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

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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.

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 about 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...
effected by an ecdysone-receptor complex formed at the
initiation of the larval-pupal transition, and gene activation
results from transcription factors encoded by the early
genes. Because repression of the early puffs, like the sec-
dary 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 induc-
tion 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 distrib-
uted among the target tissues, suggesting that the tissue
specificity of the metamorphic response to ecdysone de-
rivatives is, in part, from the EcR isoforms—a suggestion that has
received support from recent evidence that the EcR iso-
forms are functionally distinct (Bender et al., 1997; Schu-
biger 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 demon-
strated 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 ecdy-
sone 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 de-
fense 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 distin-
guish 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). Immu-
nostaining of salivary gland polypeptide chromosomes
with monoclonal antibodies specific for EcR-B1 (the preva-
lent isoform in these glands) demonstrated the expected
presence of EcR-B1 at the 22C and 82F sites prior to puffing; how-
however, no staining was observed at 63E despite consider-
able effort to detect it (Talbot, 1993). The second distin-
guishing 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 nec-
essary condition for their activation, as both are inhibited
by ecdysone concentrations as low as 5 × 10⁻⁸ M (Ash-
burner, 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 re-
sembles 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 expres-
sion 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. Deletion stocks Df(3L) 663 and Df (3L) 294 and the lethal alleles B1, 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 P2-3 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 cytoplasmic positional 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+] 33D 3. 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 hsdc2 and hsdc2c stocks were previously described (Lehner and O’Farrell, 1990). The lesion of the L63Δ23 mutant was determined by isolating total mRNA from L63I/TM6b and control 1619/TM6b flies (1619 is a chromosome generated in the same mutagenesis and from the same isogenic parental chromosome as L63I), 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 L63I/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 (Takun 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 polynucle chromosomes using genomic phase clones as probes. Deletion breakpoints were determined more precisely by genomic Southern blot analysis. Df (3L) 294 has its distal breakpoint within a 7-kbp EcoRI fragment of phase 6307; Df(3L) 1-11 has its proximal breakpoint within a 3.5-kb Smal/EcoRI fragment of phase 6311; Df(3L) O has its proximal breakpoint within a 1.2-kb Smal/EcoRI fragment 3’ of the L63 B1 exon in phase 6311; Df(3L) E1 has its proximal breakpoint within a 600-bp Sall/EcoRI fragment of phase 6311; Df(3L) GN50 has its distal breakpoint within a 900-bp EcoRV/HindIII fragment of phase 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 Nai/BamHI cDNA fragment probe from an L63B1 cDNA, (middle) 1.5-kb EcoRI/Sall genomic fragment containing the entire 1.1-kb L63A1 exon, and (bottom) 2.0-kb Xhol 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., 1989).

**Plasmid subclones for P-element transformation.** The HSL63B1 transformation plasmid pSS238 was constructed by subcloning the 1.6-kb Hpal/BamHI fragment of pSS156 into the Hpal/BamHI sites of pCaSpeR-hs (Thummel et al., 1988). The HSL63B1/202 transformation plasmid was constructed by subcloning a fragment formed by the complementary oligonucleotides 5’-GAATTCCAGAAACATGGAGGCCTATGTTAAAC-3’ and 5’-GTCGAATTTAATCAGCGCTCCATGTTTCTG-3’ into the EcoRI/Xhol partial digest of pSS156 and subcloning the resulting 1.0-kb EcoRI/BamHI fragment from this plasmid into the EcoRI/BglII 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 G424A-GCT to GCA, 1249L-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
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 SalI-blunt/XhoI fragment inserted into the XmnI/Sall vector sites, while the E monoclonals derived from a 114-bp PstI/BamHI-blunt fragment inserted into PstI/HindIII-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

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 phase 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 L63b1 exon was determined by both primer extension and RNase protection. A likely transcription start site for the L63y1 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.
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 [32P]cDNA probes constructed from two poly(A)+ RNA populations—one prepared from white prepupae when the...
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

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 L63b1–3 region cDNA probe (top). An identical Northern blot hybridized with an L63a1-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.

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designations, 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 1.3-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 RNAase 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 32P-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 al., 1992; Lazzaro et al., 1997; Lorincz and Reed, 1984; Pirotta et al., 1987).

**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) PFTAIRE-1 of Homo sapiens (Meyerson et al., 1992; SWISS-PROT—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 at 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.
al., 1992) and the EcR-A and EcR-B1 transcription units that encode the respective ecysdase receptor isoforms (Talbot et al., 1993). The embryonic ecysdase 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 ecysdase 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 of transcription is seen for the 2.6-kb band of RNA 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 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 ecysdase pulses do not influence the abundance of these mRNAs in whole animals. That exception is an increase in transcript abundance during the 120–168-h interval that could result from the prepupal and/or pupal ecysdase 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 ecysdase 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...
TABLE 1
Rescue of L63 Mutants by Heat-Shock-Induced Synthesis of Wild-Type L63 Proteins

<table>
<thead>
<tr>
<th>Heat-shock treatment</th>
<th>Progeny phenotype</th>
<th>Number of adult progeny of the indicated phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>w+, Hu</td>
<td>101</td>
</tr>
<tr>
<td>None</td>
<td>w+, Hu+</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>w, Hu</td>
<td>107</td>
</tr>
<tr>
<td>None</td>
<td>w, Hu+</td>
<td>0</td>
</tr>
<tr>
<td>37°C</td>
<td>w+, Hu</td>
<td>73</td>
</tr>
<tr>
<td>37°C</td>
<td>w+, Hu+</td>
<td>76</td>
</tr>
<tr>
<td>37°C</td>
<td>w, Hu</td>
<td>116</td>
</tr>
<tr>
<td>37°C</td>
<td>w, Hu+</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. Rescue to adulthood of the L63<sup>158</sup>/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<sup>158</sup>/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.

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 (L, 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<sup>81</sup>, 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<sup>81</sup>/Df 663 and L63<sup>81</sup>/Df E1 mutants revealed 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<sup>81</sup> 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
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 L6381/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 L6381/Df GN50 and DfE1/Df GN50 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 lethaliies. 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 Df 1-11/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 Df 1-11/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 L6381 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).
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 lefmost 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 escapee adult L63 mutant animals shown in Fig. 5D (compare to wildtype leg in Fig. 5C). While such L63 mutant animals do manage to clasp 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 L63mutant 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 wild-type (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 hydrophobic interaction between the two members of the catalytic triad—K234 and D344 in the CDK2/CYCA cocrystal structure. The glutamic acid residue in human CDK2 that corresponds to the L63 E251 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 wild-type (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.
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 Preupal 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). A further 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 metamorphic 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-
The Drosophila L63 Gene

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 L63β1 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 L63β1 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; PCTAIRE, 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; Meadley 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
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 32P-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
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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