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The *L63* Gene Is Necessary for the Ecdysone-Induced 63E Late Puff and Encodes CDK Proteins Required for *Drosophila* Development

R. Steven Stowers,[1] Dan Garza,[2] Anne Rascle,[3] and David S. Hogness[4]

Department of Biochemistry and Department of Developmental Biology, Stanford University School of Medicine, Beckman Center, B300, Stanford, California 94305-5329

The pulse of ecdysone that triggers *Drosophila* metamorphosis activates six early genes in a primary response made visible by polytene chromosome puffs. The secondary response is detected by the induction of over 100 late puffs, only a few of which have been subject to molecular genetic analysis. We present a molecular and mutational analysis of the *L63* gene responsible for the late puff at 63E. This gene contains overlapping *L63A*, *B*, and *C* transcription units of which the *A* unit encodes two isoforms and the *B* unit three. The *C* unit, which exhibits little activity, encodes one of the *B* isoforms. Evidence that *L63B*, but not *L63A*, transcription is ecdysone responsive derives from their developmental transcription profiles and from P-element mutagenesis showing that ecdysone induction of the 63E puff requires sequences adjacent to the 5′ end of *L63B* but not those adjacent to the 5′ end of *L63A*. *L63*-specific lethal mutations showed that *L63* is required not only for metamorphosis, but also maternally and for embryonic and larval development. The L63 proteins contain a common C-terminal 294-aa sequence that is 71% identical to the CDK sequence of the murine PFTAIRE protein. *In vivo* tests of L63 proteins altered by site-directed mutagenesis showed that they exhibit CDK functions. L63 proteins are widely distributed among late larval and prepupal tissues and are unlikely to be involved in cell cycle functions.
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INTRODUCTION

The principal temporal determinants of *Drosophila* metamorphosis are the pulses of the steroid hormone ecdysone which emanate from the prothoracic gland in response to signals from the brain (Riddiford, 1993). Metamorphosis is triggered by the late-larval pulse which arises some 6 h prior to the larval-to-prepupal transition (pupariation), peaks at pupariation, and rapidly declines. It is further controlled by two subsequent pulses: a small prepupal pulse that peaks 10 h after the late-larval pulse, just 2 h before the prepupal-to-pupal transition (pupation), and a broad pupal pulse. The genetic response to these pulses is best defined for the late-larval pulse and least well defined for the pupal pulse. These differences have their origin in the puffing response seen in the polytene chromosomes of the larval salivary gland (reviewed by Russell and Ashburner, 1996)—a response that can be fully observed for the late-larval pulse and only partially observed for the prepupal pulse and cannot be observed for the pupal pulse due to programmed cell death induced by the preceding pulses (Jiang *et al.*, 1997).

The puffing response to the late-larval ecdysone pulse consists in the rapid induction of six early puffs in a primary response to the hormone, followed by a secondary response consisting in the regression of these early puffs and the concomitant induction of over 100 late puffs. A detailed examination of these early puffs and a subset of certain model late puffs led to a hierarchical regulatory model for the puff response in which each puff was assumed to derive from the transcriptional activation of a gene (Ashburner *et al.*, 1974). In this model, activation of the early puff genes is

[1] Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

[2] Present address: Department of Biology, Florida State University, Tallahassee, FL 32306.

[3] Present address: Cell Signaling Department, DNAX Research Institute, Palo Alto, CA 94304.

[4] To whom correspondence should be addressed at the Department of Developmental Biology, Beckman Center, B300, Stanford University School of Medicine, Stanford, CA 94305-5327.

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effected by an ecdysone–receptor complex formed at the initiation of the late-larval pulse, and late gene activation results from transcription factors encoded by the early genes. Because regression of the early puffs, like the secondary induction of the late puffs, is sensitive to protein synthesis inhibitors, early puff regression was assumed to result from repression of the early genes by one or more of the proteins they encode. The model also included the proposition that the ecdysone–receptor complex initially represses the late genes to prevent their premature induction by early-gene transcription factors.

Principal aspects of the model have been confirmed by molecular genetic analyses, which have also shown that the model is applicable to other target tissues, both larval and imaginal (see reviews by Bayer *et al.*, 1996; Thummel, 1996; and Richards, 1997). The ecdysone receptor gene, *EcR*, encodes three isoforms (EcR-A, -B1, -B2), each of which is activated by heterodimerization with an orphan nuclear receptor, USP. The EcR isoforms are differentially distributed among the target tissues, suggesting that the tissue specificity of the metamorphic response to ecdysone derives, in part, from the EcR isoforms—a suggestion that has received support from recent evidence that the EcR isoforms are functionally distinct (Bender *et al.*, 1997; Schubiger *et al.*, 1998). Molecular analyses of the early genes *BR-C*, *E74*, and *E75* responsible for the early puffs at 2B, 74EF, and 75B showed that they encode, respectively, members of the C2H2 zinc finger, ETS, and nuclear receptor families of transcription factors. Like EcR, these early genes encode multiple isoforms that exhibit distinct spatial and/or temporal expression patterns. Mutational analyses of the *BR-C* and *E74* genes have demonstrated that specific isoforms encoded by these genes are required for normal late gene puffing, and molecular analyses have demonstrated that BR-C and E74 isoforms combine to directly activate transcription of the *L71* late puff genes. Molecular analyses reported subsequent to the above reviews have shown that the early genes *BR-C*, *E74A*, and *E75A* can be repressed by a nuclear receptor encoded by another primary response gene (*DHR3*), thereby confirming the proposition that early puff genes are turned off by a negative feedback mechanism (Lam *et al.*, 1997; White *et al.*, 1997). The *DHR3* gene is associated with the 46F puff (Koelle *et al.*, 1992), a member of the early-late class of puffs defined by Ashburner and Richards (1976) as an offshoot of the late class. Early-late puffs are differentiated from late puffs [i.e., the "late-late" puffs in the nomenclature of Ashburner and Richards (1976) which has fallen out of use] not only by their earlier expression but also by absence of initial ecdysone repression, a characteristic of the late puffs that led to the aforementioned proposition that late genes are initially repressed by the ecdysone–receptor complex.

In the general model for the ecdysone response (Burtis *et al.*, 1990; Thummel *et al.*, 1990; Talbot *et al.*, 1993), it was assumed that the secondary-response genes provide effector functions and that more than 100 effector genes might be activated in a given target tissue—as estimated from the number of late puffs. Evidence for this assumption exists but is scarce. The secondary-response *Sgs* genes clearly meet the definition of effector genes as they encode proteins that are excreted from the salivary gland as a glue which protects the animal during metamorphosis by attaching it to a dry surface (reviewed by Russell and Ashburner, 1996). Several secondary-response genes activated in imaginal discs also fit this definition (reviewed by Bayer *et al.*, 1996), as do the coordinately regulated *L71* genes which encode a secreted set of small polypeptides similar to the vertebrate defensins and may therefore provide an antimicrobial defense during metamorphosis (Wright *et al.*, 1996). The paucity of data concerning the late genes is exemplified by the fact that the molecular genetic analysis of the three best characterized late puffs (at cytological positions 22C, 63E, and 82F; Ashburner and Richards, 1976) has only just begun for the 63E (this paper) and 82F (Stowers *et al.*, 1999) puffs, while the 22C puff is as yet unattended.

The 63E puff exhibits a number of features that distinguish it from the other two late puffs. The first concerns the expectation that the ecdysone receptor would bind to each of the three model puff sites prior to puff formation because the model predicts that late genes are initially repressed by the ecdysone–receptor complex (Ashburner *et al.*, 1974). Immunostaining of salivary gland polytene chromosomes with monoclonal antibodies specific for EcR-B1 (the prevalent isoform in these glands) demonstrated the expected presence of EcR-B1 at the 22C and 82F sites prior to puffing; however, no staining was observed at 63E despite considerable effort to detect it (Talbot, 1993). The second distinguishing feature is the appearance of a puff at the 63E locus during the midprepupal stage, whereas midprepupal puffing is not observed at the other two sites. This midprepupal 63E puff occurs at the same time as the well-characterized 75CD midprepupal puff, at 5 to 6 h after pupariation when the ecdysone concentration is very low—evidently a necessary condition for their activation, as both are inhibited by ecdysone concentrations as low as 5×10^{-8} M (Ashburner, 1967; Richards, 1976). These observations suggest that one gene may be responsible for both the late larval and the midprepupal puffs at 63E and, hence, that it may be a more complex gene than the simple *Sgs* and *L71* late puff genes noted above.

We show here that the *L63* gene responsible for the late larval 63E puff is indeed complex. It spans 85 kb of genomic DNA that includes three overlapping transcription units which generate nine different mRNAs encoding five different L63 isoforms. In these regards, *L63* more closely resembles the *BR-C*, *E74*, and *E75* early genes than it does the simple *Sgs* and *L71* secondary-response genes. *L63* also differs from these genes in its temporal and spatial expression patterns, again more closely resembling the early genes. Whereas *Sgs* and *L71* gene expression is limited to the terminal stages of the larval salivary gland's existence, one or more of the *L63* transcription units are expressed throughout *Drosophila* development and in many different tissues. These characteristics provide for a redundancy of

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expression in a gene that mutational analysis shows is required even before metamorphosis. Finally, the L63 isoforms contain a common 294-amino-acid region near the carboxy-terminus that shows strong homology to the cyclin-dependent kinase (CDK) protein family and which provides a CDK function critical to development.

MATERIALS AND METHODS

Fly stocks. Deletion mutant stocks *Df(3L) HR232, Df (3L) GN34,* and *Df (3L) GN50* were obtained from the Bloomington stock center. Deficiency stocks *Df (3L) 663* and *Df (3L) 294* and the lethal alleles *81, 1619,* and *2266* were gifts from Stephanie Paine-Saunders and Jim Fristrom. Lethal alleles *1, 15, 29,* and *33* were gifts from Graham Thomas. The *△2-3* transpose strain used in the P-element hopping and excision screens was previously described (Robertson *et al.,* 1988). The *P[w+] 2683* stock used for local hopping (Zhang and Spradling, 1993) contained a single *P-lacW* (Bier *et al.,* 1989) insertion at cytological position 63C6 and was generously provided by Matt Scott.

Identification and mapping of L63 mutations. Transposition of *P[w+] 2683* led to the identification of approximately 1000 new, independent transposition events, of which 271 "hops" segregated with the original third chromosome. Genomic DNA was isolated from pools of 10 hop lines each and was screened by PCR using a primer to the inverted repeat of the P-element in combination with antisense primers near the 5′ end of *L63* exons *A1, B1,* and *C1.* This led to the identification of the *P[w+] 33D* line that contains both the original *P[w+] 2683* insertion and a novel *P-lacW* insertion 1.0 kb upstream of the *L63B1* exon. The original P-element and the new insertion were separated by recombination to create the homozygous viable line *P[w+] 33D3.* The excision/deletion screen used to identify *Df(3L) O, Df(3L) E1, Df(3L) CC,* and *Df(3L) FF* was carried out using a *Df(3L) 663 △2-3* chromosome in order to eliminate the possibility of excision/repair events from the homologous chromosome. The *hscdc2* and *hscdc2c* stocks were previously described (Lehner and O'Farrell, 1990). The lesion of the $L63^{81}$ mutant was determined by isolating total mRNA from $L63^{81}$/*TM6b* and control *1619*/*TM6b* flies (*1619* is a chromosome generated in the same mutagenesis and from the same isogenic parental chromosome as $L63^{81}$), performing RT-PCR on them both using four overlapping primer sets that encompass the entire *L63* open reading frame, and sequencing a lower molecular weight RT-PCR product specific to $L63^{81}$/*TM6b*.

Identification of genomic clones in the 63E region. Yeast artificial chromosome isolation was performed as described (Garza *et al.,* 1989). Chromosome walking was performed as described (Bender *et al.,* 1983). All other methods for purifying and manipulating DNA and RNA were essentially as described (Sambrook *et al.,* 1989). The genomic phage were derived from previously published libraries (Tamkun *et al.,* 1991; Moses *et al.,* 1989).

Breakpoint mapping of deletion mutants. The deletion mutant breakpoints shown in Fig. 2A were determined by *in situ* hybridization to polytene chromosomes using genomic phage clones as probes. Deletion breakpoints were determined more precisely by genomic Southern blot analysis. *Df (3L) 294* has its distal breakpoint within a 7-kb *Eco*RI fragment of phage 6307; *Df(3L)1-11* has its proximal breakpoint within a 3.5-kb *Sma*I/*Eco*RI fragment of phage 6311; *Df(3L) O* has its proximal breakpoint within a 1.2-kb *Sma*I/*Eco*RI fragment 3′ of the *L63* B1 exon in phage 6311; *Df(3L) E1* has its proximal breakpoint within a 600-bp *Sal*I/*Eco*RI fragment of phage 6311; *Df(3L) GN50* has its distal breakpoint within a 900-bp *Eco*RV/*Hin*dIII fragment of phage 6312. The breakpoints for *P[w+]33D* w^- excision derivatives *Df(3L) CC* and *Df(3L) FF* were determined by genomic Southern analysis. DNA sequencing of PCR products obtained using primers on either side of the breakpoint shows that *Df(3L) CC* breaks approximately 1.0 kb upstream of the 5′ end of *L63* exon B1 and 284 bp downstream of the 3′ end of *L63* exon B1.

Northern blot hybridization. RNA for Northern analysis was purified as described (Thummel *et al.,* 1990). Purified RNA was fractionated by formaldehyde agarose gel electrophoresis, transferred to Hybond N+ membranes, and hybridized as described (Karim and Thummel, 1991). The probes used for the Northern blot in Fig. 3 were (top) 459-bp *Nae*I/*Bam*HI cDNA fragment probe from an *L63B1* cDNA, (middle) 1.5-kb *Eco*RI/*Sal*I genomic fragment containing the entire 1.1-kb *L63A1* exon, and (bottom) 2.0-kb *Xho*I common region cDNA fragment from an *L63B1* cDNA. A probe from the *rp49* gene (O'Connell and Rosbash, 1984) was used as a loading control.

cDNA isolation. *L63* cDNAs were identified by hybridization screening of cDNA libraries. Approximately equal numbers of cDNAs were isolated from each of three different cDNA libraries: 0- to 2-h and 8- to 12-h white prepupal libraries (Stowers *et al.,* 1999) and an ecdysone–cycloheximide-treated third-instar internal tissue library (Burtis *et al.,* 1990). The following plasmids contain the indicated *L63* cDNAs: pSS177-*L63A1,* pSS178-*L63A2,* pSS285-*L63A3,* pSS156-*L63B1,* pSS175-*L63B2,* pSS167-*L63B3,* pSS158-*L63B4,* pSS290-*L63C1,* and pSS160-*L63C2.*

Polytene chromosome squashes and in situ hybridizations. These were performed essentially as described (Langer-Safer *et al.,* 1982).

Plasmid subclones for P-element transformation. The HS*L63B1* transformation plasmid pSS238 was constructed by subcloning the 1.6-kb *Hpa*I/*Bam*HI fragment of pSS156 into the *Hpa*I/*Bam*HI sites of pCaSpeR-hs (Thummel *et al.,* 1988). The HS*L63B1△202* transformation plasmid was constructed by subcloning a fragment formed by the complementary oligonucleotides 5′-GAATTCAGAAACATGGAGGCCTATGTTAAAC-3′ and 5′-GTCGAGTTTAACATAGGCCTCCATGTTTCTG-3′ into the *Eco*RI/*Xho*I partial digest of pSS156 and subcloning the resulting 1.0-kb *Eco*RI/*Bam*HI fragment from this plasmid into the *Eco*RI/*Bgl*II sites of pCaSpeR-hs. Site-directed mutagenesis was used on the parental plasmid pSS238 to introduce mutations into *L63* using the Unique Site Elimination kit (Pharmacia, Piscataway, NJ). The codon changes are G243A-GCT to GCA, I249L-CGA to CTT, E251Q TCG to GAC, and S359A GTG to GCC. P-element-mediated transformation was performed as described (Rubin and Spradling, 1982).

Mutant rescue experiments. These were performed by taking 24-h embryo collections in vials, removing the adult flies, and placing the vials in a circulating water bath whose water temperature was maintained at 25°C by a circulating water heater. Every 12 h, a second circulating water heater in the water bath under the control of an automatic timer heated the water to 37°C for 30 min. The water bath was kept in a cold room to facilitate rapid return of the water temperature to 25°C after the 37°C heat shocks. Vials were left in the water bath until adults started to eclose after which time adults were scored on a daily basis until all adults had eclosed.

Antibody generation, Western blotting, and immunohistochemistry. Specific *L63B1* cDNA restriction fragments were cloned into the pMalc expression vector (New England Biolabs, Cambridge, MA), expressed and purified as per the manufacturer's

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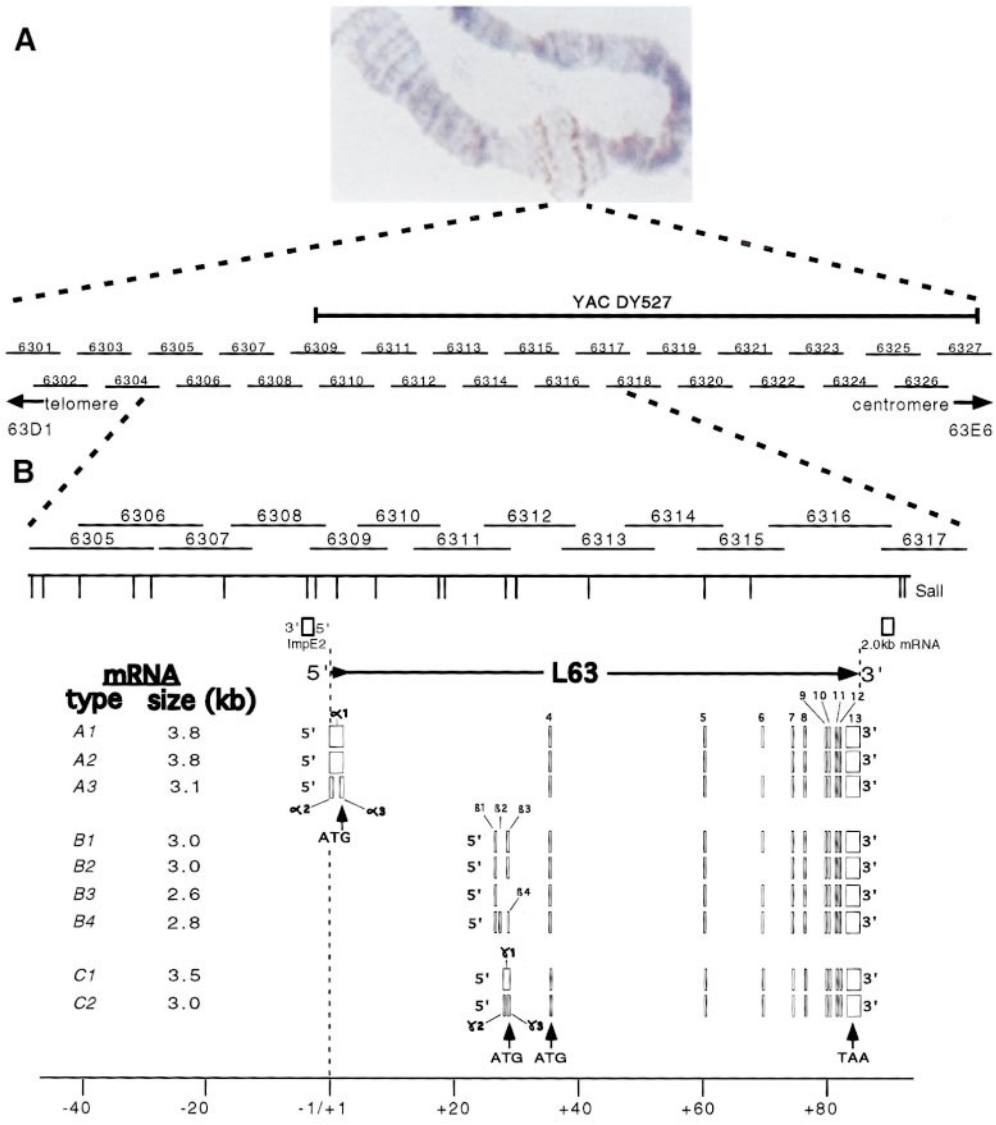

FIG. 1. The structure of the *L63* gene. (A) *In situ* hybridization to white prepupal-stage salivary gland polytene chromosomes with probes transcribed from the end phage of the contig (6301 and 6327). The hybridization signals flank either side of the active 63E puff. Shown below is an expanded diagram of the ~300-kb 63D1 to 63E6 chromosomal region including the 27 genomic phage that comprise the contig as well as the yeast artificial chromosome (YAC) DY 527. (B) Expanded view of the phage 6305–6317 portion of the contig. The *L63* gene spans nearly 90 kb of genomic DNA and contains three nested transcription units that together encode nine distinct *L63* mRNAs. The transcription start site for the L63β1 exon was determined by both primer extension and RNase protection. A likely transcription start site for the L63γ1 exon was determined based on RNase protection experiments. The exon structure of the nine distinct *L63* cDNA types is shown below the phage (exons are shown at approximately twice the scale at the bottom). The predicted ATG translation start codons are marked with arrows as is the TAA stop codon. Exon locations were determined by sequencing across the intron/exon boundaries. The GenBank accession numbers for the *L63* mRNAs are *L63A1*—AF152398, *L63A2*—AF152399, *L63A3*—AF152400, *L63B1*—AF152401, *L63B2*—AF152402, *L63B3*—AF152403, *L63B4*—AF152404, *L63C1*—AF152405, and *L63C2*—AF152406.

instructions, and injected into Balb/c mice. The resulting A–E fusion proteins contain the following L63B1 amino acid residues: A, 54–209; B, 210–309; C, 310–411; D, 375–484; and E, 484–522. Only A and E produced a significant immune response. The A monoclonals derived from a 465-bp *Sal*I-blunt/*Xho*I fragment inserted into the *Xmn*I/*Sal*I vector sites, while the E monoclonals derived from a 114-bp *Pst*I/*Bam*HI-blunt fragment inserted into *Pst*I/*Hin*dIII-blunt vector sites. Immunization of mice and hybridoma procedures were as described (Harlow and Lane, 1988). The monoclonal antibodies isolated were further distinguished into epitope classes A1–A4 and E1–E3 based on recognition of distinct patterns of partially degraded fusion proteins on Western blots. The

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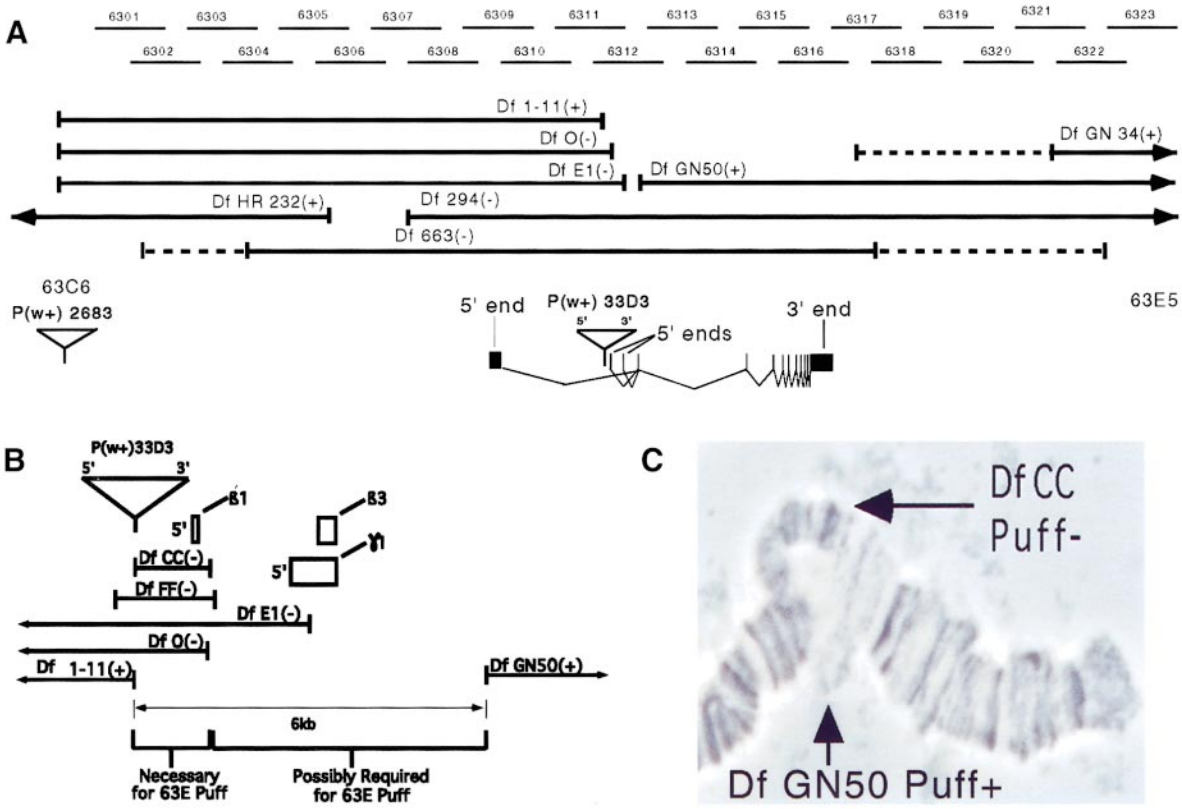

FIG. 2. Genetics of the 63DE region. (A) Low-resolution genetic map of the 63C6–63E5 region showing the relative locations of the genomic phage (top), the breakpoints of deletion mutants (middle), and the *L63* gene (bottom). Solid lines indicate regions missing in the deletion mutants while dotted lines indicate regions of breakpoint uncertainty. A (+) sign next to a deletion mutant indicates that the 63E puff does form in that mutant while a (−) sign indicates that the 63E puff does not. The location of the starting P-element *P[w+] 2683* used in the local hopping screen is to shown at the far left. The location of the *P[w+] 33D3* P-element that was hopped into the *L63* gene is indicated in the middle. (B) High-resolution map of the 6-kb region surrounding the *L63B1* transcription start site showing the locations of the deletion mutant breakpoints relative to the L63β1 and L63γ1 transcription start exons. (C) Polytene chromosome squash of *Df CC*/*Df GN50* white prepupal animal. The 63E puff is present on the *Df GN50* chromosome but is absent from the *Df CC* chromosome. This result establishes that sequences distal to the *Df GN50* breakpoint are sufficient for 63E puff formation and that the 1.0 kb of sequence missing in *Df CC* that contains the L63β1 transcription start exon is essential for 63E puff formation.

A1 epitope class consisted of three independently isolated monoclonals, A1-1, A1-29, and A1-31; the A2 class of four, A2-6, A2-8, A2-20, and A2-34; with only one for each of the remaining two A classes: A3-17 and A4-26. Similarly, the three E classes yield the E1-42, E1-44, E2-50, and E3-53 monoclonals. Western blotting and immunohistochemistry were performed as described (Talbot *et al.*, 1993). For Westerns, the monoclonals were used at 1:10 while for tissue stains they were used at 1:3.

RESULTS

The L63 Gene Consists of Three Nested Transcription Units That Map to the Center of the 63E Late Puff

Figure 1A shows that the 27 overlapping *Drosophila* genomic DNA segments 6301–6327 span the ecdysone-induced 63E late puff. Nineteen of these segments (6309–6327) were obtained by screening λ phage libraries of *Drosophila melanogaster* genomic DNA segments for those that hybridized to the DNA of YAC DY527, a yeast artificial chromosome containing ~220 kb of DNA from the 63E region (Garza *et al.*, 1989). These 19 segments covered all but the distal side of the puff (i.e., the side closest to the telomere)—a region that was subsequently covered by a short chromosomal walk to obtain the remaining 6301–6308 segments. The 27 segments span ~300 kb of genomic DNA that corresponds to the polytene chromosome region 63D1–E6.

Low-resolution mapping of potential ecdysone-inducible genes within this region was carried out by differential hybridization of genomic DNA restriction fragments with [^{32}P]cDNA probes constructed from two poly(A)$^+$ RNA populations—one prepared from white prepupae when the

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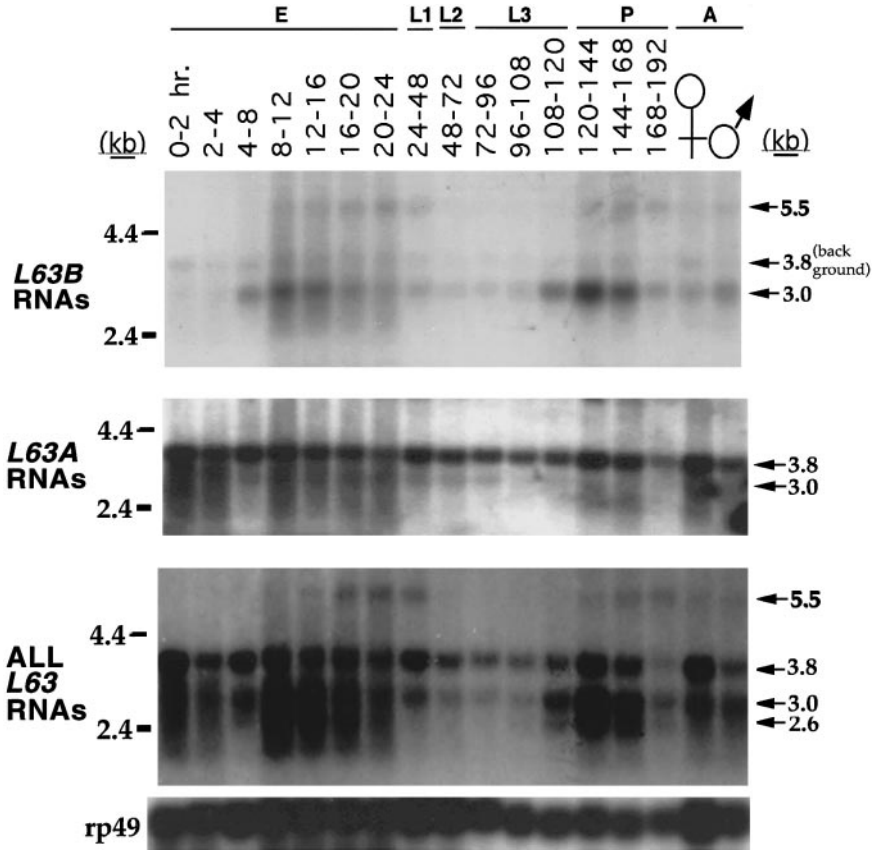

FIG. 3. Developmental mRNA expression of the *L63* gene. Northern blot of total RNA isolated from whole animals at the indicated developmental stages hybridized with an L63β1–3 region cDNA probe (top). An identical Northern blot hybridized with an L63α1-specific exon genomic probe (middle). Another identical Northern blot hybridized with an L63 common region cDNA probe (bottom). An *rp49* control hybridization of one of these blots is shown below the common region Northern. Hours of development are at 25°C. Abbreviations: E, embryogenesis; L1, first-instar larvae; L2, second-instar larvae; L3, third-instar larvae; P. prepupae and pupae; A, adult.

63E puff is active, the other from mid-third-instar larvae when the puff is inactive. Genomic restriction fragments that hybridized preferentially with the white prepupal probe were then used to identify cloned cDNAs derived from putative ecdysone-induced mRNAs (see Materials and Methods). Figure 1B shows the three putative ecdysone-induced genes that were identified in this manner: *ImpE2*, *L63*, and a gene with a 2.0-kb poly(A)$^+$ transcript. As will become apparent, *L63* contains the DNA required for the formation of the 63E late puff. *ImpE2* is a previously defined gene that exhibits a primary response to the late-larval ecdysone pulse in imaginal discs, but is not responsive to that pulse in salivary glands (Paine-Saunders *et al.*, 1990), and the gene with the 2-kb transcript was not analyzed further.

A large set of cloned *L63* cDNAs (~150) from three independent libraries (Materials and Methods) provided the source for extensive restriction and selective sequence analyses that defined the overlapping *L63A*, *B*, and *C* transcription units from which the nine mRNAs shown in Fig. 1B are formed by alternative splicing. These mRNAs contain 10 common exons (Nos. 4–13), except that the *A2* and *B2* mRNAs lack exon 6 and are thereby distinguished from the *A1* and *B1* mRNAs, respectively. Otherwise, the mRNAs are distinguished by exons specific to each transcription unit, i.e., exons α1–α3, β1–β4, and γ1–γ3 for transcription units *L63A*, *B*, and *C*, respectively (Fig. 1B).

Deletion Mapping Shows That the 63E Late Puff Requires DNA Adjacent to the L63B Transcription Initiation Site

Molecular mapping of deficiency mutations was used to identify the DNA required for the 63E late puff. Figures 2A and 2B show the molecular maps of the five existing deficiencies known by polytene chromosome mapping to have at least one breakpoint in the 63DE region—namely, *Df GN34*, *Df GN50*, *Df HR232*, *Df 294*, and *Df 663*. [As all deficiencies used in our experiments derive from chromosome arm 3L, the (3L) notation normally included in the

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designation, and which appears under Materials and Methods (e.g., Df(3L) GN34), has been eliminated under Results and Discussion.] While at least one breakpoint in each deficiency mapped to the DNA defined by the cloned segments 6201–6323 shown in Fig. 2A, only the *Df GN50* breakpoint mapped within the *L63* gene. The position of that breakpoint is of considerable interest as it results in the elimination of virtually all *L63* coding sequences while leaving all three *L63* transcription initiation sites and their surrounding sequences intact. (The coding sequences not eliminated by *DfGN50* consist of only 19 N-terminal codons in the α1 and β3 exons.) As the 63E late puff is activated in the *Df GN50* chromosome (Fig. 2C), the DNA required for that puff must be distal to the *Df GN50* breakpoint, providing a useful approximation for the proximal limit for the DNA required for the 63E late puff.

As the breakpoints of the other four deficiencies are too remote to provide a likely distal limit for that DNA (Fig. 2A), P-element mutagenesis was used to generate additional deletion breakpoints in the *L63* region. To that end, the w^+ P-element of the *P[w$^+$] 2683* strain was hopped (Zhang and Spalding, 1993) from its position at 63C6–D1 into the *L63* gene at a site 1.0 kb upstream of the *L63B* transcription initiation site (Fig. 2B), yielding the *P[w$^+$] 33D* strain containing w^+ P-elements at both the original and the new insertion sites (Fig. 2A). The *Df 1-11*, *Df 0*, and *Df E1* deletions were generated as *w*-excision derivatives of *P[w +] 33D* and extend from the 63C5–D1 site to breakpoints located 1.0 kb upstream and 0.3 and 2.3 kb downstream of the *L63B* transcription initiation site, respectively (Fig. 2B). The observation that the 63E late puff is present in *Df 1-11*, but not in *Df 0* and *Df E1*, demonstrates that the 1.3 kb of DNA between the proximal breakpoints of *Df 1-11* and *Df 0* contains sequences required for the 63E late puff (Fig. 2B).

The same conclusion was derived from the properties of two short, overlapping deletions—the 1.3-kb *Df CC* and the ~2-kb *Df FF* (Fig. 2B). These deletions were generated as w^- excision derivatives of *P[w$^+$] 33D3*, a recombination derivative of *P[w$^+$] 33D* that retains the w^+ P-element in the *L63* gene, but not that in the 63C6–D1 region. Figure 2C shows that the 63E late puff is not expressed in the *Df CC* chromosome. This result is comparable to the above observation that this puff is expressed in *Df 1-11* but not in *Df 0* because the 1.3 kb of DNA retained in *Df 1-11* but not in *Df 0* is essentially the same as the DNA deleted in *Df CC* (Fig. 2B).

These results show that expression of the 63E late puff requires sequences in the *L63* gene. Furthermore, the localization of some, if not all, of these sequences to a 1.3-kb segment that contains the β1 exon of the *L63B* transcription unit (Fig. 2B) suggests that induction of the 63E late puff is correlated with induction of *L63B* transcription. It should be noted in this regard that both RNase protection and primer extension analyses indicated that the *L63B* transcription initiation site and the 5′ end of the β1 exon are the same—an indication that is confirmed by comparison to consensus sequence data for transcription initiation sites (Cherbas and Cherbas, 1993; Hultmark *et al.*, 1986; Pirotta *et al.*, 1987).

Temporal Patterns of L63A and L63B Transcription during Development

The deletion mapping of sequences required for the ecdysone induction of the 63E late puff suggested that *L63B* transcription, and possibly that of *L63C*, is ecdysone responsive. Here we use Northern analyses to show that the temporal profile of *L63B* transcription mimics that of known ecdysone-responsive genes and that the *L63A* profile is more complex, while *L63C* transcription is too weak to be detected by these analyses.

The top of Fig. 3 shows the relative abundance of the *L63B* mRNAs detectable by a probe consisting of ^{32}P-labeled sequences in the β1 and β3 exons defined in Fig. 1B. While sequence overlap exists between β3 and the γ1 exon of *L63C*, a probe specific for γ1 sequences failed to detect any mRNAs on an identical Northern blot, even though it contained more *L63C* nucleotides than are present in the β3 exon (data not shown). Among the nine *L63* mRNAs defined in Fig. 1B, the β1 + β3 probe will therefore detect only the *L63B* mRNAs. Consequently the 3.0-kb mRNA band in the top of Fig. 3 should represent the *L63B1* and/or *L63B2* mRNAs, while the 5.5-kb band cannot be accounted for by the known *L63* cDNAs.

This temporal profile of the *L63B1*/*B2* mRNAs is like that of several ecdysone-responsive genes. Thus, the *L63B1*/*B2* midembryonic expression is like that observed for the ecdysone-inducible early-late *DHR3* gene (Koelle *et*

FIG. 4. Primary structures of the L63 proteins and comparisons to those of other CDK proteins. (A) Exon distribution of the coding sequences for the five L63 proteins. The names of the L63 proteins are given at the left and the *L63* mRNAs that give rise to them (see Fig. 1) are indicated at the right. The predicted molecular weights of the L63 proteins are (in kDa) L63A1, 59.763; L63A2, 58.338; L63B1, 59.938; L63B2, 58.512; and L63B3, 57.736. (B) The L63 amino acid sequences of the five L63 proteins aligned with the common region of three CDK proteins: (i) PFTAIRE of *Mus musculus* (Lazzaro *et al.*, 1997; GenBank—U62391), (ii) PCTAIRE-1 of *Homo sapiens* (Meyerson *et al.*, 1992; PIR—S23384), and (iii) CDC28 of *Saccharomyces cerevisiae* (Lorincz and Reed, 1984; Swiss Prot—P00546). The boundaries of sequences encoded by individual L63 exons are indicated by short vertical lines above the sequence with the exon designations of Fig. 1B noted to each side of such a line. Other vertical lines above the sequence designate sites of L63 mutation, while the blue bars indicate L63 sequences equivalent to those involved in binding of cyclin A to CDK2 in humans. Vertical lines below the CDC28 sequence indicate residues in that protein but not in the others. Asterisks indicate endogenous stop codons.

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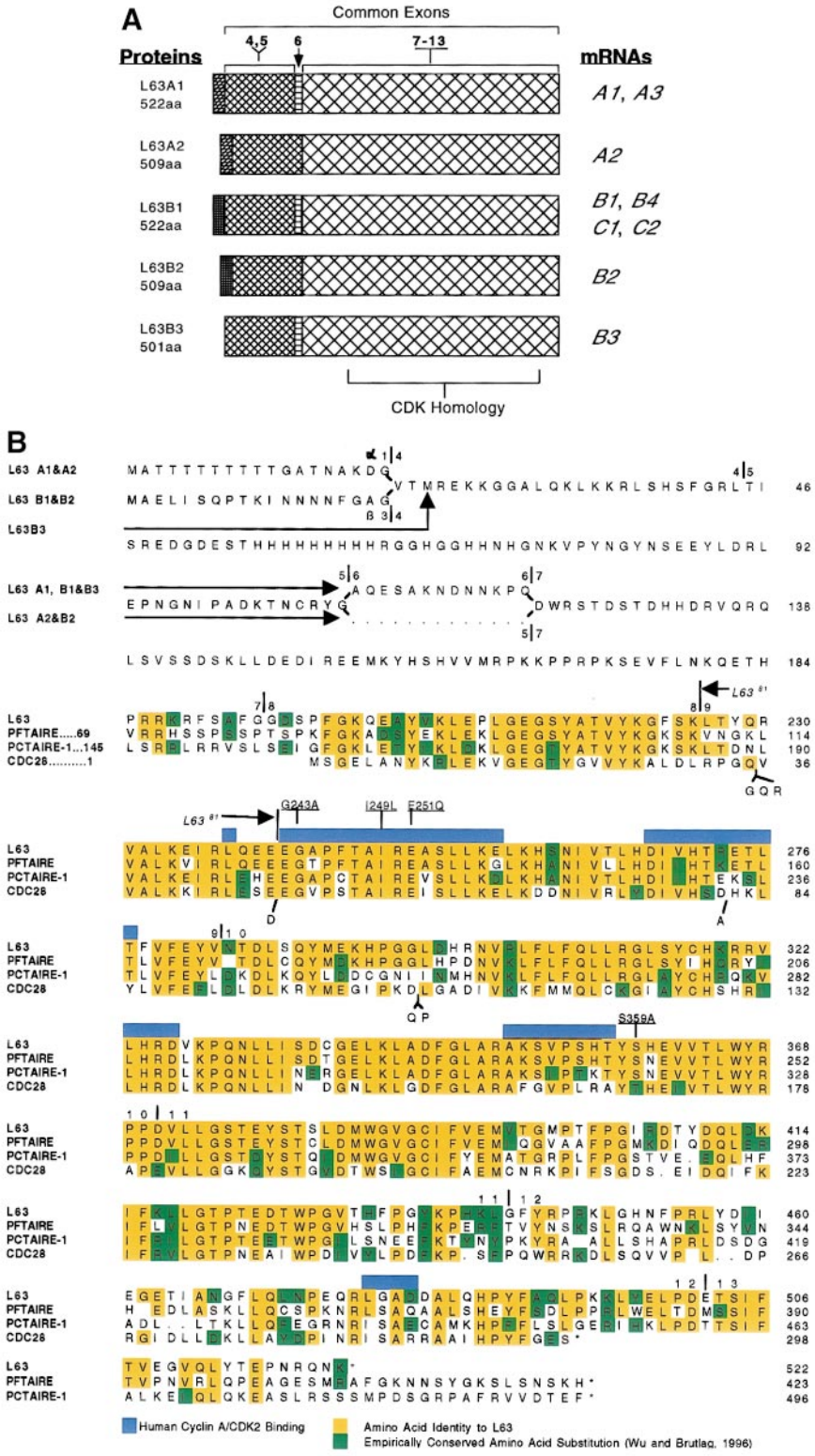

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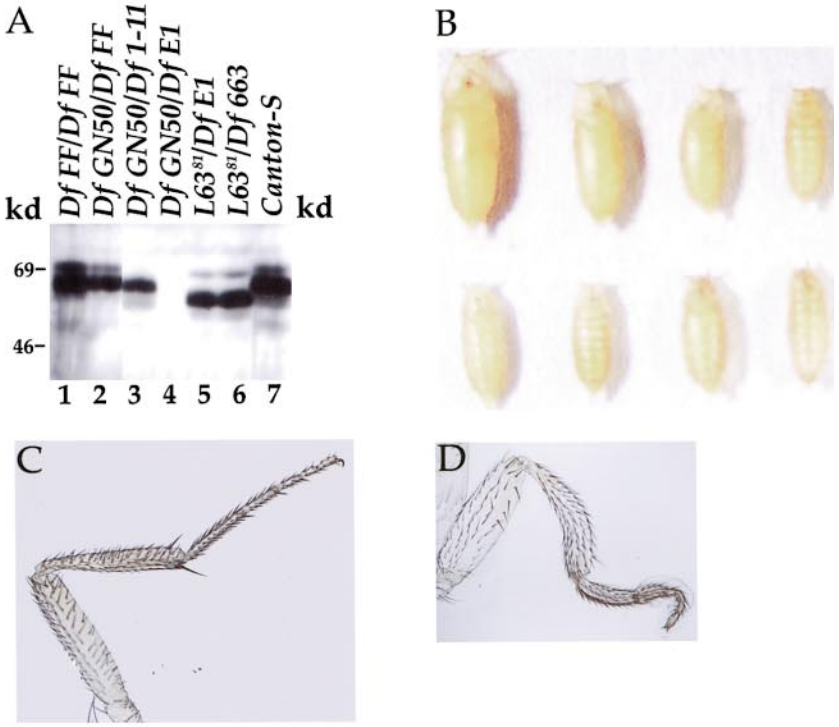
A
Df FF/Df FF
Df GN50/Df FF
Df GN50/Df 1-11
Df GN50/Df E1
L63 81/Df E1
L63 81/Df 663
Canton-S
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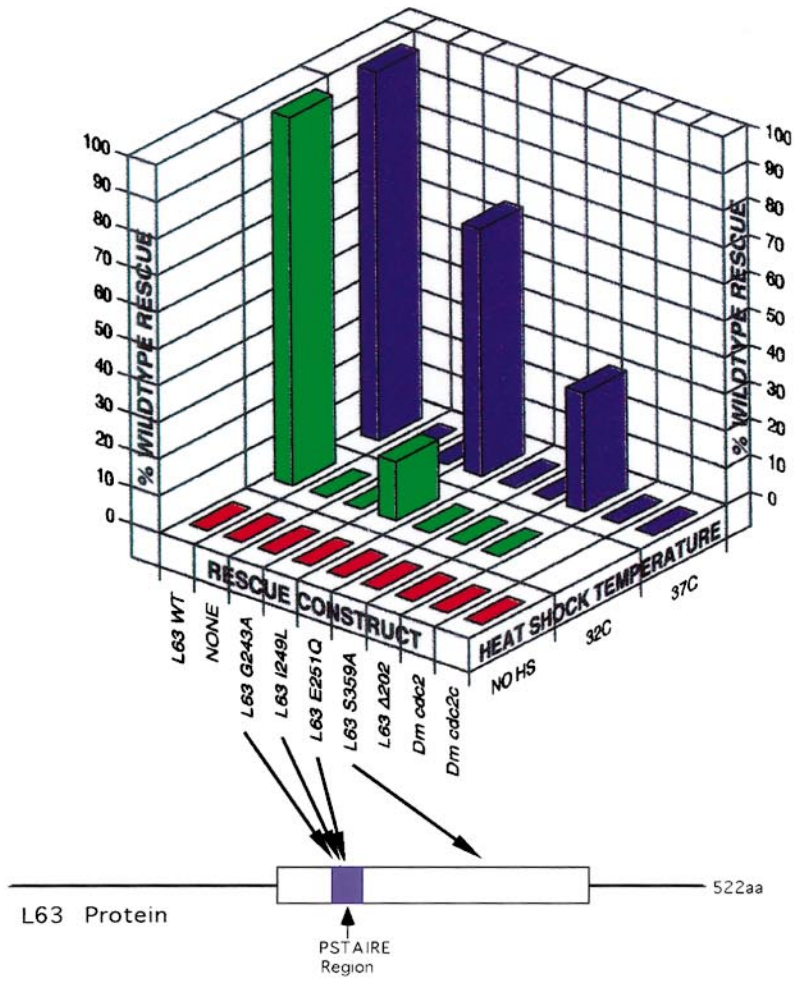
kd
69-
46-
1 2 3 4 5 6 7
B
C
D

5


% WILDTYPE RESCUE
100
90
80
70
60
50
40
30
20
10
0
RESCUE CONSTRUCT
L63 WT
NONE
L63 G243A
L63 I249L
L63 E251Q
L63 S359A
L63 Δ202
Dm cdc2
Dm cdc2c
HEAT SHOCK TEMPERATURE
NO HS
32C
37C
L63 Protein
522aa
PSTAIRE
Region

6

al., 1992) and the *EcR-A* and *EcR-B1* transcription units that encode the respective ecdysone receptor isoforms (Talbot *et al.,* 1993). The embryonic ecdysone pulse presumed to induce these genes peaks at 10 h (Kraminsky *et al.,* 1980; Geitz and Hodgetts, 1985) and is well suited to generating the *L63B* embryonic expression pattern. Similar arguments apply to the strong *L63B1*/*B2* expression that extends from late third instar (L3) to the middle of pupal development (108–168 h) when *DHR3* and *EcR-A* are also strongly expressed in conjunction with the late-larval, prepupal, and pupal ecdysone pulses that peak at 120, 130, and 150 h, respectively (Riddiford, 1993). The same temporal pattern of transcription is seen for the 2.6-kb band of RNA in the bottom of Fig. 3, for which the probe consisted of sequences in the common 8–13 exons. We have assigned this band to the 2.6-kb *L63B3* mRNA because the match of lengths and pattern are unique. The probable reason why this transcript was not detected in the top blot is that this *L63B3* mRNA does not contain the β3 exon, which makes the largest sequence contribution to the β1 + β3 probe used in that blot.

In contrast to *L63B* transcription, the observed temporal profile for the 3.8-kb *L63A1* and *L63A2* mRNAs seen in the middle of Fig. 3 after hybridization with labeled α1 exon sequences indicates that, with one exception, the ecdysone pulses do not influence the abundance of these mRNAs in whole animals. That exception is an increase in transcript abundance during the 120- to 168-h interval that could result from the prepupal and/or pupal ecdysone pulses. It should, however, also be noted that this increase in the *L63A* mRNA might also result from the same regulatory mechanisms that cause a reappearance of the 63E puff during the middle of the prepupal period when the ecdysone levels are very low (Richards, 1976; see Introduction). Given that the 3.1-kb *L63A3* mRNA is represented by the 3.0-kb band of the middle blot, then its expression is largely confined to embryogenesis. The large abundance of all of the *L63A* mRNAs during the first few hours of embryogenesis is probably of maternal origin, particularly because the abortion of transcription during the short nuclear division cycles of this period makes it highly unlikely that the long *L63A* primary transcript could be zygotically produced (Shermoen *et al.,* 1991).

The Nine L63 mRNAs Encode Five CDK-like Protein Isoforms

Determination of the nucleotide sequences of the nine *L63* cDNAs shown in Fig. 1B, and of genomic DNA sequences sufficient to define the exon boundaries within these cDNAs (GenBank Accession Nos. AF152398–AF152406), showed that only five *L63* protein isoforms are encoded by the nine *L63* mRNAs. The deduced primary structures of these proteins are represented in Fig. 4A by block diagrams in which each block represents the amino acid sequence encoded by a particular exon or a set of consecutive exons. For example, the N-terminal block of the L63A1 isoform represents the amino acids encoded by the 3′-terminal sequence of the overlapping α1 and α3 exons of the *L63A1* and *L63A3* mRNAs, respectively (Fig. 1B). Because the splicing patterns downstream of α1 and α3 are identical for these two mRNAs, they encode the same L63A1 protein. In contrast, the *L63A2* mRNA, which differs from *L63A1* only by the absence of exon 6, encodes the shorter L63A2 isoform.

The *L63B* transcription unit encodes the three other protein isoforms (L63B1, 2, 3), one of which (B1) can also be encoded by the *L63C* transcription unit. Indeed, this isoform can be encoded by four mRNAs, two from each unit (Fig. 4A). The L63B1 isoform differs from L63A1 only in respect to the N-terminal amino acids that are encoded by the α1 or α3 exons for L63A1 and by the β3, β4, γ1, or γ3 exons for L63B1 (Fig. 1B). The L63B2 and L63A2 isoforms also differ only by these two N-terminal amino acid sequences; in this case, however, each isoform appears to be encoded by a single mRNA. The *L63B3* mRNA that encodes the remaining isoform, L63B3, is unique in that all of its codons derive from the common exons 4–13. As there is no in-frame AUG in the β1 exon of the *L63B3* mRNA, translation is presumed to begin with the first in-frame AUG in exon 4 (see Fig. 4B).

Figure 4B shows the deduced amino acid sequence for the five isoforms, each of which includes a 294-residue region (residues 199–492 of isoforms L63A1 and B1) that exhibits strong similarity (51% amino acid identity) to the CDC28 CDK of *Saccharomyces cerevisiae.* The L63 isoforms differ from CDC28 in having long N-terminal extensions of 176

FIG. 5. L63 protein expression in *L63* mutants. (A) Western analysis of protein extracts from white prepupae of the indicated *L63* mutant genotypes. The major L63 protein runs at 66 kDa as indicated by the arrow. This blot was probed with L63 monoclonal antibody A2-6, which recognizes the 54–209 aa region of the L63B1 protein (Materials and Methods). Essentially the same result was obtained by probing a similar Western blot with L63 monoclonal antibody E1-44, which recognizes the 484–522 aa region of the L63B1 protein. Ponceau S staining of the blot after transfer to nitrocellulose indicated that similar amounts of protein were loaded in each lane. (B) Canton-S wild-type control (top leftmost) and *L63 DfE1*/*Df GN50* mutant (all others) pupae. The *L63* mutant pupae exhibit a small-size phenotype. Adult legs of wildtype (C) and $L63^{81}$/*Df 663* mutant (D). *L63* mutants express a bent-leg phenotype, indicating a role for *L63* in epithelial morphogenesis.

FIG. 6. Rescue of *L63* mutant animals by L63 mutant proteins and other *Drosophila* CDKs. Rescue to adulthood was attempted with the indicated rescue constructs under conditions of no heat shock or 32 or 37°C heat shocks given every 12 h throughout development. The locations of the site-directed mutants in the L63 protein are indicated with arrows. Data are presented as the ratio of rescued mutants to heterozygous siblings compared to this ratio obtained with the wild-type *L63B1* cDNA.

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TABLE 1
Rescue of *L63* Mutants by Heat-Shock-Induced Synthesis of Wild-Type L63 Proteins

Heat-shock treatment	Progeny phenotype	Number of adult progeny of the indicated phenotype	
		w;L63^{81}/TM6b × *w;HS L63/+;Df GN50/TM6b*	*w;Df E1/TM6b* × *w;HS L63/+;Df GN50/TM6b*
None	w+, Hu	101	98
None	w+, Hu+	0	0
None	w, Hu	107	107
None	w, Hu+	0	0
37°C	w+, Hu	73	68
37°C	w+, Hu+	76	49
37°C	w, Hu	116	78
37°C	w, Hu+	0	0

Note. Rescue to adulthood of the *L63^{81}/Df GN50* and *Df E1/Df GN50* progeny from the above crosses was attempted by heat-shock induction of an *L63B1* expression construct that was inserted into the *HSL63* chromosome (Materials and Methods) via a P-element vector containing a *w+* marker and an *L63B1* cDNA linked to the *hsp70* promoter. Because this *HSL63* is the only one in the above crosses that contains a *w+* gene, only *w+* progeny contain the *L63B1* expression construct. Similarly, since *Hu* (humeral) is a dominant marker on the *TM6b* balancer chromosome, all *Hu+* progeny are *L63^{81}/Df GN50* and should not yield adults unless supplied with L63B1 protein via heat shock. Incubation was at 25°C except for 30-min heat shocks every 12 h. Results are given in the number of flies of the indicated phenotypes.

to 197 residues. In this they are akin to certain human CDK proteins, called PCTAIRE-1, -2 and -3 (Meyerson *et al.*, 1992), as exemplified by the PCTAIRE-1 sequence shown in Fig. 4B. It has a 158-residue N-terminal extension followed by a CDK-homology region (residues 159–449) with 61% identity to that of L63. Of even more interest is a murine CDK protein called PFTAIRE that also has an N-terminal tail followed by a CDK region (residues 83–376; Fig. 4B) with a remarkable 70% identity to L63 (Lazzaro *et al.*, 1997). The L63, PFTAIRE, and PCTAIRE-1 proteins are also similar in having C-terminal tails that extend beyond the C-terminus of CDC28. As distinguished from the N-terminal extensions, which exhibit little or no sequence similarity, the first 24 residues of the murine PFTAIRE and the human PCTAIRE-1 C-terminal tails exhibit, respectively, 54 and 42% identity to the equivalent L63 residues.

By the above criteria, the L63 isoforms most closely resemble the murine PFTAIRE protein, which derives its name from the sequence of 7 amino acids found in the highly conserved PSTAIRE motif of the proven CDKs, such as the human CDK2 and the yeast CDC28 (residues 52–58, Fig. 4B). In the human CDK2, the PSTAIRE sequence is contained within the α1 helix (De Bondt *et al.*, 1993), which undergoes a conformational change in the cyclin A–CDK2 complex that is critical to kinase activation (Jeffrey *et al.*, 1995). A single amino acid change from serine to cysteine, or to phenylalanine, in this signature sequence is responsible for the nomenclature of the human PCTAIRE-1 (residues 205–211) and murine PFTAIRE (residues 129–135) proteins, respectively. Here again, the L63 isoforms, which exhibit the PFTAIRE sequence motif (residues 245–251), are most similar to the mouse protein. Accordingly, the L63 isoforms could be called PFTAIRE proteins; indeed, this terminology was used for the L63A2 isoform, as defined by the sequence of a single cDNA (Sauer *et al.*, 1996).

The N-terminal sequences of the L63 isoforms, while showing little or no similarity to other CDK proteins with N-terminal extensions, do exhibit certain interesting characteristics. One of these is the 9-residue polythreonine tract near the N-terminus of the L63A1 and A2 isoforms, and it is at least curious that the quite different N-terminal sequences encoded by the α1 and β3 exons each consist of 19 residues (Fig. 4B). Finally, we note that a stretch of 19 residues encoded by exon 6 (residues 56–75 in Fig. 4B) contains 13 histidines (68%), which includes a polyhistidine tract of 9 residues.

L63 Is an Essential Gene

Evidence that *L63* is an essential gene was obtained from the analysis of seven lethal EMS mutations in the 63E region. Complementation mapping of these EMS alleles (*1, 15, 29, 33, 81, 1619, 2266;* Materials and Methods) defined three complementation groups, of which groups I (*15, 33*) and III (*1, 29, 1619, 2266*) are proximal and distal to *L63*, respectively, with group II (*81*) in between. Molecular analysis of the *81* mutation showed that it is a mutant allele of the *L63* gene, hereafter denoted *L63^{81}*, consisting of an in-frame deletion of the DNA sequences encoding residues 226–241 within the conserved CDK region of the L63 isoforms (Fig. 4B). Western analyses of proteins in the *L63^{81}/Df 663* and *L63^{81}/Df E1* mutants reveal the expected shift in mobility (Fig. 5) resulting from the deletion of 16 amino acids. One of these is the highly conserved lysine (K234) that forms an essential part of the ATP-binding catalytic triad in the human CDK2 protein (Jeffrey *et al.*, 1995). Hence, it is likely that the *L63^{81}* mutation is null for *L63*.

The *Df E1/Df GN50* transheterozygote is an even better candidate for an *L63* null because *Df E1* eliminates all three

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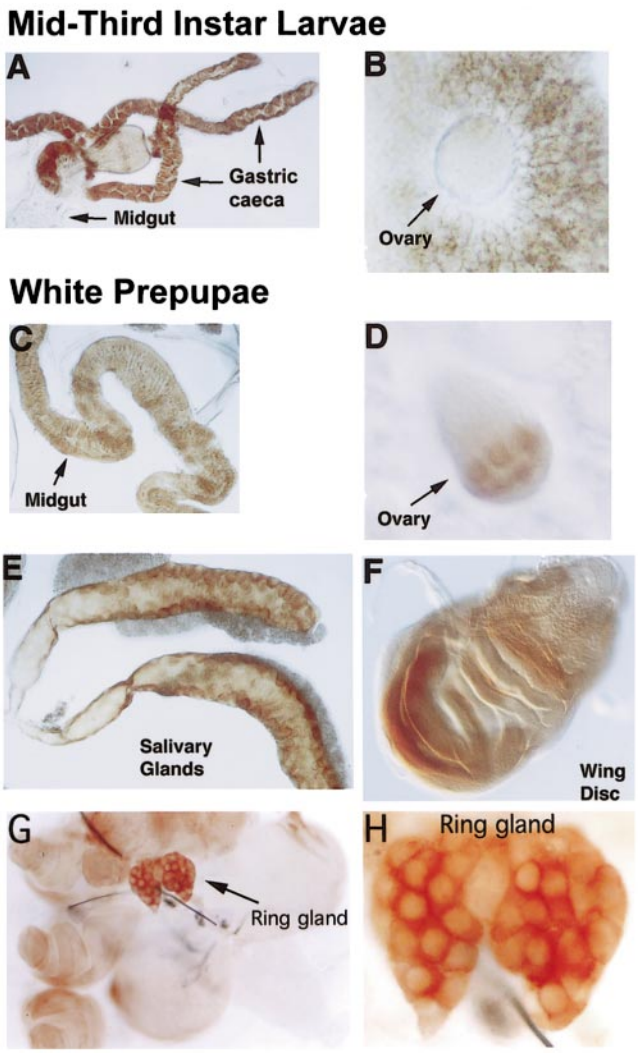

FIG. 7. Expression of L63 protein. Mid-third-instar gastric caeca and midgut (A) and ovary (B). White prepupae midgut (C), ovary (D), salivary glands (E), wing disc (F), CNS and attached ring gland (G), and close-up of ring gland (H). L63 monoclonal antibody A1-29 was used for the staining in A, G, and H while all other samples were stained with L63 monoclonal antibody E3-53. Similar spatial expression staining was observed with each of these antibodies, which recognize nonoverlapping epitopes of the L63 protein (Materials and Methods) while no staining was observed in the absence of goat anti-mouse HRP secondary antibody.

of the *L63* transcription initiation sites along with adjacent, presumably regulatory DNA, while *Df GN50* eliminates virtually all *L63* coding sequences (Fig. 2). The supposition that the *Df E1/Df GN50* heterozygote is an *L63* null is further confirmed by the inability to detect any L63 protein by Western analysis of this mutant genotype at metamorphosis (Fig. 5). As expected, the *Df E1/Df GN50* transheterozygote exhibits a lethal phase much like that of the $L63^{81}$*/Df GN50*. Both mutant combinations show a heterogeneous lethal phase with the majority of mutant animals dying during larval development and a small percentage living to pupation with an occasional adult escaper. Further evidence that the lethality of these heterozygotes results from alteration of *L63* is given in Table 1, in which we show that the lethal $L63^{81}$*/DfGN50* and *DfE1/DfGN50* heterozygotes can be rescued to adulthood by heat-shock-induced expression of the *L63B1* isoform. This result provides strong evidence that defective *L63* genes cause the above lethalities. No aberrant phenotypic consequences of the ectopic *L63B1* expression were observed in these rescue experiments.

L63 Isoforms Are Functionally Redundant

The redundancy of the *L63* isoform functions is indicated not only by the ability of a single isoform, *L63B1*, to rescue *Df E1/Df GN50* embryos to adulthood, but also by the viability of deletion mutants in which the production of a particular isoform is eliminated. The most striking illustration of this redundancy is the finding that *Df 1-11/Df GN50* animals are viable and can be perpetuated as a stock. *Df 1-11* removes all of the exons specific to the L63A proteins (exons α1-3; Figs. 1 and 2), as well as potential regulatory regions both upstream and downstream of the *L63A* transcription initiation site (Figs. 1 and 2). Given that the *Df GN50* is null for *L63*, the viability of *Df1-11/Df GN50* animals indicates that the L63A1 and 2 proteins are not required when the L63B proteins are present. The reduction of L63 protein levels in *Df1-11/Df GN50* late larvae, compared to the Canton-S wildtype (Fig. 5), may result not only from the *Df GN50* deletion, but also from the absence of L63A proteins due to the *Df 1-11* deletion. If this is the case, then the protein band seen above the *Df FF/Df GN50* main band, but not seen in *Df 1-11/Df GN50*, may represent one of the L63A isoforms. Indeed, the absence of such a band is specific to *Df 1-11/Df GN50* among the genotypes shown in Fig. 5. (Even the $L63^{81}$ heterozygotes exhibit such a band at an appropriately reduced apparent molecular weight.)

Figure 2B shows that the *Df FF* and *Df CC* deficiencies eliminate the B1 exon and hence the *L63B* transcription initiation site. The observation that *Df CC* can be maintained indefinitely as a homozygote indicates that the *L63B* transcription initiation site and neighboring sequences are not required for viability. One cannot, however, conclude that the L63B1, B2, and B3 proteins are not required in the presence of the L63A1 and A2 proteins because the L63B1 protein is encoded by the *L63C1* and *2* mRNAs as well as the *L63B1* and *B4* mRNAs (Figs. 1B and 4A). Furthermore, there is no obvious reason why an additional *L63C* mRNA encoding the L63B2 protein might not be made by the same exon 5/7 splice used for the *L63A2* and *L63B2* mRNAs (Fig. 1B). Indeed, the fact that we did not detect such an *L63C* cDNA may well result from the low abundance of the *L63C* mRNAs in the wildtype (Fig. 3).

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L63 Displays a Zygotically Rescuable Maternal Effect

The rescue of *Df E1/Df GN50* animals to adulthood (presented in Table 1) provided an opportunity for carrying out a self-cross of *w; HSL63/+; DfE1/DfGN50* animals. The observation that none of the embryos from this cross were observed to hatch, although such embryos can be rescued to adulthood by the protocol given in Table 1, demonstrates an embryonic requirement for the L63 protein. Moreover, this observation raises the possibility of a maternal effect because *Df E1/Df GN50* mutants derived from females not maternally deficient for L63 die later in development. The viability of *Df 1-11/Df GN50* animals (maternally deficient for L63A) allowed us to confirm this possibility by showing that the direction of the cross *Df 1-11/Df GN50* × *Df E1/TM6b* affects the lethal phase of the *L63* mutant genotype. When female *Df 1-11/Df GN50* are crossed to male *Df E1/TM6b,* all *Df E1/Df GN50 L63* mutant progeny die prior to the third-instar developmental stage, while if the reciprocal cross is performed a significant percentage of *Df E1/Df GN50* mutant progeny survive to third instar or further, exhibiting a lethal phase similar to that of *Df E1/Df GN50* animals from the cross *Df E1/TM6b* × *Df GN50/TM6b*. Notably, when L63 maternally deficient *w; HSL63/+; DfE1/DfGN50* mothers are crossed to *Df E1/TM6b* males, TM6b progeny survive to adulthood. This result indicates that the embryonic requirement for *L63* that is absent in the *L63* maternal deficient females is zygotically rescuable by the wild-type *L63* function present on the TM6b chromosome.

L63 Phenotypes Indicate Multiple Roles during Development

Phenotypic analysis of rare *L63* null mutant animals that survive past the third instar stage reveal developmental roles for *L63.* One such role is a general effect on the overall size of the animal. This can be observed by comparing the upper leftmost pupa in Fig. 5B (wildtype) with any of the seven others (*L63* mutants). *L63* mutant pupae range in size from approximately one-third to two-thirds that of wildtype under uncrowded conditions and take 2–3 days longer than their heterozygous siblings to reach pupariation. A second and more specific developmental role for *L63* is an involvement in epithelial morphogenesis. This is evidenced by the bent-leg phenotype in rare escaper adult *L63* mutant animals shown in Fig. 5D (compare to wildtype leg in Fig. 5C). While such *L63* mutant animals do manage to eclose they almost immediately fall into the food and die soon thereafter. Both the small pupal size and the bent-leg phenotypes were rescued along with viability in the heat-shock-induced *L63* cDNA rescue experiments presented in Table 1. In contrast to the morphogenetic defect observed in *L63* mutant legs, no morphogenetic defects were observed in $L63^{81}$ mutant eyes composed exclusively of mitotic clones of L63 generated by the EGUF/*hid* technique (Stowers and Schwarz, 1999). Furthermore, analysis of the electrophysiological properties of *L63* mutant eyes by electroretinogram revealed no defects in phototransduction or synaptic transmission (R.S.S., unpublished data).

In Vivo Tests of L63 Site-Directed Mutants Indicate That L63 is a CDK

The transgene rescue technique described in Table 1 was adapted to test for CDK function by attempting to rescue *Df E1/Df GN50* animals with heat-shock-induced expression of mutant L63 proteins in place of wild-type *L63B1* used in Table 1. Thus, in these experiments a mutant version of the *L63B1* cDNA was linked to the *hsp70* promoter in the context of a P-element vector that was germ-line transformed to generate a mutant *HS L63* chromosome. The only other change in the Table 1 protocol was to employ 32°C, as well as 37°C, heat shocks—thereby testing for rescue at different *in vivo* concentrations of the mutant L63 protein. Residues known to be important for cyclin binding, kinase activity, and phosphorylation in CDK proteins were chosen for site-directed mutagenesis. If these functional hallmarks of the CDK family contribute to the *in vivo* function of L63, it would be predicted that mutating these residues would adversely affect the ability of the altered L63 protein to rescue the *Df E1/Df GN50* mutant.

The conserved glycine (G243) and isoleucine (I249) residues of L63B1 (Fig. 4A) correspond to human CDK2 residues known to be crucial for binding cyclin A. In the human CDK2/CYCA cocrystal structure, the glycine corresponding to the L63 G243 is adjacent to the cyclin-interacting PSTAIRE domain (PFTAIRE in L63). It is thought to be essential at that position because it is the only amino acid that will allow the two residues directly adjacent to it to form hydrogen bonds with the human cyclin A (Jeffrey *et al.,* 1995). The isoleucine residue in human CDK2 that corresponds to the L63 I249 (Fig. 4A) is crucial because it fits tightly into a hydrophobic pocket of the human cyclin A. Figure 6 shows that the G243A L63 mutant protein failed to rescue the *Df E1/Df GN50* animals exposed to either the 32°C or the 37°C heat shocks and that the I249L mutant provided only partial rescue compared to the L63B1 wildtype (17% with 32°C heat shock and 68% with 37°C heat shock). These differences cannot be attributed to differences in the heat-shock response as the wild-type and mutant protein levels were similar for a given temperature (Stowers, 1997). We therefore take these results as strongly supportive of the proposition that L63 protein interacts with cyclins.

The glutamic acid residue at 251 in L63B1 (E251) corresponds to a highly conserved residue in both cyclin-dependent kinases and other, more distantly related, protein kinases. In the human CDK2, this residue assists the highly conserved lysine and aspartic acid residues (the other two members of the catalytic triad—K234 and D344 in L63B1) in correctly positioning the γ phosphate of ATP for nucleophilic attack by serine and threonine substrates (Jeffery *et al.,*1995). Mutation of the glutamic acid to

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glutamine (E251Q; Fig. 4B) results in complete loss of the rescue function at both temperatures (Fig. 6)—a result in strong support of the minimal proposition that L63 is a protein kinase.

The serine residue S359 in L63B1 corresponds to a conserved threonine phosphorylation site in human CDK2 (T160) and in other CDKs, including the T169 site in the yeast CDC28 (Fig. 4B). Phosphorylation of this residue is critical for full CDK activity. The L63B1 mutant in which this serine is replaced by alanine (S359A; Fig. 4B) cannot rescue *Df E1/Df GN50* whether induced at 32 or 37°C (Fig. 6).

An attempt was made to see if the N-terminal tail plays an essential role in L63 function by constructing a mutant, Δ202 L63, in which the N-terminal 202 residues of L63B1 were eliminated. No rescue by Δ202 L63 was observed when the heat shocks were carried out at 32°C, but 31% rescue occurred with 37°C heat shocks. This result cannot, however, be directly compared with those of the other L63 rescue constructs because the amount of Δ202 L63 stably produced by the 37°C heat shocks was only 15 ± 5% of that obtained for the other L63 constructs (Stowers, 1997). What can be said about the nonconserved N-terminal tail is that it is not necessary for L63 function, although it may enhance that function or protect L63 from denaturation and/or destruction. Finally we carried out an ancillary experiment in which we asked whether either of the bona fide *Drosophila* CDKs [cdc2 and cdc2c (Lehner and O'Farrell, 1990; Jimenez *et al.*, 1990)] could rescue the *Df E1/Df GN50* null. The answer was a clear no (Fig. 6), even though both proteins were produced at high levels by the 37°C heat shocks (Stowers, 1997).

L63 Proteins Are Widely Distributed among Late Larvae and Prepupal Tissues

Our collection of monoclonal anti-L63 antibodies divides into two classes, one class against epitopes in the N-terminal tail and the other class against epitopes in the C-terminal tail, with all epitopes present in all five L63 isoforms (Materials and Methods). Specificity criteria for L63 in the tissue staining shown in Fig. 7 is that the pattern obtained with an antibody from one class (A1-29; N-terminal epitope) is the same as that obtained with antibody from the other class (E3-53; C-terminal epitope). The antibodies used for Western analyses in Fig. 5 (in which the evidence for specificity was the absence of antigens in the *Df E1/DfGN50* mutant) elicited too weak a tissue response to be useful—perhaps because their epitopes are fully revealed only under the denaturing conditions of Western analysis.

L63 is expressed throughout development and in many tissues (Fig. 7 and data not shown). However, some tissues show different L63 expression levels at different developmental stages, especially at metamorphosis. Comparison of the larval midgut in Figs. 7A and 7C shows that the L63 protein increases from little or nothing in feeding mid-third-instar larvae (i.e., ~18 h before pupariation) to high levels in white prepupae (i.e., at pupariation), implying that one or more of the *L63* transcription units is activated in response to the late-larval pulse of ecdysone. The same developmental correlation of L63 with ecdysone titer is observed in ovaries which show no staining in mid-third-instar larvae (Fig. 7B) but by pupariation contain high levels of L63 (Fig. 7D). The gastric caeca, which exhibit high levels of L63 at mid-third instar (Fig. 7A), show signs of programmed cell death by pupariation and disappear a few hours later (Jiang *et al.*, 1997). Another pattern of *L63* expression is seen in both the larval salivary gland and the imaginal discs. In mid-third instar, both exhibit significant levels of L63 (not shown) which increase severalfold by pupariation (Figs. 7E and 7F), as if *L63* is induced in two phases. Interestingly, a similar developmental expression profile for L63 was observed in the ring gland, the site of ecdysone biosynthesis, with moderate expression levels at mid-third instar (not shown) and high expression levels at white prepupae (Fig. 7G). A higher magnification view of the ring gland staining reveals that L63 is primarily localized to the cytoplasm. A similar conclusion can also be drawn from close examination of the salivary gland staining (Fig. 7E). The observation that L63 protein is present in some tissues before the later larval ecdysone pulse is consistent with temporal patterns of *L63A* and total *L63* mRNA expression shown in Fig. 3 and the predominantly premetamorphic lethal phase of L63 mutants.

DISCUSSION

The 63E Puff and the L63 Ecdysone Response

The data presented in this paper suggest that the *L63B* unit is ecdysone responsive. The temporal pattern of *L63B* transcription shown in Fig. 3 is consistent with this premise. Stronger evidence that the *L63B* transcription unit is ecdysone responsive, however, consists in the observation that the *Df CC* deletion eliminates the ecdysone-responsive 63E late puff (Fig. 2C) since this deletion eliminates only the *L63B* transcription initiation site and approximately 1.0 and 0.4 kb of upstream and downstream sequences, respectively (Fig. 2B). In contrast, the temporal expression of L63A is not suggestive of a positive ecdysone response, with the possible exception of the strong expression of L63A mRNA during the 120–144 h interval (Fig. 3). That expression, however, might be associated with the midprepupal 63E puff noted in the Introduction. This puff peaks at 128 h, some 8 h after the late larval 63E puff peaks (Ashburner, 1967), and is further distinguished from that puff by its inhibition by very low concentrations of ecdysone (Richards, 1976; Ashburner and Richards, 1976). In these regards, it is very similar to the midprepupal puff at 75CD, except that there is no late larval puff at this site (Richards, 1976). The *β-FTZF1* gene is responsible for the 75CD puff (Lavorna *et al.*, 1993; Woodard *et al.*, 1994) and is activated at the appropriate time in prepupae by the com-

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bined action of the *DHR3* and *E75B* genes, both of which are directly induced by the late larval ecdysone pulse and encode orphan nuclear receptors (Lam *et al.*, 1997; White *et al.*, 1997). The similarity in the expression kinetics of the 63E and 75CD midprepupal puffs suggests that the *L63A* and *β-FTZF1* genes may be induced by similar mechanisms. If *L63A* transcription is responsible for the 63E midprepupal puff then one would expect that the *Df 1-11* deletion would eliminate that puff, as it removes the L63A transcription initiation site as well as ~25 kb downstream and even more upstream of that site (Figs. 1 and 2).

These speculations about the relationship between 63E puffing and *L63A* and *B* transcription are based on two assumptions: that the midprepupal 63E puff is associated with the *L63* gene and, more generally, that a transcription unit which is normally correlated with a puff will be inactivated by a mutation that inactivates the puff. The first of these assumptions derives from two observations, of which the first is that the *L63* gene occupies a central and considerable fraction of the DNA represented in the 63E late-larval puff (Fig. 1). The second observation is that the late-larval and midprepupal 63E puffs are of the same size and position in *D. melanogaster* (Ashburner, 1967) and that they are decreased equivalently in the sibling species *D. simulans* to 40% of their size in *D. melanogaster*—a change that Ashburner remarks is "the most remarkable difference in puffing noticed between the two species."

While the second assumption has been validated for several mutations, in some instances a puff and the activity of the associated gene can be uncoupled by mutations that define DNA segments necessary for puffing but not for transcription (reviewed for the *Sgs* genes and their intermolt puffs by Russell and Ashburner, 1996). Such an uncoupling could account for the small size of the 63E late-larval and midprepupal puffs in *D. simulans* without requiring a change in the activity of any of the *L63* transcription units. Clearly, the first steps in sorting out these possibilities are to determine *L63A* and *L63B* transcription in *L63* deletion mutants (particularly *Df 1-11* and *Df CC*) and to examine the 63E midprepupal puff in such mutants.

L63 Is Likely to Be a CDK That Does Not Regulate the Cell Cycle

Given the strong sequence similarity of L63 to established CDKs, the observation that the $L63^{81}$ mutant, which removes a key catalytic site, is a likely null, and the results of the rescue experiments with altered L63 proteins generated by *in vitro* mutagenesis, we argue that the L63 proteins function as cyclin-dependent kinases. However, there is no evidence that L63 is involved in the control of cell division as is the case for many CDKs. In fact, several observations suggest otherwise. First, qualitative examination of the brains of wild-type and *Df E1/Df GN50* larvae at pupariation revealed no significant difference in the fraction of cells undergoing mitosis (Stowers, 1997). Second, no differences in polytenization in the salivary gland polytene chromosomes were seen in wandering third-instar larvae of *Df E1/Df GN50* compared to wild-type animals (Stowers, 1997). Finally, eyes composed entirely of $L63^{81}$ mitotic clones were indistinguishable from control mitotic recombinant eyes, indicating that L63 is not required for cell division in the eye (R.S.S., unpublished data). These results suggest that the CDK functions of the L63 proteins are required for functions other than the regulation of cell division. A similar proposal has been made for the closest mammalian relatives of L63—namely, the PFTAIRE and PCTAIRE proteins (Fig. 4B), which may play a role in cell differentiation rather than in cell division (PCTAIRE, Hirose *et al.*, 1997; Besset *et al.*, 1999; PFTAIRE, Lazzaro *et al.*, 1997; Besset *et al.*, 1998).

Given the above observations, the wide spatial (Fig. 7) and temporal (see below) distributions of the L63 proteins, the functional redundancies indicated by the *L63* mutants, and the observation that L63B1 overexpression appears to have no phenotypic consequences, we propose that the functional specificity of L63 proteins derives in large measure from associated cyclins and/or other proteins that regulate L63 activity. For instance, an interaction between L63 and one cyclin in an imaginal tissue, like the leg disc whose metamorphic fate is to form an adult structure, could result in L63 controlling epithelial morphogenesis, while an interaction between L63 and a different cyclin in a strictly larval tissue, such as the salivary gland whose fate during metamorphosis is histolysis, could result in L63 playing a role in programmed cell death. This scenario is consistent with the functions observed for other CDKs. Such a functional specificity has been observed for the mammalian CDK5, a key regulator of neuronal differentiation (Tsai *et al.*, 1993; Ohshima *et al.*, 1996). CDK5 is widely expressed in human and mouse tissues, but its kinase activity is restricted to the brain where its specific activator p35/p25 is expressed (Lew *et al.*, 1994; Tsai *et al.*, 1994). Another example of specific control of CDK activity by different cyclins is that of the yeast PHO85 CDK protein in *S. cerevisiae* (Huang *et al.*, 1998; Tennyson *et al.*, 1998; Measday *et al.*, 1997). It is essential for the proper regulation of phosphate and glycogen metabolism as well as for cytokinesis. To accomplish these roles it appears that PHO85 may well interact with as many as 10 different cyclins. Mutation of some of these cyclins has shown that they can produce mutant phenotypes that are a subset of those of the PHO85 mutant. Apparently these cyclins exhibit overlapping but unique functions. Using these results as a model, we can imagine that L63 proteins interact with quite different cyclins or other proteins to provide different developmental functions. We are currently investigating this possibility using yeast two-hybrid assays to identify candidates for such proteins.

L63 Is Required at Most Stages of Development

Evidence that *L63* is required for embryogenesis consists in our observation that *L63* mutant embryos that are

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maternally and zygotically deficient for *L63* activity do not complete embryogenesis. Interestingly, recent observations make it plausible that *L63* maternal expression may be ecdysone regulated during oogenesis because the same set of early genes induced by ecdysone at the onset of metamorphosis is coordinately regulated by ecdysone during oogenesis (Buszczak *et al.*, 1999). Maternal *L63* expression may therefore be part of an ecdysone-regulated hierarchy of gene expression in oogenesis that is used repeatedly during development.

Our observation that the majority of zygotically deficient *L63* mutants die as larvae indicates that *L63* is also required during larval development. From the small pupal phenotype of the few *L63* mutants that survive to pupariation (Fig. 5B) we infer that *L63* may participate in the regulation of organism size. The possibilities by which the loss of *L63* activity indirectly reduces organism size are numerous and could include a role for L63 proteins in digestion, muscle contraction, or the nervous system, to name a few. An interesting possibility is that L63 proteins act as direct determinants of organism size. One possible scenario is that these proteins are part of a system that regulates progression from one developmental stage to the next, so that when L63 is absent, precocious developmental transitions occur.

The bent-leg phenotype of rare escaper adult *L63* mutants (Fig. 5C) indicates a third function of L63 in development—namely an involvement in epithelial morphogenesis. Two possibilities come to mind as to how *L63* could be involved in this developmental process: (1) regulating shape changes of individual epithelial cells and (2) as a component of a communication system between epithelial cells. One mechanism by which L63 proteins could alter the changes in cell shape due to remodeling of the cytoskeleton is by phosphoregulating critical components of the cytoskeleton, such as actin. Alternatively, if *L63* is part of a cell–cell communication system, the bent-leg phenotype of *L63* mutants could be explained by a failure of the epithelial cells to communicate to each other their relative spatial positions, thereby altering the evagination process that occurs in the leg disc during pupal development. A role for L63 in the morphogenesis of the leg disc is further supported by recent genetic results that show that *L63* mutations genetically interact with genes involved in leg morphogenesis (von Kalm and Garza, unpublished results). It should be emphasized that whatever role *L63* plays in epithelial morphogenesis, it cannot be generalized to all discs given our finding that eyes composed exclusively of L63 mitotic clones showed no morphological defects (R.S.S., unpublished data).

The tissue distributions of L63 proteins just before and during metamorphosis (Fig. 7) also suggest a role for *L63* in the cell death of the histolysing larval tissues. In this regard, we note that the larval midgut, which expresses little or no L63 protein in mid-third-instar larvae (~18 h before pupariation), exhibits strong L63 expression at pupariation while in the midst of the process of cell death and histolysis (Fig. 7; Jiang *et al.*, 1997). In this connection we also note that the gastric caeca, which undergo histolysis before the midgut, exhibit strong L63 expression earlier.

The L63 and L82 Late Genes Share a Number of Properties That Contrast Them to the Previously Cloned L71 Late Genes

L63 and *L82* (Stowers *et al.*, 1999) are the first late puff genes to be isolated by positional cloning directed at the sites of model late puffs defined by Ashburner and Richards (1976). Prior to their isolation, molecular information about the late puff genes derived mostly from the *L71* genes, cDNA clones of which were first isolated in this laboratory by a method not directed toward a particular late puff (Wolfner, 1980). Thus, cDNA clones were isolated from libraries obtained from cultured larval salivary glands before and after their exposure to ecdysone, using stage-specific ^{32}P-labeled cDNA probes from the same set of glands for their identification. Subsequent analyses of the corresponding genomic DNA showed that the 71E late puff results from the activation of five pairs of oppositely oriented, short *L71* genes each of which encodes a small polypeptide thought to be secreted from the gland as a protection against infection during metamorphosis (Restifo and Guild, 1986; Wright *et al.*, 1996). The tissue- and temporal-specific expression of these genes is in stark contrast to the broad expression profiles of the early puff genes and generated an emphasis on such a distinction between these two classes (Thummel, 1996). The molecular analyses of the *L63* and *L82* genes shows that these late genes exhibit similarly complex structures and expression patterns as the early genes and raises the question of whether differences between the *L71* and the *L63*/*L82* genes derive, in part, from differences in the methods of isolation. Would positional cloning of genes responsible for other large well-defined late puffs tend to yield additional functionally and structurally complex genes?

The functional significance of the complex structures of *L63* and *L82* is not clear, as in both genes overexpression of a single isoform can rescue mutations that eliminate gene function, and genetic analysis indicates at least partial internal functional redundancy. Conversely, the widespread temporal and spatial expression of these genes is consistent with the phenotypic complexity of their mutants. Third, molecular and genetic analyses suggest that transcriptional regulation of both *L63* and *L82* has ecdysone-dependent and ecdysone-independent aspects, so that steroid hormone regulation may be superimposed upon, or even interact with, other regulatory signals. Finally, while most previously characterized secondary-response genes encode proteins that are consistent with their being "effectors" of the ecdysone response (see for example Bayer *et al.*, 1996), the proteins encoded by the *L63* and *L82* genes do not fit easily into this category. The *L63* gene encodes members of the CDK family, and mutational analysis of transgene rescue (Fig. 6) suggests that L63 may

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interact with one or more cyclins. Given that such cyclin/CDK complexes are generally accepted as having regulatory functions, this suggests that L63 proteins have a regulatory rather than an effector function. Similarly, the developmental delays associated with the *L82* mutations are not consistent with a simple effector function. A more detailed analysis of both the regulation and the function of these two complex late genes will undoubtedly lead to further insights into the regulation of late genes within the ecdysone hierarchy and the role of these complex genes in development and metamorphosis.

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