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Molecular Genetic Analysis of the Nested *Drosophila melanogaster* Lamin C Gene

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ABSTRACT

Lamins are intermediate filaments that line the inner surface of the nuclear envelope, providing structural support and making contacts with chromatin. There are two types of lamins, A- and B-types, which differ in structure and expression. Drosophila possesses both lamin types, encoded by the *LamC* (A-type) and *lamin* Dm_0 (B-type) genes. *LamC* is nested within an intron of the essential gene *ttv*. We demonstrate that null mutations in *LamC* are lethal, and expression of a wild-type *LamC* transgene rescues lethality of *LamC* but not *ttv* mutants. Mutations in the human A-type lamin gene lead to diseases called laminopathies. To determine if Drosophila might serve as a useful model to study lamin biology and disease mechanisms, we generated transgenic flies expressing mutant LamC proteins modeled after human disease-causing lamins. These transgenic animals display a nuclear lamin aggregation phenotype remarkably similar to that observed when human mutant A-type lamins are expressed in mammalian cells. LamC aggregates also cause disorganization of lamin Dm_0, indicating interdependence of both lamin types for proper lamina assembly. Taken together, these data provide the first detailed genetic analysis of the *LamC* gene and support using Drosophila as a model to study the role of lamins in disease.

LAMINS belong to a family of structural proteins known as intermediate filaments (Stuurman *et al.* 1998). All intermediate filaments, except lamins, localize to the cytoplasm where they impart physical strength to a cell. In contrast, lamins localize to the inner surface of the nuclear envelope, providing structural support for the nucleus and making contacts with other nuclear components (Zastrow *et al.* 2004). Lamins possess an N-terminal head domain, a central α-helical rod domain, and a C-terminal globular domain (Stuurman *et al.* 1998). Dimerization occurs through interactions in the rod domain; dimers associate in a head-to-tail fashion to form protofilaments, which then align in antiparallel orthogonal arrays to form a meshwork called the lamina (Stuurman *et al.* 1998). The nuclear lamina possesses additional protein components, including the so-called "LEM" domain proteins, named after a conserved domain found in *L*AP2, *E*merin, and *M*AN1 (Laguri *et al.* 2001). Components of the nuclear lamina make connections with histones (Goldberg *et al.* 1999), transcription factors (Ozaki *et al.* 1994), and nucleic acids (Rzepecki *et al.* 1998). These interactions are likely to play a role in spatially organizing the genome and regulating gene expression.

There are two types of lamins, A- and B-types, which differ in their protein structure and expression patterns (Hutchison 2002). In humans, B-type lamins are encoded by the genes *LMNB1* and *LMNB2*. B-type lamins are ubiquitously expressed and possess a C-terminal CaaX box (C, cysteine; a, an aliphatic amino acid; X, any amino acid) that is isoprenylated and carboxy methylated, serving as a membrane anchor. Mammalian cells require at least one B-type lamin for viability (Harborth *et al.* 2001). In humans, A-type lamins are encoded by the *LMNA* gene. Alternative splicing generates messages encoding two isoforms: lamin A and lamin C. Expression of A-type lamins is limited to terminally differentiated somatic cells. Human lamin A possesses a CaaX box that is proteolytically removed to form the mature protein, whereas no CaaX box is encoded in the primary amino acid sequence for lamin C.

A-type lamins are not required for viability in mammalian cell culture (Harborth *et al.* 2001); however, in humans, mutations in *LMNA* are associated with a range of diseases known as laminopathies. Many of these diseases have tissue-restricted phenotypes, such as Emery-Dreifuss muscular dystrophy (EDMD) (Bonne *et al.* 2000) and familial dilated cardiomyopathy (Brodsky *et al.* 2000) that affect skeletal and cardiac muscle; Dunnigans's familial partial lipodystrophy (Shackleton *et al.* 2000) that affects adipose tissue; and Charcot-Marie Tooth syndrome type 2 that is a neuropathy (Chaouch *et al.* 2003). For a few laminopathies, the phenotype appears to be more systemic; these diseases include Hutchinson-Gilford progeria syndrome (HGPS) (Eriksson *et al.* 2003) and

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atypical Werner's syndrome (Chen *et al.* 2003), both diseases of premature aging. It is unclear how different mutant forms of A-type lamin cause these various disease phenotypes (Burke and Stewart 2002). With the possible exceptions of Dunnigan's familial partial lipodystrophy and HGPS, the amino acid substitutions responsible for a given laminopathy do not map to a specific domain of the lamin protein (Burke and Stewart 2002). Therefore, functional studies are required to determine how distinct molecular defects arise from particular amino acid substitutions in specific protein domains.

To address the molecular defects associated with expression of mutant A-type lamins in disease, genetically tractable model organisms are invaluable. Analyses of *LMNA* gene knock-out and mutant *LMNA* gene knock-in transgenic mice have provided important insights into disease progression. Knocking out *LMNA* or *LMNB1* in mice causes lethality shortly after birth (Sullivan *et al.* 1999; Vergnes *et al.* 2004). Knock-in mice expressing mutated lamin A/C proteins (H222P and L530P) exhibit phenotypes similar to EDMD and HGPS (Mounkes *et al.* 2003; Arimura *et al.* 2005). Nevertheless, a comprehensive study of the numerous lamin mutations associated with these diseases would benefit from the rapid generational analysis that is possible in a well-characterized invertebrate model organism. Most invertebrates, however, do not possess the two distinct lamin types found in humans. An exception is *Drosophila melanogaster*, which possesses two genes encoding nuclear lamins. *lamin* Dm_0 encodes a B-type lamin on the basis of its constitutive expression pattern, possession of a CaaX box, and *in vitro* assembly properties (Riemer *et al.* 1995; Klapper *et al.* 1997). Mutations in *lamin* Dm_0 show defects in locomotion, tracheal development, and nuclear positioning in the oocyte and eye (Lenz-Bohme *et al.* 1997; Guillemin *et al.* 2001; Patterson *et al.* 2004). In contrast, *Lamin C* (*LamC*) encodes an A-type lamin, on the basis of its developmentally regulated pattern of gene expression and lack of a CaaX box (Riemer *et al.* 1995).

To understand the function of the A-type lamins in Drosophila, we undertook a formal genetic and molecular analysis of *LamC*. We demonstrate that *LamC* is an essential gene nested within another essential gene called *tout-velu* (*ttv*). *LamC* null mutants die at the prepupal stage, consistent with a critical role in differentiating tissues. To assess whether *LamC* functions similarly to its human counterpart, we have generated transgenic stocks expressing mutant forms of *LamC* modeled after mutations linked with human disease. These transgenic animals exhibit nuclear phenotypes that are remarkably similar to those observed in mammalian cell culture upon expression of mutant human A-type lamins. Collectively, these data provide a foundation for using an insect model to dissect the biology of nuclear lamins and their role in disease.

MATERIALS AND METHODS

Drosophila culture and imprecise excision: Drosophila stocks were raised at room temperature on standard sucrose/cornmeal medium. All crosses were performed at room temperature unless otherwise stated. Crosses for complementation tests, rescue experiments, and transgene expression assays were carried out in vials. For heat-shock-induced expression, 45-min heat-shock treatments were administered daily throughout development by placing vials in a 37° water bath and then returning the cultures to room temperature for recovery.

To generate deletions of *LamC*, a *P*-element excision scheme (Adams and Sekelsky 2002) was performed as follows: females bearing the *G00158* green fluorescent protein (GFP) exon-trap allele (Morin *et al.* 2001) over a *CyO* balancer chromosome (*w/w*; *G00158/CyO*; +/+) were mated to males carrying a second chromosome balanced over *SM1* and a transposase source, Δ*2-3*, marked with *Sb* on the third chromosome (*w/Y*; +/*SM1*; Δ*2-3Sb/TM6, Tb*). Individual F_1 male progeny that were heterozygous for the *G00158* allele and the transposase source (*w/Y*; *G00158/SM1*; Δ*2-3Sb/+*) were outcrossed to a homozygous *white* stock with dominantly marked second chromosomes (y^1w^{67c23}/ y^1w^{67c23}; *Sco/SM1*). Resulting *white* mutant F_2 male progeny carrying a marked second chromosome were individually backcrossed to flies of the maternal genotype ($y^1w^{67c23}/y^1w^{67c23}$; *Sco/SM1*); the resulting flies carrying potential deletions of *LamC* were balanced over *SM1*.

Lethal phase analysis: To determine the lethal phase of a *LamC* deletion, the putative null excision allele, $LamC^{EX296}$, and a deficiency for the region, *Df(2R)trix*, were used. $LamC^{EX296}$/*CyO-GFP* males were crossed to *Df(2R)trix/CyO-GFP* virgin females. Resulting embryos were collected in cornmeal/yeast bottles overnight at room temperature and aged for 5 days. A total of 200–250 larvae were collected from the food and scored for GFP using a Leica MZ12 dissecting microscope equipped with a fluorescent light source (Kramer Scientific, Yonkers, NY). Approximately 60 GFP-negative and GFP-positive larvae were collected per assay. Larvae were transferred to 350-mm petri dishes containing Whatman filter paper moistened with water and pulvarized cornmeal/sucrose media. The number of individuals at each developmental stage was recorded daily. Larval stages were identified by the morphology of mouth hooks and/or anterior spiracles. As a control, $LamC^{EX296}$/*CyO-GFP* and *Df(2R)trix/CyO-GFP* siblings were scored in parallel. As an external control, +/*CyO-GFP* males were crossed to *Df(2R)trix/CyO-GFP* virgin females, and the +/*Df(2R)trix* progeny were scored.

Transgene design: A full-length *LamC* cDNA was amplified by PCR from 18- to 21-hr embryonic RNA [purchased from CLONTECH (Palo Alto, CA)]. The cDNA was cloned into pCR2.1-TOPO (Invitrogen, San Diego) and sequenced. A comparison of the *LamC* DNA sequence to that present in FlyBase (http://flybase.bio.indiana.edu/) revealed seven silent mutations; these could represent naturally occurring strain polymorphisms and/or errors generated during PCR. The full-length *LamC* cDNA was used as a template for *in vitro* mutagenesis (QuikChange, Invitrogen) to generate the R401K mutant transgene. For the N-terminal truncation, a 33-amino-acid deletion generated in the human A-type lamin (Spann *et al.* 1997) served as a model. Alignment of human lamin A/C and Drosophila LamC amino acid sequences identified amino acid position 48 in the Drosophila protein as the equivalent end point of the truncation. The following primers were designed for *Pfu*-Ultra (Stratagene, La Jolla, CA) amplification of the N-terminal deletion mutant: 5′-CAAA CATGGAACTGCAGCATTTGAACGATC-3′, which encodes a

consensus translation start sequence, and 5′-CTAGAAGAG CAGGGAGAAGAG-3′, which includes the last 6 amino acids and a termination codon. cDNAs encoding wild-type LamC, R401K, and the N-terminal truncation were cloned into the two *P*-element germline transformation vectors pUAST (GAL4 inducible) and pCaSpeR-hs/act (heat-shock inducible). Constructs were injected according to standard procedures. For each transgenic line, Southern analysis was performed to examine transgene integrity and copy number; Western analysis was performed to examine LamC expression levels (see below).

Molecular characterization of the excision alleles: For Southern analysis of *LamC* alleles, genomic DNA was isolated from 100 adult flies (Bender *et al.* 1983). For each sample, 3 μg of DNA was digested for 3 hr by the appropriate restriction endonucleases (New England Biolabs, Beverly, MA). The DNA was ethanol precipitated, resuspended in dH_2O, separated by agarose gel electrophoresis, and transferred to positively charged nylon membrane (Hybond N; Amersham, Arlington Heights, IL). Hybridization was carried out using a nonradioactive *LamC* cDNA digoxygenin (DIG)-labeled probe (DIG High Prime DNA labeling and detection kit II; Roche, Indianapolis). Anti-DIG-AP conjugate antibody was used to detect hybridization, followed by CSPD chemiluminescence reaction and exposure to X-ray film. All procedures were carried out according to manufacturer's guidelines.

To generate adults homozygous for a given excision allele, chromosomes carrying excisions were balanced over a *CyO* chromosome possessing a *GFP* reporter gene and crossed into a background containing an X-linked wild-type *Lamin C* transgene. Daily heat shocks of 45 min in a 37° water bath were administered throughout development, and DNA was extracted from individual homozygous (straight wing) adults (Gloor *et al.* 1993). PCR was performed to determine the integrity of genomic exons, using primers that would distinguish them from the sequences within the *LamC* transgene present in the background. The regions encompassing the deletion breakpoints in the excision stocks $LamC^{ex187}$ and $LamC^{ex296}$ were amplified by *Pfu*-Ultra (Stratagene), cloned into pCR2.1-TOPO (Invitrogen), and sequenced (University of Iowa DNA Core Facility).

Western analysis: To determine the expression levels of LamC, proteins were extracted from third instar larvae or adults (Friedman *et al.* 1992) and separated by size on 10–12% polyacrylamide gels, transferred to nitrocellulose membrane, and incubated with anti-LamC LC28.26 anti-mouse IgG (Riemer *et al.* 1995) used at 1:5000–1:8000 dilution or anti-α-tubulin anti-mouse IgG1 [Sigma (St. Louis) no. T5168] used at 1:400,000 dilution. An HRP-conjugated anti-mouse IgG [Pierce (Rockford, IL) no. 31446] used at a 1:20,000 dilution served as a secondary antibody. Detection was carried out using the SuperSignal West Pico chemiluminescent substrate (Pierce no. 34080). Signal from the membranes was collected from an Epi Chemi II darkroom unit fitted with a CCD camera (UVP, San Gabriel, CA) and the resulting data were quantified using LabWorks Image Acquisition software (UVP) and/or Image J software (http://rsb.info.nih.gov/ij/). Three independent protein isolations were performed for each genotype. Means and standard deviations were calculated; the formula shown below was used to calculate the standard error of the variance between the expression level in a mutant relative to that in the normalized wild-type controls:

$$\mathrm{SE}(\bar{X}_\mathrm{m}/\bar{X}_\mathrm{w}) \approx \left(\frac{\bar{X}_\mathrm{m}}{\bar{X}_\mathrm{w}}\right)\sqrt{\frac{S_\mathrm{m}^2/n_\mathrm{m}}{\bar{X}_\mathrm{m}^2}+\frac{S_\mathrm{w}^2/n_\mathrm{w}}{\bar{X}_\mathrm{w}^2}}.$$

SE represents the standard error, $\bar{X}_\mathrm{m}$ represents the mean of the LamC:tubulin ratio in the mutant, S_m^2 represents the square of the variance of the LamC:tubulin ratio in the mutant, $\bar{X}_\mathrm{w}$ represents the mean of the LamC:tubulin ratio in the wild type, and S_w^2 is the square of the variance of the LamC:tubulin ratio in the wild type. n is the number of independent experiments performed.

Nuclear staining and quantitation: For nuclear morphology studies, third instar larvae were raised at room temperature in vials, administered a heat shock (45 min, 37°), and allowed to recover for ~2 hr. Salivary glands ranging in age from early second instar to late third instar larval were dissected in phosphate-buffered saline solution (PBS). Additional tissues released upon salivary gland dissection were also stained; these included epithelial tissues, imaginal discs, brain, and gut. The total dissection time for an experiment was not >1.5 hr. For salivary glands, three to five pairs per genotype were placed in welled slides for fixation in 2% paraformaldehyde for 15–20 min, followed by 3 × 5-min washes in PBS^{2+} (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 10 mM EGTA, 0.1% Triton-X). The glands were blocked in PBS^{2+} + 0.1% BSA for 60 min and then incubated with 1:500 dilution of primary antibody (LC28.26 for LamC and ADL84.12 or ADL67.10 for lamin Dm_0, University of Iowa Hybridoma Core Facility) in PBS^{2+} + 0.1% BSA. Incubation was carried out overnight at 4° in rotating 1.5-ml microfuge tubes. Following incubation with primary antibody, the salivary glands were washed 3 × 5 min in PBS^{2+}, followed by blocking in PBS^{2+} + 0.1% BSA for 60 min, and then incubated for 1 hr in the dark with a 1:1000 dilution of Rhodamine-conjugated goat anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA, no. 115-025-068). The glands were then washed for 3 × 10 min in PBS^{2+} and treated with DAPI at a concentration of 250 ng/μl for ~30 sec. The glands were washed for 3 × 10 min in PBS^{2+} and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). The slides were placed in a dark box and left at 4° overnight. The nuclei were examined using a Leica DMLB compound microscope with fluorescent capabilities, and the images were collected and processed with a Spot RT-Slider CCD camera (Diagnostic Instruments) and Spot Advanced software. To determine the percentage of nuclei showing a lamin localization defect, nuclei within the first third of a gland (where visibility and antibody adsorption are best, due to reduced thickness of the tissue) were counted. The percentage of nuclei showing abnormal localization was calculated for at least three independent preparations per genotype.

RESULTS

Mutations in *Lamin C* are lethal: To determine the function of A-type lamins in Drosophila, a genetic analysis of the Drosophila *LamC* gene was carried out. The *LamC* gene maps to cytological position 51B1 on the right arm of the second chromosome, spans ~5 kb of genomic territory, and is nested within the fifth intron of the essential gene *ttv*, which encodes a protein involved in heparin sulfate proteoglycan biosynthesis (Bellaiche *et al.* 1998). Three lethal *P*-element transposon insertions, *EP(2)2199*, *UM-8373*, and *G00158*, all located within the first intron of *LamC* (Figure 1A), are available through stock centers and academic collections [*EP(2)2199*, http://expbio.bio.u-szeged.hu/fly/; *UM-8373*, http://www.drosdel.org.uk/; *G00158*, http://flytrap. med.yale.edu/). Due to the nested arrangement, it is unclear whether the lethality results from

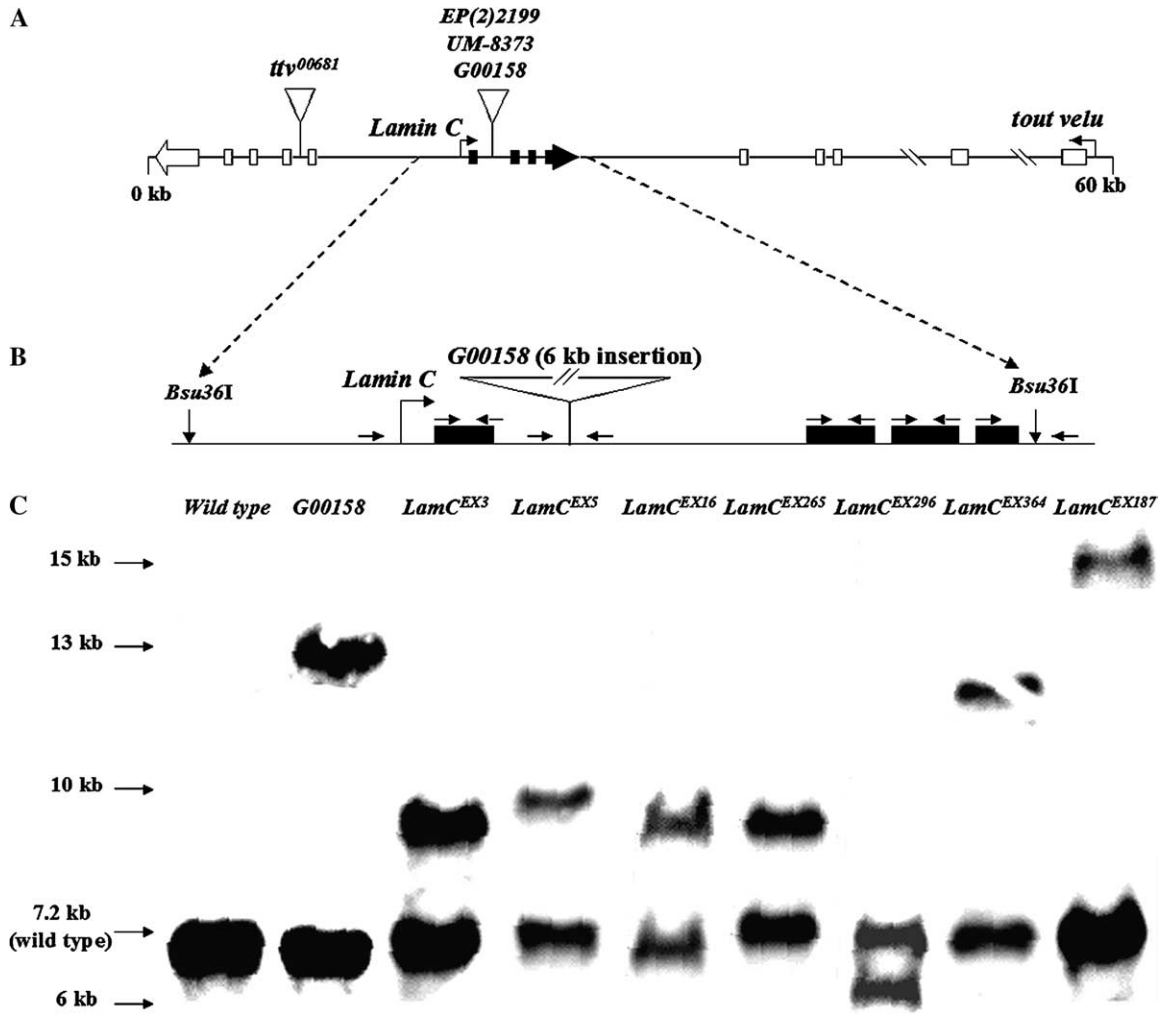

FIGURE 1.—(A) Diagram of the *LamC* and *ttv* genomic region. *LamC* (solid exons) is nested within the fifth intron of the essential gene *tout velu* (open exons). *EP(2)2199*, *UM-8373*, and *G00158* are lethal *P*-element insertions in *Lamin C*. ttv^{00681} is a lethal insertion into the sixth intron of *tout velu*. (B) Diagram of *LamC* with the location of the *P*-element insert in stock *G00158* indicated. *G00158* is mutant for *LamC* and *ttv*. The diagram shows the position of the primers (arrows) used for PCR and sequencing (Table 3). (C) Southern analysis of *LamC* alleles. *Bsu36*I cleavage sites flank the *LamC* genomic region and generate a 7.2-kb fragment containing the wild-type *LamC* gene. The membrane was hybridized with full-length *LamC* cDNA.

disruption of *LamC*, *ttv*, or both. To determine whether *LamC* and *ttv* encode genetically separable functions, we generated additional mutant *LamC* alleles and carried out complementation tests.

Initial complementation tests were performed with *Df(2R)trix* (a stock carrying a deletion of ~60 genes including *LamC* and *ttv*), the three *LamC P*-element insertions, and the well-characterized ttv^{00681} null allele (THE *et al.* 1999). Complementation between lesions in different, noninteracting genes results in *trans*-heterozygote adult viability and fertility. As anticipated, *Df(2R)trix* failed to complement all three *LamC P*-element insertion alleles and ttv^{00681} (Table 1). The *P*-element insertion stocks *UM-8373* and *G00158* also failed to complement *EP(2)2199* and ttv^{00681}, suggesting that *UM-8373* and *G00158* are doubly mutant for *LamC* and *ttv*. *EP(2)2199*, however, does complement ttv^{00681}, indicating that the *P*-element insert in this stock specifically disrupts *LamC* function.

To generate additional mutant alleles of *LamC*, imprecise excision (ADAMS and SEKELSKY 2002) was performed using the *P*-element insertion stock *G00158*. The *P* element in this stock contains coding sequences for GFP flanked by splice donor and acceptor sites ("exon trap") and a $white^{+}$ reporter gene (MORIN *et al.* 2001). Insertion of this *P* element within the intron of *LamC* generates a LamC fusion protein containing GFP sequences within the rod domain; the LamC-GFP fusion protein exhibits abnormal nuclear localization (MORIN *et al.* 2001). Flies from the *G00158* stock were crossed to flies expressing transposase. Mobilization of the *P* element was scored by the presence of a *white* eye phenotype and/or loss of GFP fluorescence. From a total of 236 independent *P*-element excision events, 24

TABLE 1

Complementation analysis of *Lamin C* alleles

	Df(2R)trix	ttv^{00681}	*EP(2)2199*	*UM8373*	*G00158*	$LamC^{EX3}$	$LamC^{EX5}$	$LamC^{EX16}$	$LamC^{EX187}$	$LamC^{EX265}$	$LamC^{EX296}$	$LamC^{EX364}$
Df(2R)trix		−	−	−	−	−	−	−	−	−	−	−
ttv^{00681}			+	−	−	+	+	+	+	+	+	+
EP(2)2199				−	−	−	−	−	−	−	−	−
UM8373					−	−	−	−	−	−	−	−
G00158						−	−	−	−	−	−	−
$LamC^{EX3}$							−	−	−	−	−	−
$LamC^{EX5}$								−	−	−	−	−
$LamC^{EX16}$									−	−	−	−
$LamC^{EX187}$										−	−	−
$LamC^{EX265}$											−	−
$LamC^{EX296}$												−
$LamC^{EX364}$												

All alleles and the *Df(2R)trix* are balanced over the *CyO* chromosome.

generated a genetic lesion that was homozygous lethal. Of these 24, a complementation test showed that 10 were lethal for *LamC* and not for *ttv*. These lethal excision alleles failed to complement *Df(2R)trix* and the *LamC P*-element insertions; however, they all complemented ttv^{00681} (Table 1). Therefore, 10 novel mutant *LamC* alleles were generated.

Wild-type *Lamin C* transgenes rescue lethality of *LamC* mutants: To confirm that *LamC* and *ttv* encode unrelated essential functions, we performed experiments to rescue the lethality of mutant *LamC* alleles by supplying a source of wild-type LamC protein. To this end, we generated transgenic stocks expressing wild-type *LamC* under control of either a heat-shock *hsp70* promoter or a GAL4/UAS-driven promoter (Duffy 2002). Rescue data for heat-shock-induced expression of wild-type *LamC* are shown in Table 2. Viable, fertile adults were obtained for the *trans*-heterozygous combination of *LamC P*-element alleles *UM-8373* and *EP(2)2199* under heat-shock conditions (applied 45 min daily, with recovery at room temperature). Rescue of this combination of mutant alleles required heat-shock treatment; in addition, no rescue was observed when *trans*-heterozygous combinations included alleles that were lethal for *ttv* (*UM-8373* and ttv^{00681}). Only partial rescue was observed (27% of the expected class), possibly due to inappropriate levels of LamC produced by the daily heat-shock treatment. Rescue using GAL4/UAS-driven *LamC* gave similar results (data not shown). Thus, the lethality associated with mutations in *LamC* can be rescued by transgenic wild-type *LamC* expression, confirming that *LamC* is an essential gene.

Molecular characterization of *LamC* lethal excision alleles: The molecular structure of the *LamC P*-element excision alleles was first determined by performing Southern analysis. Genomic DNA from flies heterozygous for a *LamC* excision allele and a *CyO* balancer chromosome (wild type for *LamC*) was digested with the restriction enzyme *Bsu36*I, which cleaves immediately upstream and downstream of the *LamC* coding region, but not within the *P* element present in stock *G00158* (Figure 1B). A 7.2-kb fragment representing the wild-type *LamC* genomic region from the *CyO* balancer chromosome is present in all heterozygotes (Figure 1C).

TABLE 2

Rescue of *Lamin C* lethality: *Hsp70-Lamin C/Y; m^1/CyO × w/w; m^2/CyO*

	No heat shock		Heat shock (37°/45 min)	
F_1 genotype (m^1/m^2)	Proportion of expected class	Total progeny scored	Proportion of expected class	Total progeny scored
UM-8373[a]*/EP(2)2199*	0	171	25/94[b] (27% rescue)	399
UM-8373[a]$/ttv^{00681}$	0	288	0	369

[a] *UM-8373* is doubly mutant for *Lamin C* and *tout velu*.

[b] The expected rescued class (*trans*-heterozgous females for two different mutant *Lamin C* alleles) represents one-sixth of the total progeny. Note that no *trans*-heterozygous males could be rescued (as the transgene was X-linked), which would represent another one-sixth of the total progeny. Balanced progeny represent two-thirds of the total, and this proportion was used to estimate the theoretical total number of progeny scored assuming complete viability for both sexes. Therefore, the expected number of rescued progeny is 94 (564/6). As anticipated, 0/94 males survived and 25/94 females survived, reflecting a 27% rescue.

TABLE 3

Molecular structure of *Lamin C* excision alleles

Mutant name	Coding region	No. of insertions	Insertion size (kb)	*GFP*[a]	*white* [+/−b]
G00158 (parent stock)	Intact	1	6	+	+
$LamC^{EX3}$	Intact	1	1.5	—	—
$LamC^{EX5}$	Intact	1	1.8	—	—
$LamC^{EX16}$	Intact	1	1.5	—	—
$LamC^{EX187}$	357-bp deletion in first exon	2	ND	+	—
$LamC^{EX265}$	Intact	1	1.4	—	—
$LamC^{EX296}$	560-bp deletion in first exon	0	NA	—	—
$LamC^{EX364}$	Intact	1	ND	+	—

ND, not done; size exceeded standard PCR. NA, not applicable.
[a] Determined by PCR.
[b] Determined by eye color phenotype indicating presence of white reporter (+, red; −, white).

A second fragment, varying in size among the stocks, represents the *LamC* genomic region of the mutant chromosome. Six of the seven alleles analyzed exhibited a larger-sized fragment than that produced from the wild-type endogenous *LamC* gene, indicating these excision stocks have retained a portion of the original *P*-element insertion. $LamC^{EX296}$ appears to have lost a considerable portion of the *LamC* genomic region, making it the best candidate for a *LamC* deletion mutant.

To more precisely determine the nature of the genetic lesions resulting from imprecise *P*-element excision, a high-resolution molecular characterization of the *LamC* excision stocks was performed using PCR and sequence analysis (Table 3). To obtain genomic DNA from adults homozygous for each excision allele, a wild-type *LamC* heat-shock-inducible transgene was used to rescue individuals homozygous for a particular excision allele. Seven novel *LamC* excision alleles were rescued by the wild-type *LamC* transgene, indicating that no second site lethal mutations exist in these stocks. Therefore, the only lethal mutations on these excision chromosomes are in *LamC*. PCR analysis revealed that six of the seven excision alleles retained a partial *P*-element insertion, while five of seven possessed intact *LamC* coding regions. Sequence analysis confirmed that two alleles, $LamC^{EX296}$ and $LamC^{EX187}$, possess deletions within the first exon of *LamC*, making them the best candidates for protein nulls.

Mutations in *LamC* show reductions in LamC protein levels: To determine the LamC protein levels in the various mutant stocks, Western analysis was performed using antibodies that specifically recognize LamC (Riemer *et al.* 1995). Initially, larvae heterozygous for the *LamC* alleles used in rescue were assayed for LamC protein expression levels. All three *LamC P*-element insertion stocks, the *LamC* excision allele, and the deficiency, showed at least a 50% reduction in LamC levels compared to the control stock. In contrast, ttv^{00681} exhibited wild-type levels of expression for LamC (Figure 2). Thus, at least one endogenous wild-type copy of *LamC* is required for viability in Drosophila.

Western analysis was performed on selected excision stocks to determine which mutants were likely to be protein nulls for LamC. Stocks containing the excision alleles $LamC^{EX296}$, $LamC^{EX265}$, and $LamC^{EX5}$ and the deletion *Df(2R)trix* were placed over the *CyO* chromosome possessing a *GFP* reporter gene and mated to one another. Individuals homozygous for an excision allele ($LamC^{EX}/LamC^{EX}$) or hemizygous for an excision allele ($LamC^{EX}/Df$) were selected by loss of GFP fluorescence; heterozygotes ($LamC^{EX}/CyO\text{-}GFP$) were selected on the basis of the presence of GFP fluorescence. LamC protein was not detectable in the $LamC^{EX296}$, $LamC^{EX265}$, and $LamC^{EX5}$ homozygotes and hemizygotes in this assay (Figure 3), suggesting they are all protein nulls.

***LamC* lethal phase is prepupal:** The identification of the lethal phase for *LamC* mutants would determine when LamC function is essential for development. *LamC* is not maternally supplied (Riemer *et al.* 1995); therefore, null alleles were used in this assay. $LamC^{EX296}$ is the best candidate for a protein null given that it possesses a deletion in the first exon (Table 3) and produces no detectable LamC protein on the basis of Western analysis (Figure 3). Therefore, the lethal phase was determined for individuals *trans*-heterozygous for $LamC^{EX296}$ and *Df(2R)trix*. These mutants were placed over the *CyO-GFP* chromosome and mated to each other. The number of resulting progeny lacking GFP fluorescence [$LamC^{EX296}/Df(2R)trix$] was scored for developmental stage for 14 days (for control genotypes, see materials and methods). The $LamC^{EX296}/Df(2R)trix$ individuals die predominantly as late third instar larvae or white prepupae. The lethal phase analysis also showed that development was delayed by ~2 days for all stages in the hemizygote (data not shown). Therefore, loss of LamC produces a developmental delay and ultimately causes death at the prepupal stage, a time in development in which significant apoptosis and tissue differentiation occur (Ashburner 1989).

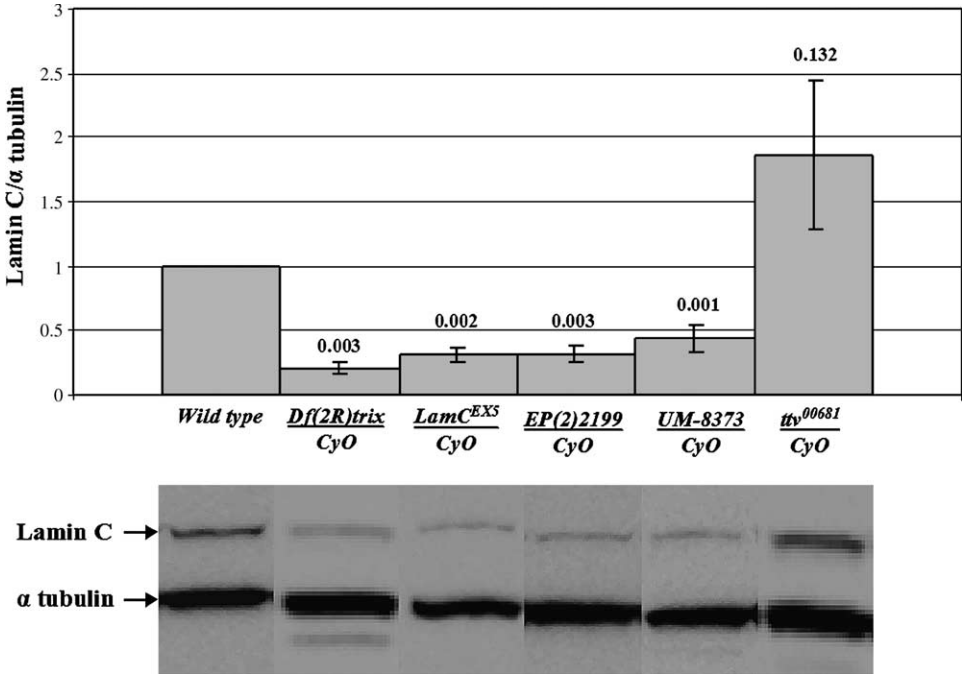

FIGURE 2.—Western analysis of protein from *LamC* mutants used in rescue experiments. Western analysis of heterozygous adults carrying one mutant allele for *LamC* over the *CyO* balancer chromosome is shown. Protein extracts from a wild-type stock (y^1w^{67c23}) and heterozygous tty^{00681} adults are also shown. LamC was detected with the antibody LC28.26 (RIEMER *et al.* 1995). Quantitation and *P*-values (Student's *t*-test) for LamC levels from three independent samples per genotype are indicated on the histogram, and representative Western blots are depicted below. Values are normalized using the levels of α-tubulin and expressed as a ratio to the levels of LamC in the wild type, set at 1.0 (see MATERIALS AND METHODS).

Expression of mutant *LamC* transgenes causes lethality and nuclear defects: In mammals, expression of specific A-type lamin mutants causes nuclear phenotypes that include lamin aggregations and nuclear envelope blebbing (OSTLUND *et al.* 2001). To examine functional similarities between human and Drosophila lamins, we generated transgenic animals expressing mutations in *LamC* that correspond to disease-causing mutant forms of A-type lamin in humans. A Drosophila *LamC* heat-shock-inducible transgene encoding an R401K amino acid substitution within the rod domain was generated (Figure 4). This substitution is homologous to the R386K missense mutation in human lamin A/C that leads to EDMD (BONNE *et al.* 2000). In addition, structural studies suggest that this residue plays a role in higher-order lamin assembly (STRELKOV *et al.* 2004).

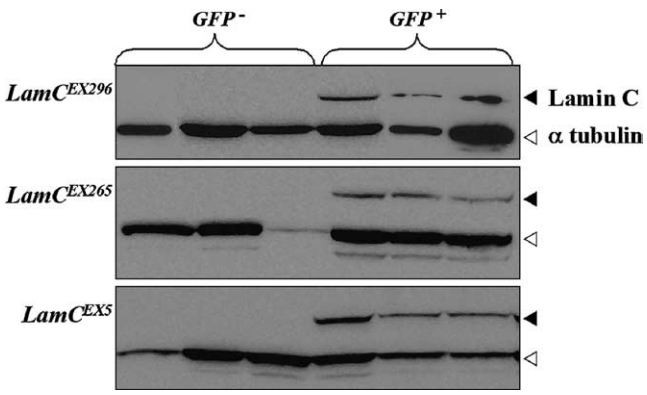

FIGURE 3.—Three *LamC* excision alleles appear to be protein nulls. Larval protein extracts were isolated from *LamC* excision alleles or the *Df(2R)trix* over the *CyO-GFP* chromosome. GFP⁻ samples represent $LamC^{EX}/LamC^{EX}$ or $LamC^{EX}/Df(2R)trix$); GFP⁺ samples represent $LamC^{EX}/CyO\text{-}GFP$. Experimental triplicates are shown.

To examine the nuclear localization of the R401K mutant in Drosophila, a mixture of second instar larval tissues that included salivary glands, epithial tissue, imaginal discs, and gut was stained with antibodies against LamC. In all tissue types examined LamC aggregates appeared (Figure 5A and data not shown). We subsequently focused our cytological studies on third instar larval salivary glands due to their large size and ease of manipulation. Prominent nuclear rim staining was observed with LamC antibodies in control stocks (nontransgenic and transgenic stocks expressing wild-type LamC) with or without heat shock (Figure 5B). In contrast, daily heat-shock-induced expression of the R401K mutant resulted in a reduction in LamC at the nuclear periphery and a distinct LamC aggregation phenotype resembling O-rings (Figure 5B). The O-ring structures occurred in 50–100% of the nuclei examined, depending on the transgenic stock (see MATERIALS AND METHODS). Interestingly, flies exhibiting this LamC localization defect are viable and have no obvious defects. Western analysis showed that the R401K protein was expressed at levels similar to that of the wild-type LamC transgenic control, indicating that the nuclear phenotype is a consequence of the specific mutation, and not merely resulting from high levels of transgene expression (Figure 6). A similar O-ring phenotype, but with reduced penetrance (~40–50% of the nuclei scored), is evident in the *G00158* heterozygote containing a *GFP* insertion within the rod domain (Figure 5B). Thus, an insertion or an amino acid substitution within the rod domain gives rise to lamin aggregation defects in Drosophila that are similar to those reported for rod

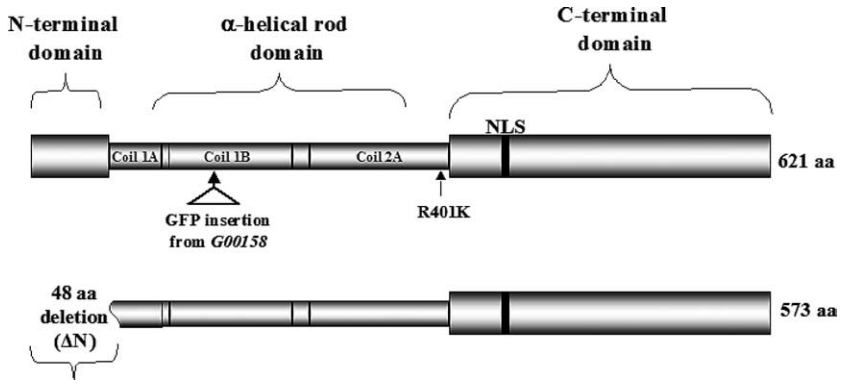

FIGURE 4.—Diagram of the mutant forms of LamC with principal domains labeled. NLS represents a putative nuclear localization signal. The top of the diagram shows the location of the *P*-element-encoded *GFP* insertion (present in stock *G00158*) and of the R401K amino acid substitution. The bottom of the diagram shows the extent of the N-terminal deletion; the first 48 amino acids are deleted, which removes the entire head domain and 8 amino acids from the first part of the rod domain.

domain mutations in human A-type lamins (OSTLUND *et al.* 2001).

Mutations within the N-terminal globular domain of human lamin A/C have been associated with EDMD, familial dilated cardiomyopathy, and Charcot-Marie-Tooth type 2 disease (WALTER *et al.* 2005). We generated an N-terminal deletion of the Drosophila LamC protein that removes the first 48 amino acids, including 8 amino acids of the rod domain (Figure 4). This mutant was modeled after an N-terminally deleted version of human lamin A/C that has been functionally tested (SPANN *et al.* 1997). Daily heat-shock-induced expression of the N-terminal LamC truncation was lethal; individuals died at the prepupal stage. A similarly lethal phenotype results when the same N-terminally deleted protein was overexpressed by ubiquitous GAL4 induction (data not shown). Prior to death, at the third instar larval stage, O-ring LamC aggregates were apparent in ~50–70% of the salivary gland nuclei examined (Figure 5B). Western analysis showed that the N-terminal truncated protein is expressed at levels similar to that of the wild-type transgenic LamC and R401K mutant (Figure 6), suggesting that the phenotype is due to the specific mutant and not simply due to overexpression of lamin protein. Thus, expression of an A-type lamin lacking the N-terminal head domain is toxic to Drosophila.

A- and B-type lamins interact during *in vitro* assembly (GEORGATOS *et al.* 1988). Therefore, we tested whether expression of mutant A-type lamin in a wild-type *LamC*

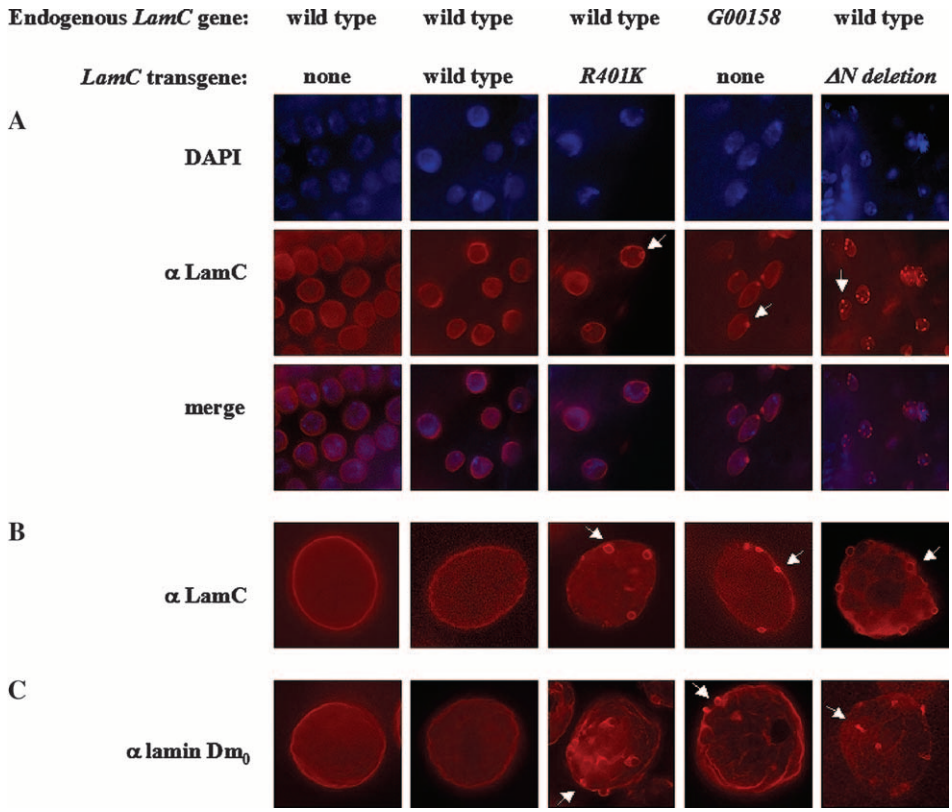

FIGURE 5.—Nuclear defects associated with expression of mutant forms of LamC. (A) Tissues (salivary gland and epithelial) from second instar larvae stained with antibodies to LamC (red). Nuclei are indicated by DAPI staining (blue). (B) Salivary gland nuclei from third instar larvae stained with antibodies to LamC (red). (C) Salivary gland nuclei from third instar larvae stained with antibodies to lamin Dm_0 (red). Arrows indicate representative examples of nuclear defects (LamC O-ring aggregates and lam Dm_0 protrusions). Images in A were photographed using a 10× ocular and a 100× oil objective on a Leica DMLB microscope equipped with a Spot RT-Slider CCD camera (Diagnostic Instruments). B and C were photographed the same way, except using a 40× oil objective rather than the 100× objective.

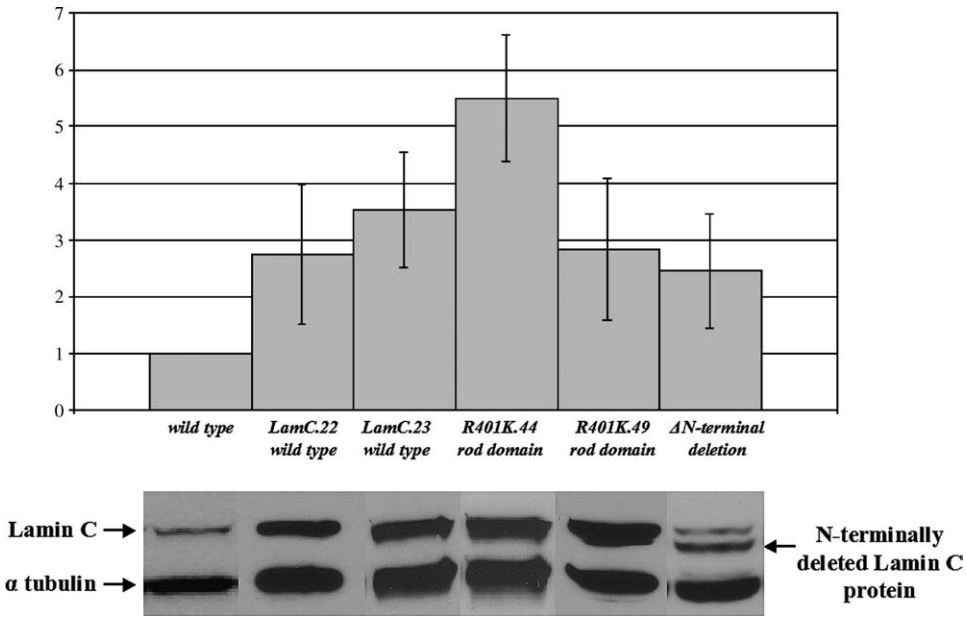

FIGURE 6.—Western analysis of LamC levels in transgenic stocks used in rescue experiments and nuclear morphology assays. Protein extracts from a wild-type stock (y^1w^{67c23}) were used for normalization. Quantitation was carried out as described in Figure 3 and MATERIALS AND METHODS.

genetic background disrupts B-type lamin organization *in vivo*. Third instar larval salivary gland nuclei from individuals expressing the R401K and N-terminal truncated form of LamC were stained with antibodies specific for lamin Dm_0, the Drosophila B-type lamin (RIEMER *et al.* 1995). In nontransgenic and transgenic larvae expressing wild-type LamC, nuclear envelope staining characteristic of wild-type lamin Dm_0 was apparent (Figure 5C). In transgenic larvae expressing mutant forms of LamC, lamin Dm_0 exhibited aggregate structures that appear to bleb from the nuclear lamina (Figure 5C). A similar lamin Dm_0 aggregation defect can be observed in the *G00158* stock (Figure 5C). Thus, disruption of LamC organization affects other components of the nuclear lamina.

DISCUSSION

To our knowledge, Drosophila is the only well-characterized invertebrate model organism that appears to have both A- and B-type lamins. *lamin* Dm_0 encodes the B-type lamin and has been the subject of several genetic and molecular studies (RIEMER *et al.* 1995; LENZ-BOHME *et al.* 1997; GUILLEMIN *et al.* 2001; PATTERSON *et al.* 2004). *LamC* encodes the A-type lamin (RIEMER *et al.* 1995; STUURMAN *et al.* 1999) and is nested within an intron of the essential gene *ttv* (BELLAICHE *et al.* 1998; THE *et al.* 1999), which complicates the genetic analysis of both genes. In this report, we provide data that functionally separate *LamC* and *ttv*. First, a previously identified lethal *P*-element insert in *LamC*, *EP(2)2199*, complements ttv^{00681}, a confirmed null mutant allele of *ttv*. Second, a wild-type *LamC* transgene rescues *trans*-heterozygous combinations of *LamC* mutants, but not combinations of mutant alleles of *ttv*. Third, the lethal phases of *LamC* and *ttv* are distinct. Lethality due to loss of *LamC* occurs at the prepupal stage, whereas lethality due to the loss of *ttv* can occur during early embryogenesis, when the maternal contribution is removed (THE *et al.* 1999).

Prepupal lethality of *LamC* is consistent with the timing of expression for this gene, as transcript levels peak during larval development (RIEMER *et al.* 1995). This lethal phase is also consistent with a role for *LamC* in apoptosis and tissue differentiation. During the prepupal stage, larval tissues begin to histolyze via apoptotic pathways (JIANG *et al.* 1997) while precursors of adult structures (the imaginal discs) proliferate and initiate differentiation programs (ASHBURNER 1989; SEMPERE *et al.* 2002). Lamins are targets for caspases (LAZEBNIK *et al.* 1995), suggesting a role in apoptosis. In addition, A-type lamins may play important roles in differentiating tissues, accounting for the tissue specificity of laminopathic diseases in humans (BURKE and STEWART 2002). Thus *LamC* is an essential gene that may play a conserved role in apoptosis and tissue differentiation.

Having determined that the A-type lamin in Drosophila encodes an essential function, we undertook a transgenic approach in Drosophila to study the effects of mutant A-type lamins *in vivo*. Mutations in human A-type lamins lead to inherited diseases, and the effects of mutant lamins on nuclear architecture and envelope integrity in mammalian cells have been investigated. In patient tissues, nuclear aberrations correlate with disease, but are also found at low frequency in control cells (VIGOUROUX *et al.* 2001). In mammalian cell culture, overexpression assays of mutant human A-type lamins produce conflicting results. Some experiments show nuclear aggregation and abnormal nuclear shapes,

while other studies using the same mutant lamin show no nuclear phenotypes (Broers *et al.* 1999; Ostlund *et al.* 2001; Raharjo *et al.* 2001; Holt *et al.* 2003). The discrepancies are possibly due to the cell type used, expression levels of the mutant lamin, and/or tagging of the expressed protein.

For our transgenic analysis, we selected a missense mutation in the rod domain of the Drosophila LamC protein, since mutations throughout this domain in the human protein account for almost 40% of laminopathies (Strelkov *et al.* 2004). Specifically, we chose to mutate the residue R401K, which in humans corresponds to R386K that causes EDMD. This residue is conserved in both vertebrates and invertebrates and belongs to a subdomain within the rod that has been studied by crystallography (Strelkov *et al.* 2004). We also designed an N-terminal deletion mutant since this domain is essential for the head-to-tail assembly of lamin dimers into protofilaments (Stuurman *et al.* 1996; Sasse *et al.* 1998) and has recently been implicated in a neurogenic variant of EDMD (Walter *et al.* 2005).

Heat-shock-induced expression of Drosophila LamC proteins with mutations that disrupt the rod domain and/or the N-terminal head domain results in nuclear defects including (1) reduced intensity of LamC antibody staining at the nuclear periphery, (2) aggregation of LamC into O-ring structures, and (3) aggregation of lamin Dm_0 (Figure 5). These defects are specific for the expression of mutant protein, as they are not evident when a wild-type LamC is expressed at similar levels (Figure 6). The O-ring aggregates are not specific for salivary gland nuclei or nuclei from tissues undergoing histolysis prior to pupation as they were observed in epithelial tissue, imaginal discs, brain, and gut from second and third instar larvae (Figure 5 and data not shown). The LamC O-rings bear a striking resemblance to lamin aggregations observed in mammalian cell culture upon overexpressing human lamin rod domain mutants (Ostlund *et al.* 2001; Raharjo *et al.* 2001; Holt *et al.* 2003). Lamin aggregration phenotypes, including O-ring structures, are thought to reflect defects in higher-order lamin assembly, which requires an intact rod and head domain (Stuurman *et al.* 1996; Sasse *et al.* 1998). A recent report describing the X-ray crystallographic structure of a portion of the human A-type lamin rod domain has provided evidence that higher-order assembly involves electrostatic interaction between charged residues in the N- and C-terminal portions of the rod domain (Strelkov *et al.* 2004). Interestingly, these interactions include arginine 386 (human lamin A/C), a residue that is mutated in patients with EDMD (Bonne *et al.* 2000) and is homologous to the R401K substitution in Drosophila LamC analyzed in our study.

In Drosophila, expression of the R401K LamC mutant produces nuclear lamin aggregates, but no overt phenotype in the adult. Expression of the N-terminal truncation produces a similar lamin aggregation defect; however, it also causes prepupal lethality. This lethal phase is similar to that of the *LamC* null mutants. The N-terminal truncation end point was selected on the basis of studies in mammalian systems (Spann *et al.* 1997); however, it removes eight amino acids of the rod domain. Therefore, the nuclear aggregation phenotype may be due to defects in the rod domain, while the lethality might result from the loss of the globular head domain.

Overexpression of our N-terminal LamC truncation, but not our wild-type LamC or the 401K mutant, caused lethality in Drosophila. Previously, a larval (48- to 120-hr) stage-specific lethal phenotype was reported for overexpression of a wild-type *LamC* transgene (Stuurman *et al.* 1999). One possible explanation for this discrepancy is that we did not achieve levels of expression high enough with our wild-type transgenes to cause lethality. Our transgenic lines express wild-type LamC approximately twofold over endogenous LamC levels (Figure 6), which is sufficient for rescue of lethality (Table 2). Another explanation is that the *LamC* transgene employed in the Stuurman study contained a single-base deletion in the 3′ end (nucleotide position G1781), causing a frameshift that adds 59 unrelated amino acids to the C terminus and shortens the overall length of the protein by 9 amino acids. Although this altered protein appears to localize normally (Stuurman *et al.* 1999), it does not possess assembly properties similar to that of wild-type LamC (Klapper *et al.* 1997 and associated erratum), which might contribute to the reported stage-specific lethality (Stuurman *et al.* 1999).

In summary, we have demonstrated that the Drosophila A-type lamin gene, *LamC*, encodes an essential function that is required during the prepupal stages of development. An essential developmental role for A-type lamins in differentiating tissues is thought to contribute to the tissue restriction of disease phenotypes manifested in human laminopathies. Expression of mutant Drosophila LamC protein causes nuclear phenotypes similar to those observed in human cell culture and tissue biopsies, in addition to demonstrating an essential function for the N-terminal head domain. Our results strongly suggest that the biological functions of A-type lamins are conserved between humans and Drosophila, thereby establishing Drosophila as a model to dissect the role A-type lamins play in development, nuclear architecture, and gene expression with relevance to human disease etiology.

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