communication). It was hypothesized that this hybrid minigene should rescue at least the small larvae phenotype. Indeed, third instar homozygous pit^{10} larvae (not *Tb*) were obtained in a Mendelian proportion, but with a delayed developmental schedule. Only a few larvae pupariated and no adults were recovered, probably because the larval expression pattern of *mod* does not perfectly match that

of *pit* (not shown) or because *pit* has not been expressed to an appropriate extent. Nevertheless, this result unambiguously demonstrates that the small larvae phenotype observed in pit^{10}/pit^{10} animals was specifically due to a mutation in the *pit* gene.

The larvae of holometabolous insects are composed of two types of tissues: the larval cells, which do not proliferate but grow by enlargement and polytenization, and the imaginal cells, consisting of diploid cells that have a very high proliferative activity and that give rise to most of the adult structures. Histological examination of various tissues taken from 5-day-old pit^{10} mutant larvae (compared to third instar wild-type larvae of the same age) revealed profound alterations in the growth of larval tissues and in that of imaginal progenitor cells. For instance, midgut progenitor cells or salivary glands could still be recognized but they did not significantly increase in size (Fig. 5E,G compared to D,F). Their total number remained identical to that present in wild-type larvae and they had been subjected to at most 1-2 cycles of endoreplication of their DNA. The imaginal cells and, in general, the adult precursor cells, which are diploid and divide during larval stages, did not proliferate in a pit^{10} mutant (Fig. 5E compared to D). Similarly, the imaginal discs were of the same size as those in young first instar larvae (not shown), suggesting a lack of proliferation in this tissue as well. The same types of modifications probably prevailed in other tissues of the mutants, especially in the epidermis since the larvae remained small although perfectly shaped and identical to wild-type first instar larvae. Finally, the mutant larvae did not incorporate BrdU in the nuclei of their cells indicating a failure in DNA replication (Fig. 5H-K). In conclusion, the loss of function of *pit* seems to lead to a general arrest in cell growth of larval cells and in cell proliferation of adult precursor cells in a precisely coordinated manner.

As previously mentioned (Fig. 3F), *pit* is strongly expressed during oogenesis and a maternal contribution to its expression during embryogenesis might explain an apparent lack of embryonic *pit* function. In order to investigate this issue, germline clones free of *pit* were generated using the dominant female sterile technique (DFS; Chou and Perrimon, 1992) in FRT pit^{10} / FRT P(ovo^{D1}) females but no eggs could be recovered. In contrast, many clones heterozygous for FRT P(neo; ry^{+}) / FRT P(ovo^{D1}) were obtained and served as

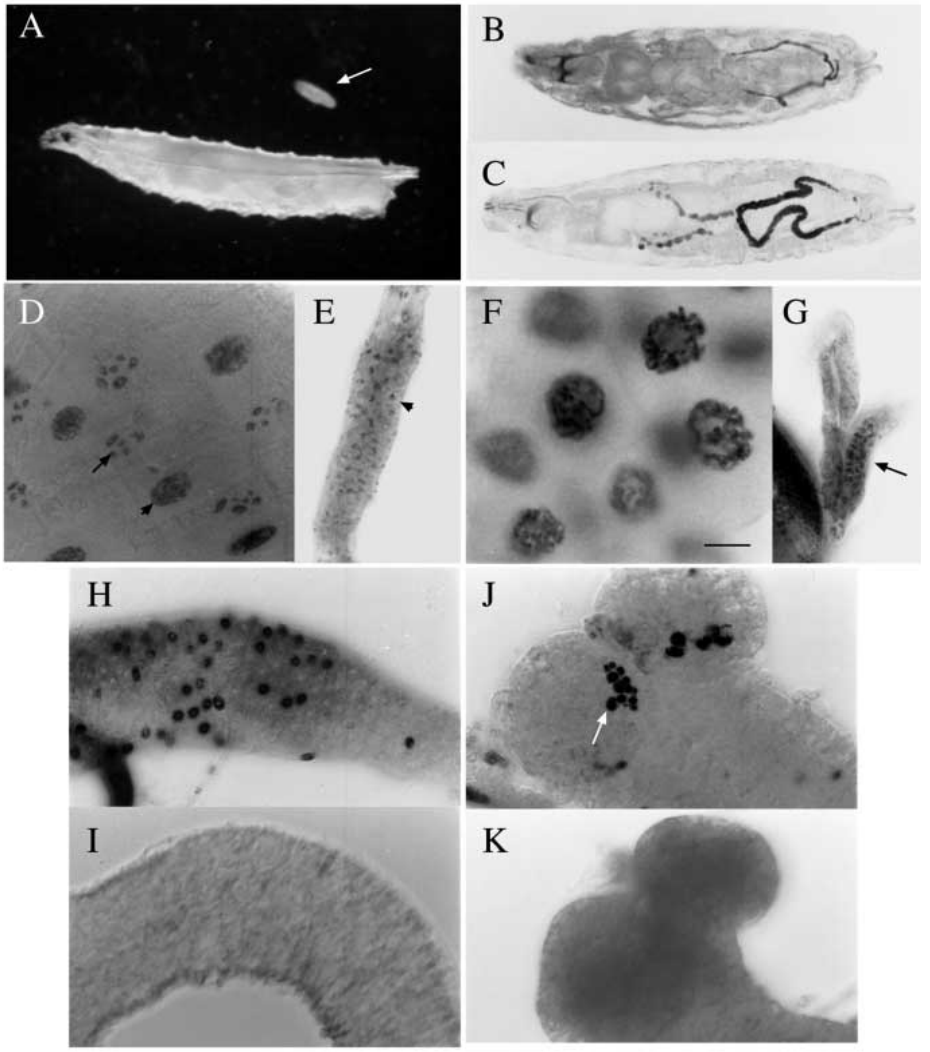


Fig. 5. Homozygous *pit* larvae stop growing after hatching. (A) Comparison of two larvae of the same age (7 days at 22°C). The wild-type larva is ready to pupariate. The small larva is still alive and can move (arrow). (B,C) Comparison of a wild-type first instar larva (B) and of a homozygous *pit* larva (C). The mutant larva (5 days old) appears similar to wild-type first instar larva. (D,E) Comparison of the midgut of 5-day-old larvae stained with fuschin. In wild-type larvae (D), the midgut epithelial cells are large and their nuclei are strongly polyploid (arrowhead). Note the presence of a large number of small diploid nuclei (arrow) corresponding to the intestine adult precursor cells. The mutant midgut (E) contains the same overall number of principal midgut epithelial cells, but they are smaller by at least two orders of magnitude. In parallel, the level of polyploidy of their nuclei (arrowhead) is considerably lower, and the number of adult precursor cells is smaller than in the third instar wild-type larvae, roughly corresponding to their number in a first instar wild-type larva.
(F,G) Comparison of salivary glands: (F) wild-type salivary glands with highly polytenized nuclei, and (G) salivary glands of homozygous *pit* larvae (arrow). (H,I) Incorporation of BrdU in midgut of first instar wild-type larvae and of 5-day-old pit^{10} larvae, respectively. In the wild type, many cells have replicated their DNA once during the period of BrdU incubation but, under the same conditions, none of the mutant cells nuclei have incorporated BrdU.
(J,K) Brains of early first instar wild-type larva and of pit^{10} larva, respectively, under the same experimental conditions as in H and I. The first cells that divide in wild-type hatching larvae are the large neuroblasts of the mushroom bodies (arrow). These cells do not incorporate BrdU in a mutant pit^{10}. Bar, 15 μm in D to G which are at the same scale.

internal controls. In pit^{10} / P(ovo^{D1}) females, the ovaries were very small and indistinguishable from those of +/P(ovo^{D1}) females. Oogenesis never proceeded through stage 6. This result was interpreted as an early requirement for *pit* activity in the germline during oogenesis. However, due to the incapacity of the females to lay eggs, pit^{10} embryos devoid of maternal contribution could not be observed.

Analysis of somatic mosaics implies a requirement of *pit* for cell growth and proliferation.

Somatic homozygous mutant clones, which were recognized by their hair and bristles phenotypes, were produced with the FLP/FRT technique (Xu and Rubin, 1993). They were *yellow* and did not carry the Sb^{63} marker (short and thick hair) in an otherwise $yellow^{+}$ and Sb^{63} environment. Only very small clones carrying the associated *yellow* marker were observed (Fig. 6A,B) and only when the recombinase was induced late in development (end of third instar larval stage). These clones were in consequence easily distinguished among the thorax chaetes being thinner and even smaller than the surrounding Sb^{63} chaetes (Fig. 6A,B). This was reminiscent of the phenotype encountered in the case of *Minute* and also of *dim* mutations, which affect cell growth and proliferation (For a review, see Kay and Jacobs-Lorena, 1987). Under the same conditions, large wild-type clones with long hair, lacking the Sb^{63} mutation were obtained in a control cross (not shown).

The cells within homozygous mutant clones were also observed in imaginal wing discs of late third instar larvae. Here again the mutant clones, which were apparent because they did not possess the clonal cellular marker *myc* (a short sequence of c-Myc recognized by the 9E10 antibody (Evan et al., 1985)), were very small and composed of only a few cells (Fig. 6D). They were at least two orders of magnitude smaller than wild-type clones which were easily recognized due to the important staining of the *myc* marker (Fig. 6C). All these observations certainly illustrate the poor ability of pit^{10} mutant cells to grow and proliferate and also suggest that mutant cells can be overtaken by their wild-type sister cells and therefore eliminated and replaced as it has been previously shown in the case of *Minute* mutant cells (Simpson and Morata, 1981). As a matter of fact, when homozygous pit^{10} clones were induced in *Minute* flies, large mutant clones were obtained (Fig. 6E) indicating that the pit^{10} mutation is not cell lethal and does not directly interfere with the cell cycle machinery but rather autonomously affects cell growth.

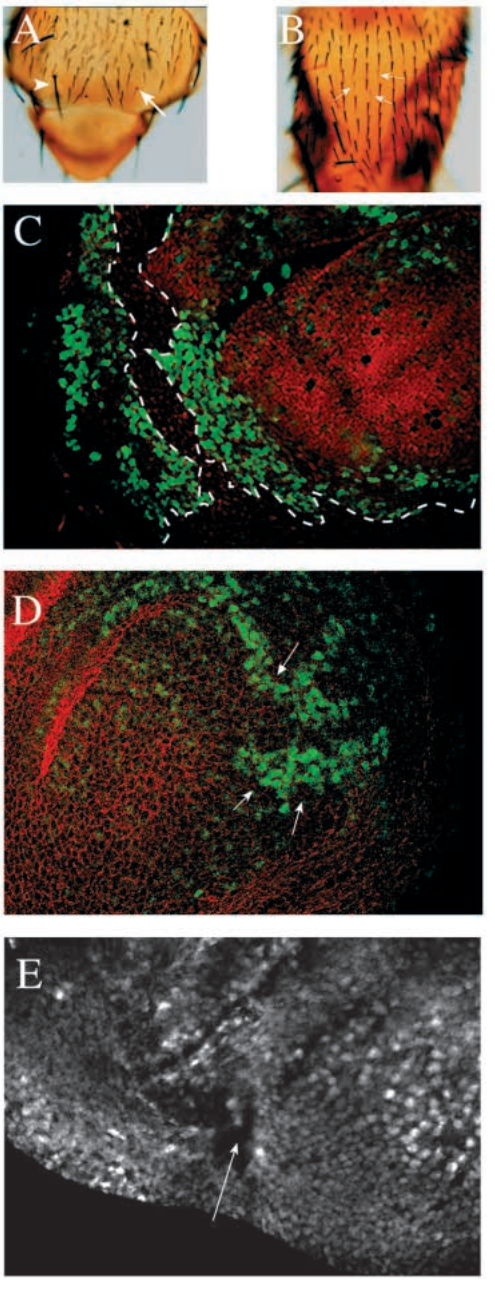


Fig. 6. Analysis of somatic mutant clones reveals a cell growth requirement for *pit*. (A,B) Thoraces of *yw*, P(ry^{+}, hsFLP); P(ry^{+}, neoFRT^{101})82B, P(w^{+}, *cmyc*)87E, Sb^{63}, P(y^{+}, ry^{+})96E / P(ry^{+}, neoFRT^{101})82B, pit^{10} females in which somatic recombination has been induced during the third instar larval stage. The mutant clones can be recognized by their *yellow* phenotype and the absence of Sb^{63} mutation. In heterozygous cells, Sb^{63} produces smaller hairs as compared to the wild type. (A) The mutant macrochaete (arrow) is smaller than the wild type (arrowhead), which is Sb^{63} and its color is yellow instead of black-brown in the case of the wild-type macrochaete. (B) *yellow* microchaetes (white arrows) are also smaller than the Sb^{63} wild-type microchaetes. (C-E) Imaginal discs of late third instar larvae in which the flipase has been heat-activated at early third instar larval stage. (C) The genotype of the larva is the same as in A and B but the chromosome P(ry^{+}, neoFRT^{101})82B does not carry the *pit* mutation. The cellular marker *c-myc* (in green) is absent from the P(ry^{+}, neoFRT^{101})82B homozygous clone whose limits are drawn with a dotted white line. Juxtaposed to this clone, the reverse clone is intense green (due to the homozygosis of the cellular *c-myc* marker). The rest of the disc is green and corresponds to the heterozygous cells. The localization of the *c-myc* tag is nuclear. The red marker, which is membrane associated, is Armadillo. (D) Despite the presence of intense green clones, which indicates that the recombination has taken place, only very small mutant clones (arrows) can be recognized. This result probably means that mutant cells either do not divide or only very slowly and/or that they are eliminated. (E) The genotype of the larva is *yw*, P(ry^{+}, hsFLP); P(ry^{+}, neoFRT^{101})82B, P(w^{+}, *cmyc*)87E, Sb^{63}, M3 (96C)/P(ry^{+}, neoFRT^{101})82B, pit^{10}. The imaginal discs were stained only for the cMyc tag and, consequently, mutant clones appeared in black in an otherwise white background. The *Minute* homozygous clones were not detected as they are cell lethal. The mutant clones (arrow) are several order of magnitude larger than in D, suggesting that cell growth is affected in pit^{10} homozygous clones.

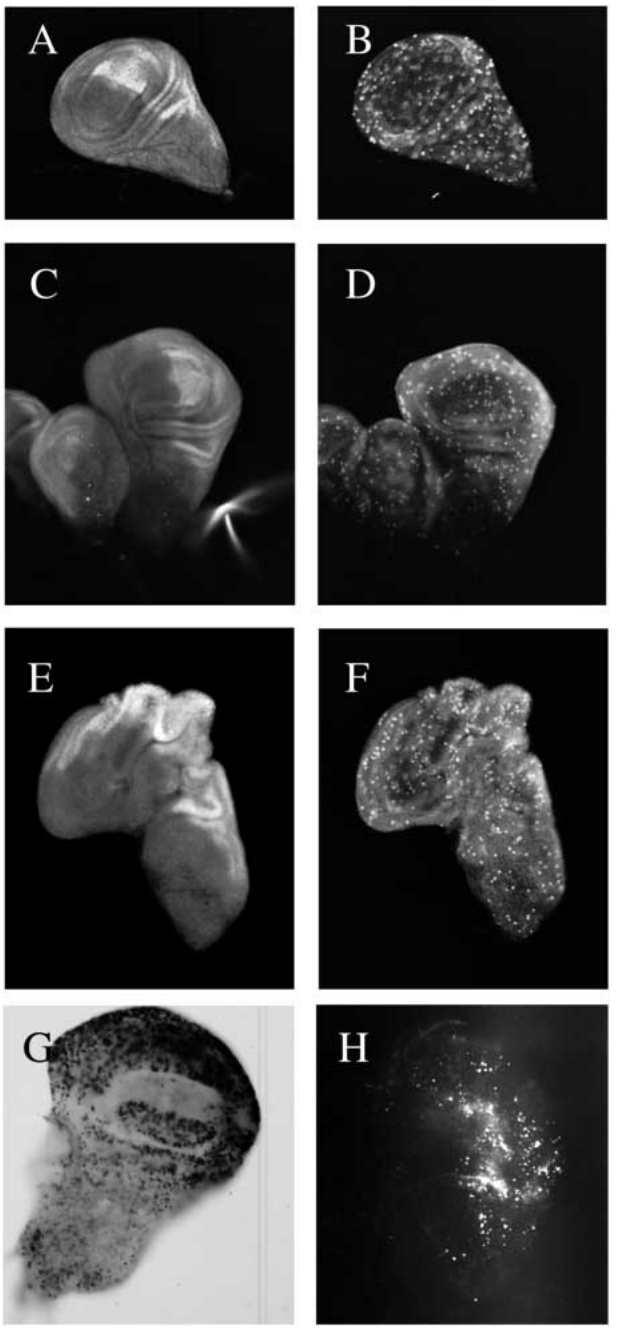


Fig. 7. Overexpression of *pit* in imaginal discs promotes cell proliferation and cell death. Anti-invected (A,C,E) and anti-Histone H3P staining (B,D,F) of wing imaginal discs from wild-type (A,B) and UAS-pit^{10}; *en*-GAL4 third instar larvae (C-F) grown at 29°C. Anti-Histone H3P reveals mitoses and anti-invected antibody stains the nuclei of the posterior compartment. Note the higher number of mitoses in the posterior compartment as compared to that in the anterior compartment in D and F. (G) Anti-BrdU staining of a UAS-pit^{10}; *en*-GAL4 wing imaginal disc from a third instar larva: a slightly higher level of BrdU incorporation can be distinguished in the posterior compartment. (H) Acridine orange staining of a UAS-pit^{10}; *en*-GAL4 wing imaginal disc revealing dying cells located principally, but not exclusively, in the posterior compartment. Very little, if any, cell death can be observed in wing imaginal discs from either wild-type or UAS-pit^{10} larvae. In all views, anterior compartment is left and posterior compartment is right.

Overexpression of *pit* can promote cell proliferation

pit overexpression into the posterior compartment of otherwise wild-type imaginal discs was analyzed in larvae resulting from a cross between UAS-*pit* flies and *en*-GAL4 flies. Larval development was allowed to proceed at 29°C. As shown in Fig. 7C,D, an increase in the number of mitoses, as judged from the expression of Phosphohistone H3 was repeatedly observed and was accompanied by a higher percentage of cell death (Fig. 7H). The number of mitoses in the posterior compartment was, however, never greater than 2 to 3 times that in the anterior compartment. Similar results were obtained by incorporating BrdU in living third instar larvae (Fig. 7G). Due to the high replicative activity prevailing in the whole disc at this developmental stage, the increase in the number of replicating nuclei in the posterior compartment was somewhat smaller than in the previous estimate but was, nevertheless significant. In rare occasions (in a small percentage of the examined discs), we noticed a clear hyperproliferative phenotype specifically affecting the posterior compartment (Fig. 7E,F).

A normal proportion of adults emerged from larvae that had overexpressed *pit* and they did not present any evident mutant phenotype, with the restriction that a few individuals might have died and thus escaped our scrutiny. This result is consistent with the idea that *pit* overexpression did not induce a permanent hyperproliferative phenotype in the imaginal discs and that some kind of compensatory mechanism (cell death etc.) may have been at work in this tissue.

Pit localizes to the nucleolus

Polyclonal antibodies directed against the C-terminal half of Pit were efficient on Western blots to detect the protein in embryos but they did not allow its subcellular localization by indirect immunofluorescence on whole-mount embryos.

To that aim, Pit was c-Myc-tagged at its N terminus by cloning of its cDNA in pCaSper hs to express the epitope-tagged protein from the hs promoter (see Material and Methods). Transfected S2 *Drosophila* cells were heat shocked and processed for immunodetection. The tagged protein was detected with anti-c-Myc antibodies and the nucleolus by the AJ1 signal using anti-AJ1 antibodies (Frasch et al., 1986). A yellow staining pattern (Fig. 8A) resulted from a complete overlapping of the expression of c-Myc-Pit and AJ1 and suggested a nucleolar localization for Pit. The predominant distribution of c-Myc-Pit within the nucleolus, in the specialized compartment for ribosome synthesis, could point towards its participation in rRNA maturation or in another step in the biogenesis of ribosomal subunits.

As the function of Pit seemed to be required in the nucleolus, an eventual perturbation of this organelle was investigated in pit^{10} mutant larvae. As a marker for the nucleolus, an antibody directed against the Modulo protein (Garzino et al., 1992) was chosen rather than AJ1 because it has recently been shown that Modulo exhibited a differential localization within the nucleolus as a function of the state of replication of the nucleus (Perrin et al., 1998). As depicted in Fig. 8C, Modulo was perinucleolar in the polyploid nuclei of the principal midgut epithelial cells whereas in diploid cells, which were able to divide, the expression of Modulo covered the whole volume of the nucleolus. This differential localization was not affected in the *pit* mutant midgut cells (Fig. 8D) thus suggesting that the nucleolus was normal in the mutant with no alteration in its

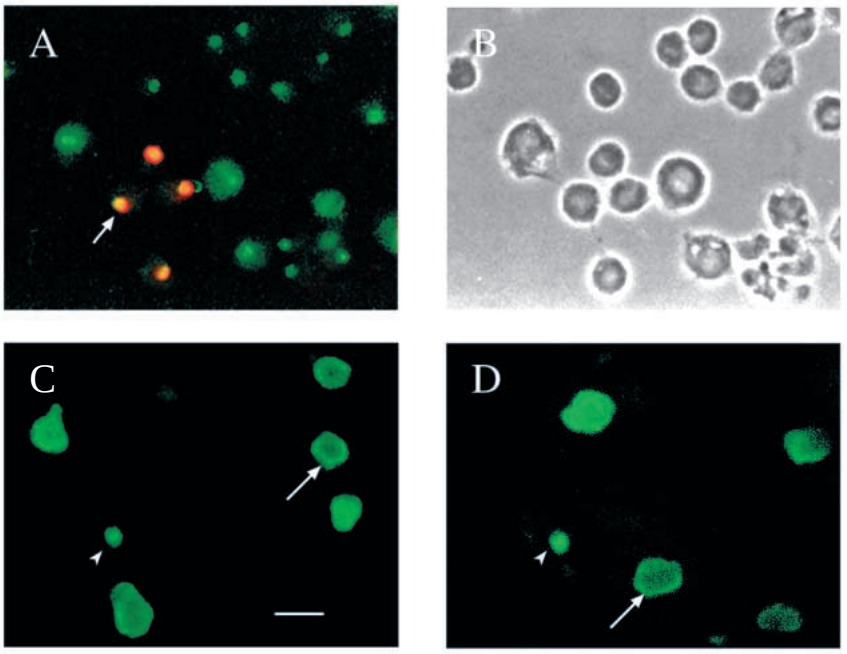


Fig. 8. Pit is localized to the nucleolus. (A) S2 cells transfected by a cDNA coding for a c-Myc-tagged Pit protein under the control of a heat-shock promoter. After induction of Pit expression, the cells are labelled with the anti-AJ1 antibody (Frasch et al., 1986) to label the nucleolus (in green) and with an antibody that recognizes the c-Myc epitope (in red). Superimposition of anti-c-Myc and anti-AJ1 stainings give a yellow color (arrow). (B) Phase-contrast view of A. (C) The midgut of a first instar wild-type larva was stained with an antibody directed against Modulo (Garzino et al., 1992). Modulo is preferentially localized at the periphery of the nucleolus in polyploid nuclei (arrow) whereas its distribution is homogeneous in the nucleolus of the diploid adult precursor cells (arrowhead). (D) Same as in C on 5-day-old *pit* mutant larvae. The bar in C represents 3.6 μm for A and B and 1.2 μm for C and D.

overall structure, at least as judged by the criterion that has been used.

DISCUSSION

We describe herein the characterization of a new *Drosophila* gene, *pit*, whose crucial function in cell growth and proliferation is supported by several observations. In the absence of this gene, larval tissues were no longer able to grow and their cells did not undergo a normal number of rounds of endoreplications. In the *pit* mutant, imaginal diploid cells, which in the wild type proliferate extensively during larval life and give rise to most of the adult structures, stopped dividing very early in the larval stages. All tissues appeared equally affected, in a very precisely coordinated manner, and growth was arrested in the whole organism. The small larvae phenotype produced by the *pit* mutation used in this study, pit^{10}, could be rescued by a transgene expressing the *pit* cDNA. *pit* expression appears ubiquitous, although to somewhat different extents, in all the investigated larval tissues (not shown), thus suggesting that this effect of the *pit* mutation is cell or tissue autonomous. However, the harmonious development of *pit* larvae is consistent with the existence of some kind of general signal that informs the different tissues to continue their growth or to stop it.

Other results support the idea that *pit* participates in protein biosynthesis by controlling some aspect of the pathway. First, the phenotype of the *pit* mutation is very reminiscent of that of homozygous *Minute* mutations, and *Minute* genes are known to encode ribosomal proteins (Kay and Jacobs-Lorena, 1987). Second, the subcellular localization of *pit* is consistent with a function in the nucleolus, an organelle in which ribosome biogenesis takes place. Finally, the proteins most closely related to Pit are DEAD-box RNA helicases, which are important players in rRNA processing or in ribosome biogenesis and which are also required for cell growth.

Based on sequences analysis, Pit belongs to the rapidly growing family of ATPase RNA-dependent helicases containing a DEAD-box. The presence in Pit of a DEVD (Val/Ala) box rather than the conventional DEAD-motif challenges, however, its ability to perform its biochemical function associated to ATP hydrolysis. Another putative RNA-dependent helicase has been described in *Drosophila* that displays a DECD sequence (Cys/Ala) (Eberl et al., 1997). However, in both *Drosophila* proteins, the methylene group in β has been preserved and the hydrogen of alanine in DEAD has been substituted by a methyl group or a sulfhydryl group in valine and cysteine, respectively. Such a conservation of the hydrophobic methylene, in the appropriate conformation, might therefore be a prerequisite to ATP hydrolysis. Further biochemical experiments will, however, be necessary to prove that Pit still behaves as an ATPase.

The DEAD-box RNA helicases family includes a large number of members belonging to a wide spectrum of organisms from bacteria to man. They have been proposed as major players in the modulations of RNA structure that provide an important means of regulating RNA function or accessibility in many physiological processes (Linder et al., 1989; Fuller-Pace, 1994). Their property of unwinding RNA homoduplexes or RNA-DNA heteroduplexes can be utilized in rRNA processing, biogenesis of the ribosome, translation, mRNA splicing, transcription etc... RNA helicase activity has, however, been formally demonstrated for only a limited number of members such as eIF-4A, p68, An3 and Vasa (Hirling et al., 1989; Gurujaran et al., 1991; Pause and Sonenberg, 1992; Liang et al., 1994) and has been assumed, essentially on the basis of sequence comparisons, for the other members.

The best characterized DEAD-box protein, eIF-4A, is a translation initiation factor required for cap-stimulated 40S ribosomal subunit recruitment (for a review see Merrick and Hershey, 1996). At least four yeast helicases, PRP5, PRP28, PRP16 and PRP22, are believed to participate in the formation of the spliceosome and in mRNA splicing (Dalbadie-

McFarland et al., 1990; Schwer and Guthrie, 1991; Company et al., 1991; Strauss and Guthrie, 1994). The human helicase A has recently been shown to regulate transcription by directly recruiting the CBP protein to RNA polymerase II (Nakajima et al., 1997) and evidence has arisen that a *Drosophila* helicase is active in the opening of chromatin structure (Eberl et al., 1997). Some RNA helicases may participate in a specific developmental event or be active only in a given tissue, especially in the germline. For example, Vasa is required for the formation of the germ cells (Lasko and Ashburner, 1988). Similarly, *C. elegans* Glh-1 is expressed in the germline although the importance of its activity in these cells has not been elucidated (Roussel and Bennett, 1993).

This non-exhaustive list of the processes in which RNA helicases might be involved illustrates the difficulties encountered in assigning a precise function to Pit on the sole basis of its structural homologies. However, the subcellular localization of Pit to the nucleolus suggests a role in this organelle. Indeed, several RNA helicases from other organisms have also been shown to be present in the nucleolus and their function in ribosome biogenesis or in rRNA processing has been well documented.

In eukaryotes, ribosome biogenesis takes place primarily in a specialized nuclear compartment, the nucleolus, in which rRNA genes are transcribed as a single long transcript or pre-rRNA. This transcript is rapidly processed by cleavages, nucleotide modifications such as methylations on base and ribose moieties and pseudouridylation and exonucleolytic degradations to generate the mature rRNAs (for a review see Mélèse and Xue, 1995). These rRNAs are assembled to the ribosomal proteins imported from the cytoplasm to form mature ribosomes which are in turn transported into the cytoplasm (Woolford and Warner, 1991). In addition, several small nucleolar ribonucleoproteins (snoRNPs) are required for many of these processing steps. The biogenesis of the nucleolus is therefore a very intricate and sophisticated process involving a large number of different proteins. The p68 human RNA helicase and An3, from *Xenopus*, are found in the nucleolus, at least transiently, and they could play a role in nucleolus assembly (Iggo et al., 1991; Gurujaran et al., 1991). Similarly, recent evidence is strongly in favour of the direct participation of yeast DEAD-box RNA helicases in rRNA processing and 40S-ribosomal subunit biogenesis (Sachs and Davis, 1990; Ripmaster et al., 1992; O'Day et al., 1996; Kressler et al., 1997; Liang et al., 1997; Venema et al., 1997; Weaver et al., 1997). Their predicted function would be to insure accessibility of the rRNAs partners to the machinery by precisely controlling RNA unwinding reactions taking place during pre-rRNA processing and ribosome assembly. It must be pointed out that the proteins of known function that are the most related to Pit in terms of sequence homologies belong to this family of helicases localized to the nucleolus. In addition, mutations in these RNA helicases as is the case for the *pit* mutation, have consequences on cell growth. It is then tempting to assume a similar function for Pit. Very preliminary results seem to indicate that *pit* mutant larvae contain approximately 4-fold less rRNA than first instar wild-type larvae and this could eventually be attributed to unprocessed pre-rRNA which is known to be rapidly degraded. Very little information is actually available on the factors controlling this process in *Drosophila*.

In conclusion, it seems very likely that Pit participates in protein biosynthesis, probably by allowing a correct maturation and functionality of the ribosomes. The absence of Pit could decrease protein synthesis to a level so low that it would be insufficient to promote cell growth and consequently cell division and proliferation. Such a coupling between growth and proliferation is well illustrated by the translational control exerted on the yeast cyclin Cln3p by the amount of ribosomes synthesized (Polymenis and Schmidt, 1997). The effects of overexpression of Pit in imaginal discs suggest a positive modification of growth rate, probably by acting as a general and rate-limiting factor in ribosome biosynthesis.

Finally, the protein sequence closest to that of Pit is that of MrDb, a human DEAD-box RNA helicase. The gene encoding that protein has been identified as a direct target of Myc because Myc-Max heterodimers were isolated as structures bound to the *MrDb* gene (Grandori et al., 1996). A canonical sequence, CACGTG, responsible for the binding of Myc was found in the coding sequence for MrDb but not in that in the *pit* gene, at least not in the transcribed part. However, *pit* transcription can be activated by ectopically expressed *d-myc* suggesting that, in *Drosophila*, *pit* could also be a potential target of Myc and it will be necessary to await complete loss-of-function mutations in *d-myc* to confirm that it directly controls *pit* transcription. Also, transgenic flies bearing both the *d-myc* and *d-max* genes will certainly be valuable tools to extend the *pit* ectopic expression experiments to tissues other than the nervous system. In regard of the known function of *myc* in cell proliferation, it is particularly interesting to note that two of its best characterized downstream targets eIF4E (Jones et al., 1996) and *pit* (this work) appear to be directly involved in protein biosynthesis thus linking *myc* activation of cell proliferation to protein biosynthesis and cell growth.

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