A Genetic and Molecular Characterization of Two Proximal Heterochromatic Genes on Chromosome 3 of Drosophila melanogaster

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ABSTRACT

Heterochromatin comprises a transcriptionally repressive chromosome compartment in the eukaryotic nucleus; this is exemplified by the silencing effect it has on euchromatic genes that have been relocated nearby, a phenomenon known as position-effect variegation (PEV), first demonstrated in Drosophila melanogaster. However, the expression of essential heterochromatic genes within these apparently repressive regions of the genome presents a paradox, an understanding of which could provide key insights into the effects of chromatin structure on gene expression. To date, very few of these resident heterochromatic genes have been characterized to any extent, and their expression and regulation remain poorly understood. Here we report the cloning and characterization of two proximal heterochromatic genes in D. melanogaster, located deep within the centric heterochromatin of the left arm of chromosome 3. One of these genes, RpL15, is uncharacteristically small, is highly expressed, and encodes an essential ribosomal protein. Its expression appears to be compromised in a genetic background deficient for heterochromatin protein 1 (HP1), a protein associated with gene silencing in these regions. The second gene in this study, Dhp80, is very large and also appears to show a transcriptional dependence upon HP1; however, it does not correspond to any known lethal complementation group and is likely to be a nonessential gene.

HETEROCHROMATIN was originally defined cytologically as those regions in eukaryotic chromosomes that appear compacted throughout the cell cycle (Heitz 1928) and has since been shown to be virtually ubiquitous in animal and plant genomes. In the fruit fly Drosophila melanogaster, it comprises ~30% of the genome, is rich in middle and highly repetitive sequences, and is gene poor. Repetitive DNA poses formidable technical difficulties for both molecular analysis and sequence assembly; therefore, it is not surprising that heterochromatin remains incompletely defined in most of the model genomes that have been sequenced to date (Mardis et al. 2002).

Heterochromatin can silence gene expression, as exemplified by the phenomenon of position-effect variegation (PEV) in Drosophila, in which the expression of a euchromatic gene is compromised when it is relocated near or within a block of heterochromatin (reviewed by Grewal and Elgin 2002). Genetic screens designed to isolate modifiers of this effect have identified genes that encode proteins involved in maintaining chromatin structure (Sinclair et al. 1983; Wustmann et al. 1989; Schotta et al. 2003). One well-characterized example, Suppressor of variegation 2-5 (Su(var)2-5), encodes heterochromatin protein 1 (HP1). This protein has been shown to localize primarily to the heterochromatin of polytene chromosomes (Fanti et al. 2003 and references therein), bind repetitive transgene arrays (Dorer and Henikoff 1994), and silence gene expression (reviewed by Eissenberg and Elgin 2000). The phenomenon of PEV suggests that heterochromatin forms a transcriptionally repressive environment within which the presence of active, resident heterochromatic genes poses something of a paradox. Moreover, these genes exhibit “reciprocal” heterochromatic PEV; i.e., a heterochromatic gene will variegate when translocated into a euchromatic environment (Eberl et al. 1993; Howe et al. 1995) and this effect is enhanced in a Su(var) mutant background. In fact, mutations in Su(var) genes appear to compromise heterochromatic gene expression in the absence of any genetic rearrangements, at least for the well-characterized heterochromatic genes light and rolled (Clegg et al. 1998; Lu et al. 2000; Sinclair et al. 2000). This suggests that heterochromatic genes have evolved a transcriptional dependence on factors that normally silence gene expression, underscoring the paradoxical nature of gene regulation in this region.

A combination of genetic, cytological, and molecular studies has been undertaken to better understand how heterochromatic genes function. Work on the organization and density of genes in Drosophila autosomal heterochromatin has identified lethal complementation groups through classical genetic screens, using chromosomal deficiencies in pericentromeric heterochromatin.
for chromosome 2 (Hilliker and Holm 1975; Hilliker 1976; Coulthard et al. 2003) and chromosome 3 (Marchant and Holm 1988a,b; Schulze et al. 2001). Complementary mutagenesis with P elements has provided "tagged" genes as well as single-copy entry points for molecular characterization of heterochromatic DNA (Zhang and Spradling 1994; Schulze et al. 2001; Konev et al. 2003). In addition, studies using in situ hybridization of mitotic chromosomes have allowed preliminary correlations between the cytological and genetic maps (e.g., Koryakov et al. 2002).

In terms of molecular characterization, heterochromatic genes tend to be relatively large, embedded within repetitive environments (Devlin et al. 1990a,b; Ringer et al. 1997; Warren et al. 2000; Tulin et al. 2002), and fall into a wide range of biological functions (Parks and Wieschaus 1991; Biggs et al. 1994; Warner et al. 1998; Rollins et al. 1999); however, relatively little is known about their expression and regulation. Release 3 of the Drosophila genome sequence has provided a large number of pieces of heterochromatic DNA sequence and allowed the prediction of ~300 genes within Drosophila heterochromatin (Hoskins et al. 2002). Complementary cytological mapping by these authors and others (e.g., Dimitri et al. 2003a,b) has also roughly defined the positions of many of these predicted genes.

An intriguing issue has been the apparent discrepancy between the small number of autosomal "vital loci," as defined genetically, and the much larger number of predicted gene models from the Release 3 sequence annotation (Hoskins et al. 2002). A recent report suggests that there may be several more proximal, essential loci than previously reported in 2R heterochromatin (Myster et al. 2004), but confirmation of this surprising new estimate may require more extensive genetic analyses to rule out the complex intragenic complementation patterns possible in these regions (Coulthard et al. 2003).

We are working toward a better understanding of the organization, expression, and regulation of genes in centric heterochromatin, with particular emphasis on chromosome 3. One focus of our efforts has been a molecular and genetic analysis of the lethal complementation group l(3)80Fi (referred to hereafter as lethal 2), the second most proximal genetic locus in 3L heterochromatin (see Figure 1 for a map of the region). On the basis of the complex phenotypes displayed by lethal 2, we initially focused on two candidate DNA sequences that might correspond to this essential gene: Dhp80, which encodes a DEAD box RNA helicase, and RpL15, which encodes a large subunit ribosomal protein.

We report here that lethal 2 corresponds to RpL15; this gene is uncharacteristically small for a heterochromatic gene, is highly expressed, and possesses regulatory features characteristic of or unique to other ribosomal protein genes. RpL15 is embedded in a repetitive environment and is located ~10 kb upstream of the other candidate gene Dhp80. The properties of this latter gene are more consistent with what has been reported for heterochromatic gene structure: it is very large, apparently due to the expansion of repetitive DNA in its many introns (Dimitri et al. 2003a,b), is moderately expressed, and appears to possess a conventional promoter structure. However, it does not correspond to any known lethal complementation group and thus may not encode an essential function. The expression of both genes is compromised in a genetic background in which the HP1 (Su(var)2-5) dose has been reduced, which is consistent with the results obtained for the well-studied heterochromatic genes light and rolled (Lu et al. 2000). Thus, heterochromatic genes may have evolved a dependence on factors that maintain heterochromatin structure, and this dependence appears to be irrespective of gene function or promoter type.

The results reported in this study provide information on two more heterochromatic genes, located deep within proximal centric heterochromatin. This work could lead to insights into the mechanisms by which genes maintain activity in an otherwise transcriptionally repressive environment and shows the utility of genetics in supporting annotation of molecularly intractable genomic regions. As more genetically mutable loci in heterochromatin are linked with annotated gene models, we will also be in a better position to address questions concerning the ratio of essential to nonessential genes in this region and the role that chromatin structure plays in regulating gene expression.

**MATERIALS AND METHODS**

**Culture conditions:** Flies were grown on standard cornmeal-sucrose medium with either tegosept or proprionic acid as a
mold inhibitor. Stocks were routinely maintained and crosses performed at room temperature unless otherwise indicated.

**Drosophila stocks and strains:** Descriptions of most mutations, special chromosomes, and deficiencies used in this work can be found at the FlyBase website (http://flybase.bio.indiana.edu:82/). Stocks, strains, and screens used to identify third chromosome heterochromatin genes are described in Marchant and Holm (1988a,b), Schulze et al. (2001), and Vilen- ski et al. (2002). Thirteen essential 3L heterochromatin genes for which we have mutant alleles are designated as lethal 1 [a simplification of l(3L)h1, from Marchant and Holm 1988b, or l(3)80F] used in FlyBase] to lethal 8, from proximal to distal relative to the centromere (see Figure 1). The three deficiencies used to map lethal 2 molecularly were Df(3L)FJX3, which removes lethal 3 to lethal 8, Df(3L)I56, which removes lethal 1 and lethal 2; and Df(3L)K2, which removes lethal 2 and lethal 3. The Su(var)2-5 alleles used in this study have all been described previously (Li et al. 2000 and references therein).

**PCR mapping:** Deficiency stocks were balanced over a TM3 chromosome bearing a GFP transgene. Genomic DNA from individual embryos from the GFP balanced stock was obtained using the method of Hatton and O’Hare (1999) and PCR was used to identify embryos homozygous for the deficiency (no GFP PCR product). PCR was then used to test for the presence or absence of RpL15 or Dhp80 coding sequences. In both cases, the unrelated X-linked gene Grip 84 was used as a DNA control. All PCR reactions were carried out in a reaction volume of 25 μl, using 2 μl of genomic template and 1 μl of 10 μM stock for each of four primers: Grip 84 (5’-ACGCTTCT CGCTGTATGAC-3’ and 5’-TGGCAGATTAAGGTAAGTACTAGTAGT-3’), GFP (5’-CAAGTGTGGATCGCCGAGG-3’ and 5’-GAGCG GCACATGCGAGC3’), Dhp80 exon 9 (5’-GAACGCT TCCTGAGGTGTCGA-3’ and 5’-ATATATATATGATAGTAGAAC CTCC-3’), or RpL15 (5’-ACGCTTCTCGCTGTATGAC-3’ and 5’-GTACCGATGAAAGCAAC-3’). In all cases, a probe for rp49 flies was collected, dechorionated, dehydrated briefly, and injected using a Leitz laborflux microscope and Eppendorf model 5242 injection controller. Survivors were crossed back to flies from the same white minus stock that was injected, and white° progeny were crossed to the double-balanced T(2;3) apb/CyO; TM3 stock for segregation analysis and homozygosity.

**RESULTS**

**lethal 2 mutants show complex complementation, and****heteroallelic combinations are Minute:** Lethal 2 is defined by six alleles: two EMS mutants, 72 and 1-166-37, behave genetically as nulls and display an L1 lethal phase, while four P alleles [natural P elements from the Birmingham strain (Robertson et al. 1988)] are denoted PΔ8, PΔ2, PΔ8, 7-1, and 8-1 and behave as hypomorphs, which typically die at later larval stages. PΔ8 and PΔ2 appear to be the weakest alleles of all six, as indicated by the occasional eclosion of sterile homozygotes in the respective stocks. All pairwise combinations display a complex complementation pattern (Schulze et al. 2001), and several hypomorphic combinations survive to adulthood, displaying a classical Minute syndrome (Figure 2), including delayed development, fine bristles, rough eyes, reduced or gapped sex combs, misrotated genitalia, and sterility. A weak Minute phenotype is also observed when a deficiency for 12is placed in trans to a wild-type chromosome (data not shown), suggesting that this gene is haplo-insufficient (see the discussion for more on the Minute phenotype). Heteroallelic combinations show variable viability, ranging from complete lethality to 82% survival and, in addition, display a distinct sex skew (Table 1) with one genotype (EMS allele/hypomorph) producing only males.

We identified two candidate genes on heterochromatic genomic scaffold AABU01002497 from the Release 3 genome sequence assembly, defects in which might show phenotypes consistent with mutations in lethal 2. The first, Dead box protein 80 (Dhp80) was identified by Eisen et al. (1998), and mutations in DEAD box helicases can be associated with a Minute-like phenotype (Dorn et al. 1993; Zaffran et al. 1998). The ortholog of Dhp80 in

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5.2 software, using the “volume analysis” option for quantitation (http://www.mdyn.com). In all cases, a probe for rp49 was used as a loading control.
yeast (Dbp5) appears to be involved in mRNA export following heat shock (Rollenhagen et al. 2004). It was mapped in Drosophila by polytene chromosome in situ to 3L heterochromatin (Eisen et al. 1998).

A schematic of Dbp80 gene structure is shown in Figure 3. From this picture it can be seen that Dbp80 shares many structural characteristics with other heterochromatic genes that have been studied. It is very large, exceeding 140 kb in length, and it is embedded within a repetitive environment. These repetitive sequences are primarily middle repetitive, consisting of degenerating transposable elements. The 61-bp intron between exons 5 and 6 may be an ancient one, as it is shared by the mammalian homologs and other Drosophila species. There are also large portions within the introns that have not yet been sequenced, and the 3′-most exon is not present within the annotated assembly. However, this last 3′ exon can be found in the trace archive (http://www.ncbi.nlm.nih.gov/BLAST/tracemb.shtml); it is therefore depicted in Figure 3 as separated from the rest of the gene by an intron of unknown length.

A second gene, RpL15, is located ∼10 kb upstream of Dbp80 and encodes a large subunit ribosomal protein. Since defects in many ribosomal proteins have been correlated with a Minute phenotype (Lambertsson 1998), this gene is also a suitable candidate for lethal 2.

TABLE 1

<table>
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<tr>
<th>Genotype</th>
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<th>SR b</th>
<th>N'</th>
<th>n c</th>
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<td>0.92</td>
<td>2080</td>
<td>567</td>
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<tr>
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<td>0.77</td>
<td>1323</td>
<td>105</td>
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<tr>
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<td>2.0</td>
<td>1551</td>
<td>62</td>
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<tr>
<td>8-1/PΔ8</td>
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<td>1.6</td>
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<td>148</td>
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<tr>
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<td>661</td>
<td>63</td>
</tr>
<tr>
<td>1-166-37/PΔ8</td>
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<td>All males</td>
<td>1296</td>
<td>61</td>
</tr>
</tbody>
</table>

a Relative viability is the observed frequency/expected frequency.

b Sex ratio: number of males divided by the number of females.

c Total number of progeny.

d Number of trans-heterozygotes. A dramatic decrease in viability correlates with sex-ratio shift.

Like Dbp80, RpL15 is characterized by the presence of middle repetitive sequences upstream, downstream, and within its introns. However, it is unusually small for a heterochromatic gene, occupying <2 kb of genomic DNA (for a schematic, see Figure 7).

We used a PCR-based strategy to position Dbp80 and RpL15 against the corresponding genetic map, using genomic DNA from embryos that were either homozygous or heterozygous for various deficiencies (Dfs) that covered this region. Results suggest that either RpL15 or Dbp80 could correspond to lethal 2 (Figure 4). So, for example, Dbp80 sequences are absent from embryos that carry Dfs removing lethal 2 (9.56/9.56, K2/K2, and 9.56/K2), but these sequences are present in other Df.

Figure 2.—Wild type (top) compared to Minute phenotypes of various lethal 2 “escapers.” Genotypes: 8-1/PΔ8 or PΔ2/PΔ8 in A–C; PΔ2/PΔ2 escapers in D and E. Note the fine bristles (A), misrotated genitalia (B), reduced or gapped sex combs (C), and wing phenotypes (D and E).
combinations. A preliminary characterization shows that both genes are single copy and expressed (data not shown), but they appear to have contrasting regulation. \textit{RpL15} is highly expressed in all tissues and stages examined, as is typical of ribosomal protein genes (data not shown), whereas \textit{Dbp80} is moderately expressed and may be developmentally regulated (Figure 5A). This correlates with the differences in their promoters: \textit{Dbp80} possesses initiator and TATA box sequences at +1 and −33 (Figure 5B), whereas \textit{RpL15} transcription appears to initiate within the vicinity of a polypyrimidine tract (see Figure 7), a feature it shares with ribosomal protein gene initiators across taxa (Barakat et al. 2001; Yoshihama et al. 2002).

\textbf{lethal 2 mutations are lesions in \textit{RpL15}}: PCR and Southern analysis of genomic DNA from the \textit{P} alleles of \textit{lethal 2} established that this gene likely encodes \textit{RpL15} (Figure 6). In all cases, complete viability was restored following reversion/precise excision of the \textit{P} element. The \textit{P} alleles were sequenced, and all the \textit{P} elements appear to have inserted into exactly the same place (Figure 7). The insertion site does not correspond to any \textit{P} element consensus (O’Hare and Rubin 1983), but this consensus is considered weak and is based on euchromatic insertions. The insertions represent, however, at least two different events, since both orientations are represented. Insertion 7-1 may have been isolated as a duplicate of 8-1, given that both insertions were isolated in the same screen and are identical in size and orientation (opposite to the direction of \textit{RpL15} transcription). However, genetic evidence suggests that they might behave differently (see, for example, in Table 1, relative viability numbers for 8-1 and 7-1 in combination with \textit{PΔ8}). Either there are subtle differences within the \textit{P} insertions themselves or years of separation of stocks have resulted in changes in genetic background (e.g., accumulated modifiers). \textit{PΔ2} and \textit{PΔ8} are inserted in the same orientation as the gene, but \textit{PΔ2} possesses a further internal deletion that may have occurred before or after mutagenesis, so it may or may not be a separate event. The EMS alleles were also sequenced and found to possess mutations in \textit{RpL15} sequences: 72 encodes a mRNA with a nonsense mutation in the second exon; 1-166-37 is a G-to-A substitution in

**Figure 3.**—Gene organization of \textit{Dbp80}: solid boxes indicate exons (sizes in base pairs); lines connecting them represent introns. This map (not to scale) was derived by aligning the cDNA sequence (Eisen et al. 1998) to genomic scaffold AABU01002497 in the Release 3 genome sequence assembly. The exception is the 3′-most exon of \textit{Dbp80}, which has not yet been linked to sequences in Release 3. It is therefore shown separated from exon 10 by an intron of indeterminate length.

**Figure 4.**—PCR mapping of \textit{Dbp80} and \textit{RpL15} under deficiencies that remove \textit{lethal 2}. \textit{FX3} is \textit{Df(3L)FX3}, which removes \textit{lethal 3–lethal 8}; 9-56 is \textit{Df(3L)9-56}, which removes \textit{lethal 1} and \textit{lethal 2}; \textit{K2} is \textit{Df(3L)K2}, which removes \textit{lethal 2} and \textit{lethal 3}. \textit{RpL15} was not tested under \textit{Df(3L)K2} since it was confirmed to be \textit{lethal 2}. \textit{Grip84} is a gene on the \textit{X} chromosome used as a DNA control. For \textit{Dbp80}, the PCR primers used derive from exon 9, which is the exon farthest to the left (and most distal to \textit{RpL15}) in Figure 3.
the 5′ splicing consensus of the first intron (Figure 7).
Both these mutants have the same lethal phase (L1) as a homozygous deficiency for the region (Df(3L)9-56).

Does Dbp80 correspond to another essential gene in the vicinity? A series of lethal excisions in RpL15 were obtained by mobilizing the P element and generating a variety of molecular lesions in this locus, and Southern blots demonstrate that at least two of these lesions appear to have removed significant portions of the Dbp80 gene (data not shown). All of these excisions, including those that affect Dbp80, are completely viable over mutations in the flanking genes lethal 1 and lethal 3; this suggests that the Dbp80 gene does not correspond to either of these essential genes. This is supported by the PCR mapping data (Figure 4): Dbp80 cannot be lethal 3, because it is present under a deficiency that removes lethal 3 (Df(3L)FX3), and cannot be lethal 1, because Dbp80 sequences are absent in a deficiency that does not remove lethal 1 (Df(3L)K2). RNA interference (RNAi) experiments also suggest that Dbp80 may not be an essential gene: when expressed in cell culture, Dbp80 RNAi constructs have no effect on cell viability (Gatfield et al. 2001). In addition, we observed no in vivo effects following either Act-5C or heat-shock GAL4-driven expression of UAS-Dbp80-RNAi transgenic lines at 18°, 25°, or 29° (data not shown).

Expression of RpL15 cDNA transgenes enhances viability of mutant lethal 2 trans-heterozygote combinations:
Germline rescue of mutant alleles with transgenic constructs would constitute further evidence establishing lethal 2’s molecular identity. To this end, three kinds of germline transformation constructs were generated. One contained a genomic insert: a 2.2-kb BglII-HindIII fragment containing 800 bp upstream and 530 bp downstream of the coding region cloned into pUAST, which possesses upstream activating sequences for the yeast transcription factor GAL4. A full-length cDNA was also cloned into both pCaSpeR-hs and pUAST. For the genomic transgene and the two cDNA constructs, a number of transgenic lines were established for each (eight, nine, and eight, respectively). Lines carrying the genomic transgene were tested in the absence of any GAL4 driver, since the few previous successful efforts to rescue ribosomal protein genes were carried out using genomic constructs in uninducible vectors (Voelker et al. 1989; Schmidt et al. 1996; Torok et al. 1999). In addition, a number of the genomic and cDNA transgenic lines were subjected to various driver protocols: by direct heat shock in the case of pCaSpeR-hs, by crossing to various driver lines expressing GAL4 under heat-shock control, or from an actin-5C (constitutive) promoter. None of these attempts was successful in rescuing the lethality of strong alleles of lethal 2.

Given these problems and the reported difficulty in rescuing mutations in many ribosomal protein genes (Lambertsson 1998), we undertook an alternative approach. Heteroallelic combinations of lethal 2 P alleles do produce a certain number of escapers, so it was possible to attempt to demonstrate partial rescue via enhanced viability—increasing the proportion of escaper offspring. The crossing scheme is shown in Figure 6.

**Figure 6.—** Genomic DNA from lethal 2 P mutants and revertants probed with the RpL15 cDNA. Rev indicates a viable, fully revertant line derived from the mutant line shown immediately to its left. So, for example, from left to right, Rev5 and Rev12 are P-revertants of the PΔ8 mutation, ry[506]/ry[506] is the wild-type control. DNA from the indicated genotypes was cut with EcoRI, blotted, and probed with RpL15 cDNA, as indicated in MATERIALS AND METHODS. In all cases the P-mutant band is absent in the revertant lines.
8A, and a statistical interpretation of the results in Figure 8B. For this particular experiment, an X-linked transgene was brought in through the male parent, so in the test generation, only the females inherit the complete complement of transgene, driver, and lethal 2 alleles, and the males serve as an internal control. The test generation was removed from a white minus background to score the eye-color mutation (rosy\textsuperscript{506}) that marks the P\textsuperscript{mutant} chromosomes. In addition, three internal controls were set up at the same time, one possessing neither transgene nor driver, one with just the transgene, and one with just the driver. Rescue is reflected by an increase in the female:male ratio of rosy/rosy flies in the test generation, and this is depicted in Figure 8B. While crosses involving other genomic and cDNA transgene lines showed similar results (consistently enhanced transheterozygote viability relative to controls; data not shown), only a cDNA transgene, UAS24-1, gave a statistically significant result (95% confidence level). As mentioned earlier, lethal 2 trans-heterozygotes show variability in relative viability (see Table 1), and this is reflected by the variability in the sex ratios for the controls.

Gene expression of both RpL15 and Dbp80 is compromised when the HP1 dose is reduced: The expression of the well-characterized heterochromatic genes light and rolled appears to be negatively affected in a genetic background in which the HP1 dose is reduced on the basis of both genetic (Clegg et al. 1998; Sinclair et al. 2000) and molecular studies (Lu et al. 2000). It is of interest to know if any other heterochromatic genes respond in a similar manner, particularly RpL15 and Dbp80, which have contrasting promoters, expression patterns, and gene organization. In addition to being essential, small, and highly expressed, RpL15 also presents a clear hotspot for transposon insertion, suggesting that its chromatin environment might be more euchromatic and might thus respond differently to reductions in HP1 dose.

Figure 7.—Composite diagram of RpL15 gene structure. The promoter region is expanded to indicate the insertion site for all four P\-alleles of lethal 2 and the position of the polypyrimidine tract (transcription initiation). Also shown are the locations and classifications of the two lethal 2 EMS alleles.

Figure 8.—Rescue (enhanced viability) of lethal 2 trans-heterozygotes with RpL15 transgenes. (A) Genetic crossing scheme for rescue experiments using P\Delta2/P\Delta8 lethal 2 trans-heterozygotes. RpL15 cDNA and genomic transgenes were cloned into pUAST and expressed under UAS regulation as a result of GAL4 expression via an ACT-5C (constitutive) driver. (B) Statistical analysis of rescue data. These results are significant (95% confidence) and derive from use of a constitutively driven X-linked cDNA transgene, UAS24-1.
Our molecular results suggest that the expression of both the RpL15 and Dbp80 genes is reduced in an HP1 mutant background. Total RNA was blotted in a Northern analysis from L3 larval genotypes trans-heterozygous for functional nulls of HP1: Su(var)2-5<sup>nl1</sup>/Su(var)2-5<sup>nl1</sup> (no functional HP1 dose), their heterozygous sibs (one functional HP1 dose), and wild-type larvae from the same developmental stage (two functional HP1 doses). When these Northern were probed with the cDNAs for RpL15 or Dbp80 and the signals quantified by phosphorimaging, the same trend was observed: gene expression was compromised. Figure 9 shows quantitative results taken from three separate total RNA Northern experiments per gene, where the ratio of Dbp80 or RpL15 signal intensity is expressed relative to a loading control (rp49). Expression of both genes is clearly reduced in the Su(var) mutant background, as has been observed for light and rolled by Lu et al. (2000).

These molecular results are supported genetically for RpL15 by two assays, which show that lethal 2 gene function is compromised in a background in which the HP1 dose has been reduced by half. The first experiment made use of an inversion that breaks in euchromatin around cytological position 62D and, in heterochromatin, in the vicinity of lethal 1, effectively relocating lethal 2 near distal euchromatin. This inversion is semilethal in combination with lesions in lethal 1 and exhibits a posterior wing-margin phenotype in combination with lesions in lethal 2. The phenotype varies in both penetrance (Table 2) and expressivity (Figure 10) and also in severity, with increasing strength of the lethal 2 mutation. It is enhanced in a genetic background heterozygous for a mutation in HP1 (Su(var)2-5<sup>nl1</sup>), which is consistent with the effects observed for heterochromatic PEV of other translocated heterochromatic genes (Eberl et al. 1993; Howe et al. 1995).

In a similar experiment, lethal 2 mutations can be shown to enhance the wing-margin defect associated with the weak Notch allele N<sup>689</sup>. Mutations in Minute genes have been shown to affect wing morphogenesis gene expression (Sinclair et al. 1984; Hart et al. 1993), and it has been proposed that this is due to the sensitivity of these genes to reductions in protein synthesis levels. The effect of lethal 2 on Notch is further enhanced in a genetic background in which a single dose of HP1 has been removed (Figure 11). The more severe effects seen in this case are consistent with a further loss of lethal 2 function (resulting from the reduced HP1 dosage).

DISCUSSION

**lethal 2 encodes RpL15 and represents an example of a small heterochromatic gene:** lethal 2 is the first complementation group to be thoroughly characterized at the molecular level in a set of 10 essential 3L heterochromatic genes identified by Marchant and Holm (1988b). It is the second most proximal gene and likely resides in cytological position h51 on the basis of mitotic mapping of deficiencies that remove it (Koryakov et al. 2002). All six lethal 2 alleles are lesions in RpL15, which encodes a large subunit ribosomal protein.

Heterochromatic genes tend to be very large due to the expansion of repetitive sequences in their introns (Devlin et al. 1990a,b; Warren et al. 2000; Tulin et al. 2002). Nevertheless, although RpL15 is embedded in a repetitive environment, this does not seem to have affected its size. On the basis of its essential housekeeping function, there is likely to be a strong selective pressure to keep this gene small (Castillo-Davis et al. 2002). The ribosome plays a fundamental role in controlling cell growth and development, and its constituent parts must be tightly and coordinately regulated. In bacteria, these genes are clustered in operons, while in eukaryotes, they are widely dispersed throughout the genome. However, the requirement for coordinate expression has not been lost in eukaryotes, so they have likely evolved mechanisms that allow them to be expressed in a variety of chromatin environments. In yeast, there is evidence that ribosomal protein genes possess insulating sequences in their promoter regions, which may render them relatively resistant to position effects (Bir and Broach 1999). While insulators have not been found associated with Drosophila ribosomal protein genes, a common upstream feature is a polypyrimidine tract, which has been demonstrated to play a critical role in both transcriptional and translational regulation across taxa (Hariharan and Perry 1990; Levy et al. 1991). It remains to be seen whether this sequence...
TABLE 2
Penetrance data for genetic interaction between lethal 2 mutations and the inversion In(3L)C90

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<th>Genotype</th>
<th>Penetrance¹ males (%)</th>
<th>Penetrance¹ females (%)</th>
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<td>1-166-37/In(3L)C90</td>
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<td>72/In(3L)C90</td>
<td>13</td>
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</tbody>
</table>

¹ Only third chromosome genotypes are shown. 1-166-37 and 72 are EMS point mutations in lethal 2 (see Figure 7); 1-16-0 is an allele of the more distal 3L heterochromatic gene SNAP25, used as a non-lethal 2 control.

Calculated as the number of observed trans-heterozygous progeny divided by the expected number for a given population scored (which, for each cross, consisted of at least 150 flies).

Difficulties in rescuing lethality for Rpl15 may be explained by the lack of an appropriate heterochromatic environment for the genomic construct in the various transgenic lines tested. However, GALA-driven expression was also problematic; therefore, failure to rescue lethality is more likely to be attributable to the above-mentioned extreme dose sensitivity. This may be a common feature of mutations in ribosomal protein genes, for which relatively few successful transgene rescues have been reported (Lambertsson 1998).

We attempted to address this issue by quantitating Rpl15 expression in various genetic contexts; however, doing this to within statistically significant margins proved difficult, due to the very high levels of expression of this gene. For example, Northern analysis of wild-type and mutant mRNA levels showed less mRNA present in mutant combinations, but these results were not statistically significant, except in the case of PΔ2/PΔ2 adult escapers (data not shown). A precise molecular analysis of haplo-insufficiency was therefore not possible, although a subtle phenotypic effect can consistently be observed in the adult (lethal 2 null/Df; data not shown). Similarly, the high endogenous expression levels for Rpl15 made it difficult to accurately quantitate transgene expression, and we could see a demonstrable increase in Rpl15 mRNA levels only in pUAST lines expressing via heat-shock-driven GAL4 (data not shown).

![Figure 10](image-url)

Figure 10.—Wing-margin phenotypes resulting from interaction between mutations in lethal 2 and In(3L)C90. This inversion breaks in or near lethal 1 and in distal euchromatin at cytological position 62D, likely placing lethal 2 close to a large block of euchromatin. The phenotype is more severe in females than in males, in terms of both penetrance (Table 2) and expressivity (shown here). (A) Wild type (female); (B) In(3L)C90/72 (female); (C) Su(var)2-501/+; In(3L)C90/+ (female); (D) Su(var)2-501/+; In(3L)C90/72 (male); (E) Su(var)2-501/+; In(3L)C90/72 (female). The results suggest a strong heterochromatic position effect on lethal 2, which is enhanced in the Su(var) background.
We conclude that problems with rescue arise from some combination of dose sensitivity and/or other peculiarities of ribosomal protein gene expression in Drosophila.

Global (organism-wide) phenotypic manifestations due to defects in ribosomal protein function appear to be unique to Drosophila (although one report identifies a Minute-like phenotype caused by an Arabidopsis ribosomal protein gene mutation [Weijers et al. 2001]). This may in part be due to the fact that, with two exceptions (Brown et al. 1988; Yokokura et al. 1993), Drosophila appears to have only single-copy ribosomal protein genes, while yeast, plants, and humans have multiple copies, including pseudogenes (Zhang et al. 2002). Defects in human ribosomal protein genes have been implicated in a number of inherited disorders [for example, diamond blackfan anemia (RpS19), Turner syndrome (RpS4), and Noonan syndrome (RpL6) (Zhang et al. 2002 and references therein)], implying that mammalian ribosomes may exist in tissue/stage-specific isoforms. However, no general Minute syndrome as such appears in organisms with multiple-copy ribosomal protein genes. It is not clear why Drosophila has retained only single copies, but this may be explained in part by a growing body of evidence that suggests that the evolution of the Drosophila genome may have been marked by considerable DNA diminution (Petrov 2002).

Is Dbp80 an essential gene? We believe that Dbp80 is unlikely to be an essential gene for a number of reasons. In terms of genetics, PCR deficiency mapping suggests that Dbp80 can be neither lethal 1 nor lethal 3, and this result is supported by the genetics of lethal excisions from RpL15 affecting Dbp80, which are completely viable in combination with mutations in lethal 1 and lethal 3. This proximal region has been subjected multiple times to extensive mutagenesis screens (Marchant and Holm 1988a,b; Schulze et al. 2001; M. Syrzycka, unpublished results) and we have found no other lethal mutations in the region that might be candidates for Dbp80.

In terms of gene function, although the ortholog of Dbp80 encodes an essential gene in yeast that plays a role in mRNA export (Rollenhagen et al. 2004), attempts to show a similar function in insect cell culture (by RNAi depletion) indicate that it is not essential for this pathway in flies (Gaffield et al. 2001). The Caenorhabditis elegans ortholog also appears to be nonessential, as there is no phenotype from an RNAi knockout experiment (http://www.wormbase.org/db/seq/rnaip?name=JA%5 AT07D4.4;class=RNAi). The functional role of Dbp80 protein may be important, but a number of related DEAD box genes could provide functional redundancy. Our results, showing no effects of RNAi in vivo, are also consistent with this view. Finally, the WGS heterochromatin assembly (Hoskins et al. 2002) places many more gene models in 3L heterochromatin than there are lethal complementation groups. While not all of these models may be confirmed as genuine after repeated rounds of annotation, these results would be consistent with the presence of a significant number of genes that would be nonessential in heterochromatin (Dbp80 and others).

Are there other candidates for essential genes in the region? The current annotation of Drosophila heterochromatin does list two small gene models nested within Dbp80 (CG40336 encoding 98 amino acids and CG40337 encoding 103 amino acids) and while we cannot formally rule out the possibility that these might encode essential functions, we think it is unlikely. A BLAST search against a nonredundant protein database produces little homology with genes of known function; it is interesting to note that a third gene model, CG40353, was initially described, but subsequent annotation has identified it as a repeat sequence. It is possible that all three represent degenerate transposable element sequences; this provides an example of the difficulties in annotating gene models in a repetitive environment.

Both Dbp80 and RpL15 are negatively affected when the HP1 dose is reduced: These two genes have very different promoters and expression patterns, suggesting that there may be no simple, common mechanism to regulate their expression. However, our molecular evi-
idence suggests that expression of both is compromised in a genetic background deficient for HP1. This is consistent with results observed for the well-characterized genes light and rolled (Li et al. 2000) and provides further evidence that genes in heterochromatin may have evolved a transcriptional dependence on factors that are known to silence gene expression. This effect can also be shown genetically for lethal 2, using a sensitized background. Ribosomal proteins have been shown to enhance wing morphogenesis mutations (Sinclair et al. 1984; Hart et al. 1993), and this appears to be the case for lethal 2 in combination with a weak Notch allele. The severity of the interaction with Notch is enhanced when only one copy of HP1 is removed. We also report results consistent with this interpretation, using the inversion In(3L)C90, which breaks proximally in the lethal 1 gene, and distally in euchromatin at position 62D, placing lethal 2 relatively close to a block of euchromatin. In(3L)C90 complements all lethal 2 alleles for viability and fertility, but presents a posterior wing-margin defect. This phenotype increases in severity with the strength of the mutant lethal 2 allele (i.e., the strongest phenotype in terms of penetrance and expressivity is observed with the EMS allele 72, which appears to behave as a functional null). It is not clear what the source of this particular phenotype is, since it does not resemble any of the wing defects resulting from lethal 2 hypomorph. However, In(3L)C90 also possesses a deletion for ~60 genes near the distal euchromatic breakpoint, and it is possible that lethal 2 interacts with a reduction in dose of a gene from this region in a manner similar to its observed interaction with Notch. Once again, the severity of this phenotype is enhanced in a background in which the HP1 dose has been reduced by half. This inversion has no effect above a background one on any mutants or lesions distal to lethal 2, unless combined with a reduction in HP1 dose, in which case the effect is less severe than that for lethal 2. The variegation phenotype is thus consistent with an interpretation of the PEV effects on the translocated lethal 2 gene and is similar to what has been observed genetically for both.

It is tempting to speculate that positive transcriptional regulation by HP1 may be a diagnostic feature of heterochromatic genes on the basis of doubling of the sample size to four genes so analyzed to date; previously, these effects had been demonstrated only for the genes rolled (Eberl et al. 1993) and light (Howe et al. 1995) in chromosome 2 heterochromatin. This generalization is given further credence by a study showing that particular euchromatic genes are indeed negatively influenced by a normal complement of two expressed copies of the HP1 gene (Hwang et al. 2001). However, the complete picture is likely to be rather less clear cut, as indicated by Piacentini et al. (2003), who showed that HP1 can in fact associate and positively regulate sites of intense gene activity in euchromatin (polytene chromosome puffs), in particular, those loci that encode the heat-shock proteins (87A, 87C, and 95D). Formaldehyde crosslinked chromatin immunoprecipitation experiments using primers to either the promoter or the coding regions of the Hsp70 gene (which maps to 87A and C) show that, after heat-shock induction, HP1 protein is enriched in the coding region, and not in the promoter of this gene. Moreover, this enrichment appears to depend on the presence of RNA and an intact chromodomain in HP1. Thus, HP1 in fact may act as a regulator of transcription by controlling the stability of the transcript (elongation, processing, etc.) rather than by inducing or repressing gene expression per se, and its function as a repressor or activator may well depend on a combination of factors, including but not limited to, chromatin environment.

Our work provides two more informative gene models to study the expression and regulation of genes in heterochromatin. In addition, it provides an example of how genetic and molecular analyses can complement and enrich the annotation of this difficult genomic region. Comparative evolutionary studies have also been informative in studying the structure and regulation of gene expression. In a parallel report (S. R. Schulze, B. F. McAllister, D. A. R. Sinclair, K. A. Fitzpatrick, M. Marchetti, S. Pimpinelli and B. M. Honda, unpublished results), we have observed dramatically different chromosomal localizations for Rpl15 and Dhp80 in other Drosophila species, showing that the same gene can evolve and function in contrasting chromatin environments. Taken together, these complementary approaches lay the foundation for further research into the relationship between chromatin structure and gene regulation.

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LITERATURE CITED


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