A STUDY OF THE COMPOSITION AND FUNCTION OF TELOMERIC CHROMATIN IN *Drosophila melanogaster*.

by

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ABSTRACT

The telomeres of most organisms are characterized by a protein-capping complex that protects chromosome ends, a series of repetitive subtelomeric sequences known as Telomere-Associated Sequences (TAS), and a behavioral phenomenon known as Telomere Position Effect (TPE). TPE is a phenomenon whereby normally active genes become repressed and silenced if relocated near to telomeres, and is thought to be a property of the proteins that constitute telomeric heterochromatin. Genetic dissection was used to exploit this phenomenon in order to identify components of telomeric heterochromatin in Drosophila melanogaster. Using genetic dissection, followed by a chromatin analysis technique known as Chromatin ImmunoPrecipitation (ChIP) I was able to identify three proteins, HDAC1, SU(VAR)3-9, and HP1c, as integral components of telomeric heterochromatin in Drosophila. HDAC1 and SU(VAR)3-9 are both believed to be involved in the gene-silencing process, and thus, their presence at telomeres could explain the phenomenon of TPE. Furthermore, I found that these proteins were specifically associated with the TAS region on the centromere-proximal side of the HeT-A transposable elements that maintain telomere length in Drosophila. As a result of this, I proposed a model, which I call the ‘pairing-sliding model of telomere length control in Drosophila,’ which proposes that temporary incorrect pairing of Drosophila telomeres results in the deacetylation and subsequent methylation of the nucleosomes associated with the HeT-A and TART elements by TAS-associated HDAC1 and SU(VAR)3-9, resulting in these elements being transcriptionally silent. Thus, I propose that the TAS region, and the HDAC1 and SU(VAR)3-9 associated with it play a role in the negative regulation of telomere length in Drosophila.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>abo</td>
<td>Abnormal oocyte (mutation)</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs of DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methane sulfonate</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>H3</td>
<td>histone protein H3</td>
</tr>
<tr>
<td>H3K9</td>
<td>lysine 9 of histone protein H3</td>
</tr>
<tr>
<td>H3K9ac</td>
<td>the acetylated form of lysine 9 of histone protein H3</td>
</tr>
<tr>
<td>H3K9me</td>
<td>the methylated form of lysine 9 of histone protein H3</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HDAC1</td>
<td>histone deacetylase one protein (encoded by the Hdac1 locus)</td>
</tr>
<tr>
<td>Hdac1</td>
<td>histone deacetylase one (mutation)</td>
</tr>
<tr>
<td>HIS-C</td>
<td>histone complex, containing multiple tandem copies of the histone genes</td>
</tr>
<tr>
<td>HMTase</td>
<td>histone methyltransferase</td>
</tr>
<tr>
<td>HP1</td>
<td>heterochromatic protein 1</td>
</tr>
<tr>
<td>HP1a,b and c</td>
<td>three homologs of the HP1 protein</td>
</tr>
<tr>
<td>Irbp</td>
<td>inverted repeat binding protein</td>
</tr>
<tr>
<td>kis</td>
<td>kismet (mutation)</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases of DNA</td>
</tr>
<tr>
<td>l(2)gl</td>
<td>lethal (2) giant larva (mutation)</td>
</tr>
<tr>
<td>lat</td>
<td>latheo (mutation)</td>
</tr>
<tr>
<td>LBR</td>
<td>lamin B receptor</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>msl-3</td>
<td>male sex-lethal 3 (mutation)</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex (composed of several proteins)</td>
</tr>
<tr>
<td>Pc</td>
<td>polycomb (mutation)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEV</td>
<td>position effect variegation</td>
</tr>
<tr>
<td>ph-d</td>
<td>polyhomeotic distal (mutation)</td>
</tr>
<tr>
<td>ph-p</td>
<td>polyhomeotic proximal (mutation)</td>
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<tr>
<td>Psc</td>
<td>posterior sex combs (mutation)</td>
</tr>
<tr>
<td>puc</td>
<td>puckered (mutation)</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative (or semi-quantitative) polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Su(TPE)</td>
<td>suppressor(s) of telomere position effect (mutation)</td>
</tr>
<tr>
<td>Su(var)</td>
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<tr>
<td>Su(var)3-9</td>
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<td>Su(z)2</td>
<td>suppressor of zest 2 (mutation)</td>
</tr>
<tr>
<td>Su(z)3</td>
<td>suppressor of zest 3 (mutation)</td>
</tr>
<tr>
<td>TPE</td>
<td>telomere position effect</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>----------------------------------</td>
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<tr>
<td>uL</td>
<td>microliter</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>yKu70/80</td>
<td>yeast DNA-binding heterodimer complex</td>
</tr>
</tbody>
</table>
I wish to thank my supervisor, Dr. Tom Grigliatti for much help and inspiration. Little did I know that a summer job as Grigliatti lab fly food cook in 1989 would turn into a much larger endeavor. I would also like to thank my colleagues, Pam Kalas, Randy Mottus for input and advice. I would also like to thank the members of my supervisory committee, Drs. Brock, Brown, Hieter, and Roberge, for much helpful insight into the direction this project has taken.
DEDICATION

I dedicate this and all my works to my beloved parents, who were my greatest teachers by example. To my Father, who showed me the beauty of academic endeavors, driven by curiosity, passion, and sometimes petulance; and to my Mother, who showed me that a tenacious mind is meaningless without the purpose and direction that can only be provided by a loving heart.

‘Settle thy studies Faustus, and begin
To sound the depth of that thou wilt profess.
Having commenced, be a divine in show-
Yet level at the end of every art
And live and die in Aristotle’s works.
Sweet Analytics, ’tis thou hast ravished me.
Bene disserere est finis logices.
Is to dispute well logic’s chiefest end?
Affords this art no greater miracle?
Then read no more, thou hast attained that end.
A greater subject fitteth Faustus’ wit:…’

Christopher Marlowe

Ad majorem Dei Gloriam!
CO-AUTHORSHIP STATEMENT

The following is a manuscript-based thesis. The idea for this project was conceived by me, in consultation with my supervisor, Professor Tom Grigliatti. All experiments were designed and executed by me, all data were collected by me, and all chapters and appendices of this thesis were written by me. Chapter Two was submitted as a manuscript to the journal *Genetics* (published by The Genetics Society of America) with myself and Dr. Grigliatti as authors. The article was rejected as written, with certain suggestions for revisions included in the comments. The article is currently being re-written by Dr. R. Mottus, who is a Research Associate in the Grigliatti lab. When the manuscript is re-submitted it will include Dr. Mottus in the list of authors, due to his contribution to the re-write. However, he did not write, nor contribute to the version included in this thesis.

Chapter three is intended to be submitted as a manuscript to the journal *Genetica* (published by Springer Netherlands), and was written entirely by me.
Chapter One

INTRODUCTORY CHAPTER

Putting the Study of the Telomeres of *Drosophila melanogaster* into an Appropriate Context.
Linear chromosomes and telomere maintenance.

Telomeres are specialized DNA-protein complexes that cap and protect the ends of linear chromosomes. While less is known about the composition and characteristics of Drosophila telomeres than is known about the telomeres of mammals and yeast, several characteristics appear to be common to the telomeres of most organisms. Telomeres generally have a terminal capping complex that protects the chromosome end from degradation by nucleases, and prevents fusion with other telomeres. This capping structure often includes a triple stranded loop, known as a t-loop, where a protruding single strand of DNA folds back upon, and invades the double stranded helix on the centromere-proximal side of it. This t-loop is usually stabilized by a characteristic set of proteins that appears to be unique to each organism. Moderately repetitive subtelomeric sequences are frequently found downstream of this capping structure.

The telomeres of most organisms are also associated with some sort of a reverse transcriptase complex that is able to lengthen chromosome ends de novo. The need for this complex accompanies the fact that the 5’ ends of chromosomes become shorter with each round of replication. It is necessary, therefore, to lengthen the chromosomes back to their original size, particularly in germline cells, lest they become shorter and shorter with each generation until large parts of the genome are lost, and the organism becomes non-viable. Drosophila is somewhat unusual in that it maintains the length of its
telomeres through intermittent transposition of specialized transposable elements to receding chromosome ends.

Finally, most telomeres are associated with a phenomenon known as **Telomere Position Effect (TPE)**, where normally active, euchromatic genes become repressed and silenced when relocated near to telomeres. This property of telomeres was exploited in this thesis to identify several protein components of telomeric heterochromatin in Drosophila. TPE is believed by many to be a function of the proteins that comprise telomeric heterochromatin, and removal or mutation of these proteins would, presumably, alleviate the gene repression associated with telomeres. Thus, the technique of genetic dissection can be used to identify components of telomeric heterochromatin that participate in TPE, provided a suitable reporter gene assay can be found to test for suppression of TPE. This technique was used here to identify at least three protein components of telomeric heterochromatin, and make inferences about their purpose in telomere biology, including telomere length maintenance in Drosophila.

Before embarking on a detailed description of what is currently known about Drosophila telomeres, a brief summary of what is known about yeast and mammalian telomeres may be helpful, particularly in putting the study of Drosophila telomeres into an appropriate context.
II Characteristic features of yeast telomeres.

Linear chromosome ends become shorter with each round of chromosomal replication, a phenomenon referred to as the end replication problem. In order to counterbalance this form of terminal erosion, and prevent loss of genetic material, many organisms possess a protein complex known as a telomerase complex that is able to extend chromosome ends de novo (BLASCO et al. 1999; PARDUE and DeBARYSHE 1999). Telomere length in Saccharomyces cerevisiae is maintained by such a complex (Singer and Gottschling 1994). As a result of this method of telomere length maintenance, yeast telomeres terminate in approximately 350 base pairs (bp) of highly repetitive DNA which was added by the telomerase holoenzyme, and which is associated with a characteristic set of telomere-specific proteins (TEIXEIRA and GILSON 2005). This nucleoprotein complex is collectively referred to as the telosome. Unlike the majority of chromatin, the telosome is not organized into nucleosomes (WRIGHT et al. 1992). The Rap1 protein is the major constituent of the yeast telosome (CONRAD et al. 1990; WRIGHT et al. 1992; WRIGHT and ZAKIAN 1995), but other proteins, such as the Sir2p, Sir3p, and Sir4p (BOURNS et al. 1998), and the Ku heterodimer, composed of the Hdf1 and Hdf2 proteins (GRAVEL et al. 1998) are also present.

The telosome and the subtelomeric regions adjacent to it are believed to be folded into a three-stranded looped structure, known as a t-loop (DE BRUIN et al. 2000; STRAHL-BOLSINGER et al. 1997). Some of the most elegant evidence for a looped telomere structure came from the observation that a yeast upstream activation sequence (UAS) placed downstream of a telomere-embedded reporter gene was able to partially activate
transcription of the reporter gene (De Bruin et al. 2000). This result implied that a loop had been formed, which brought the reporter gene into close proximity to the UAS enhancer. Evidence for looped telomeric structures has also been found in other organisms, including mammals (Griffith et al. 1999), but it is not clear whether t-loop formation is a widespread characteristic of telomeres or not.

Another characteristic feature of yeast telomeres is that they are clustered, and located at the nuclear periphery, an area where telomere-specific proteins are concentrated (Chikashige et al. 2006; Cockell et al. 1998; Gasser et al. 1998; Gotta and Gasser 1996; Gotta et al. 1996; Gotta et al. 1999; Gotta et al. 1997; Larocque et al. 1998). Positioning of telomeres at the nuclear periphery is believed to be a function of the telosome, and the telomere-specific proteins that comprise it. Some have even gone so far as to suggest that telomeres are ‘tethered’ to the nuclear periphery by direct physical interactions with structures embedded in the nuclear envelope, such as nuclear pore complexes (Taddei and Gasser 2004). Hence, the ability to create a physical interaction between chromosome ends and integral components of the nuclear envelope or nuclear matrix may also be an essential property of telomeres.

Less repetitive subtelomeric DNA sequences, known as Y’ and X elements (Gravel et al. 1998) are located on the proximal (centromere) side of the telosome. Unlike the telosome, these subtelomeric regions are organized into nucleosomes (Wright et al. 1992). However, the histone proteins that comprise these nucleosomes are less acetylated than the majority of the genome (Braunstein et al. 1993; De Bruin et al. 2000). Since
histone acetylation is thought to be a sign of actively transcribing chromatin, the observation that subtelomeric regions are hypoacetylated suggests that subtelomeric chromatin is transcriptionally silent. Furthermore, since the subtelomeric DNA sequences appear to be refractory to methylation by an exogenous dam methylase (GOTTSCHLING 1992), an assay used to measure DNA accessibility in vivo, telomeric and subtelomeric heterochromatin appears to be less accessible to transcription factors than other regions of the genome, and may be compacted into a more compressed structure than actively transcribed regions of euchromatin.

Both the lack of histone acetylation and the lack of accessibility have been invoked as possible explanations for another interesting and important characteristic of telomeres known as TPE. The term TPE refers to a phenomenon whereby normally active genes become transcriptionally repressed when ectopically relocated to telomeric regions. Although this phenomenon was originally documented in S. cerevisiae (GOTTSCHLING et al. 1990; PALLADINO and GASSER 1994; THAM et al. 2001; THAM and ZAKIAN 2000; THAM and ZAKIAN 2002), it appears to be more common. TPE has also been observed in Schizosaccharomyces pombe (NIMMO et al. 1994), Trypanosoma brucei (HORN and CROSS 1995), mouse embryonic stem cells (PEDRAM et al. 2006), human cells (BAUR et al. 2004; BAUR et al. 2001; KOERING et al. 2002), and Drosophila (BOIVIN and DURA 1998; CRYDERMAN et al. 1999a; DONALDSON et al. 2002; MASON et al. 2004; WALLRATH and ELGIN 1995). Thus, the phenomenon of TPE is one of a handful of telomeric properties that are shared by many organisms, including Drosophila. More importantly, since several components of the yeast telosome were initially identified by
Yeast telomeres terminate in approximately 350 bp of highly repetitive DNA, which has been added to the chromosome end by a telomerase complex. The protruding, single stranded end of the telomere is believed to fold back upon proximal double stranded sequences, forming a triple stranded t-loop. A number of well-characterized proteins are believed to form a capping complex which protects the free end of the telomere from degradation by nucleases, and prevents fusion with other telomeres. This protein-DNA complex is collectively referred to as the telosome. The telosome is not believed to be organized into nucleosomes. Sequences proximal to the telosome are organized into nucleosomes, but the histone proteins which comprise these nucleosomes are hypoacetylated, a feature characteristic of transcriptionally silent chromatin. Some of the proteins associated with the yeast telomere, such as the Sir proteins, are also believed to inhibit gene transcription. The Sir2 protein, for example, is known to have histone deacetylase activity, which could explain the observed nucleosome hypoacetylation, and the gene-silencing properties associated with telomeres. (Proteins and DNA sequences not necessarily drawn to scale.)
examining mutations that counteract TPE (Schulz and Zakian 1994; Singer and Gottschling 1994; Singer et al. 1998), this property is potentially useful, and can be exploited to identify protein components of telomeric heterochromatin.

III Characteristic features of mammalian telomeres.

Mammalian telomeres have also been studied extensively, although not to the same degree as yeast telomeres. While many features of mammalian telomeres appear to be similar to those seen in yeast, others are not. While the extension of telomeres by a telomerase complex, and the formation of terminal t-loops appear to be features of yeast telomeres that are shared by mammalian telomeres, the proteins that comprise the yeast telosome are not conserved in mammals. Indeed, the telosomes of mammals are composed of proteins (Cheong et al. 2003) that bear little or no similarity to the telomere proteins of yeast, implying that while the capping function of the telomere is conserved between organisms, the proteins that comprise such caps are not conserved.

Mammalian telomeres also terminate in highly repetitive, GC-rich repeats, that are added to the receding 5’ ends of chromosomes by a telomerase complex (Nugent and Lundblad 1998). As in yeast, less repetitive subtelomeric sequences, known as Telomere Associated Sequences (TAS) are found adjacent to these highly repetitive GC-rich repeats (Karp and Spradling 1992; Walter et al. 1995). Mammalian TAS repeats are analogous to the X and Y’ elements found proximal to yeast telomeres, although the DNA sequences are different. Indeed, most organisms are believed to carry moderately repetitive subtelomeric DNA sequences adjacent to their telomeres (Mrinal et
al. 2006; Mewborn et al. 2005; Karpen and Spradling 1992), suggesting that this may also be a common characteristic of telomeres, despite the fact that the sequences themselves are not conserved.

As in yeast, mammalian telomeres terminate in approximately 300 bp of single stranded overhang, which is believed to fold back onto the subtelomeric DNA on the proximal side of it, forming a triple stranded t-loop (De Lange 2004; Griffith et al. 1999). Two proteins, TRF1 and TRF2, bind to the single stranded overhang, and stabilize the t-loop (De Lange 2004; Smogorzewska and De Lange 2004). Loss of these or other capping proteins, or prevention of t-loop formation usually leads to the creation of fusagenic telomeres, and is associated with cancer and other biological problems (Maser and DePinho 2002; Maser and DePinho 2003). Several other proteins, such as hPOT1, PINX1, TIN2 and hRAP1 also help to stabilize the t-loop, and are known to be negative regulators of telomere elongation and length maintenance in mammals (Cenci et al. 2005). An elaborate capping complex, which includes a Ku heterodimer, as well as the ATM kinase protein helps to protect the telomere end from fusion with other telomeres, and degradation by nucleases (Cenci et al. 2005; Hsu et al. 2000; Karseder et al. 2004; Song et al. 2000).
Like yeast telomeres, mammalian telomeres terminate in a t-loop consisting of a capping complex, and a series of highly repetitive DNA sequences which were added to the chromosome end by a telomerase complex. While the function of the capping complex appears to be conserved between yeast and mammals, the proteins that comprise it do not. With a few exceptions, the proteins comprising the yeast telosome are not conserved in mammals.
Mammalian telomeres are also clustered, and located at the nuclear periphery during interphase (Scherthan 2003; Broccoli and Cooke 1994). Recent evidence also indicates that mammalian telomeres exhibit TPE. Reporter genes located near telomeres in both mouse embryonic stem cells (Pedram et al. 2006), and human cell lines (Baur et al. 2004; Baur et al. 2001; Koering et al. 2002) are transcriptionally repressed, suggesting that TPE is a characteristic common to the telomeres of both yeast and mammals.

IV Current knowledge about Drosophila telomeres.

From the above summary, it seems that at least five features are common to both yeast and mammalian telomeres, despite differences in the protein constituents of the telosomes, and differences in the DNA sequences that comprise the telomeric and subtelomeric regions. These common features include: i) a method of maintaining telomere length involving a reverse transcriptase enzyme, ii) formation of a terminal loop structure, iii) formation of a capping complex to protect the chromosome ends, iv) clustering of telomeres, and localization of telomeres to the nuclear periphery, and v) repression of reporter genes inserted in or near telomeres (TPE). While information about Drosophila telomeres is relatively sparse compared to the wealth of information available from yeast and mammals, it is evident that at least some features of Drosophila telomeres are strikingly different from their yeast and mammalian counterparts. Perhaps the most conspicuous difference is the Drosophila method of maintaining telomere length, which uses transposable elements, rather than a telomerase complex.
IVA   Telomere length maintenance in Drosophila.

Telomere length is maintained in Drosophila by the sporadic transposition of at least two classes of retrotransposons, known as HeT-A and TART elements (BIESSMANN and MASON 1997; BIESSMANN and MASON 2003; BIESSMANN et al. 1997). These elements are copied from other locations in the genome, and transpose to the receding ends of chromosomes at a rate roughly sufficient to counterbalance the rate of erosion due to the end replication problem. As a result, Drosophila telomeres are comprised of tandem copies of HeT-A and TART elements, arranged in a head to tail fashion (BIESSMANN et al. 1994; BIESSMANN and MASON 1988; BIESSMANN et al. 1992). Since these elements are approximately six and 10 kb in length, respectively, the ends of Drosophila chromosomes are not as repetitive as the GC-rich sequences that characterize telomeres maintained by telomerase complexes.

The HeT-A element is the most common of the two retrotransposons, and transposes to chromosome ends at a rate approximately eight times greater than the TART element (SAVITSKY et al. 2002). HeT-A contains a single open reading frame encoding a GAG-like protein, which is capable of binding RNA, and is believed to escort the HeT-A element mRNAs into the nucleus (RASHKOVA et al. 2003; RASHKOVA et al. 2002). HeT-A, however, does not encode a reverse transcriptase (POL) enzyme, and is thought to require the help of another retrotransposon to provide this function in trans. The larger TART element contains two open reading frames, encoding both a GAG protein and a POL reverse transcriptase protein (LEVIS et al. 1993; SHEEN and LEVIS 1994), and is believed to be the source of the reverse transcriptase used by the HeT-A element. Thus,
although the HeT-A element is the principal constituent of Drosophila telomeres, the TART element is indispensable to telomere length maintenance since HeT-A cannot transpose without it.

Although this method of telomere maintenance does not appear to be used by either mammals or yeast, it is not necessarily an anomaly. Other important organisms use similar systems, including related drosophilids (Casacuberta and Pardue 2002; Casacuberta and Pardue 2003a; Casacuberta and Pardue 2003b), the industrially important silkworm Bombix mori (Okazaki et al. 1995), and the protists Giardia lamblia (Arkhipova and Morrison 2001), and Chlorella (Higashiyama et al. 1995). Furthermore, it is possible that many other organisms use this method as a ‘backup’ system for maintaining telomere length and healing the ends of broken chromosomes, but that this system remains dormant when a functional telomerase is present. Indeed, it has even been proposed that the telomerase enzymes presently used by higher organisms evolved from the reverse transcriptase enzymes of retrotransposons, and that this system of telomere maintenance predates the one currently used by mammals (Eickbush 1997).

Another interesting consequence of using transposable elements to maintain telomere length is that Drosophila telomeres, unlike those of yeast and mammals, are transcriptionally active, and are formed into nucleosomes. The HeT-A elements that constitute Drosophila telomeres are believed to be arranged into nucleosomes (Rubin 1978). Furthermore, these elements are believed to be transcriptionally active, and are
capable of activating reporter genes placed downstream of them (Golubovsky et al. 2001; Mason et al. 2003a; Mason et al. 2003b).

IVB Evidence for t-loop formation at Drosophila telomeres as part of a telomere repair mechanism.

At present, there is no direct evidence to suggest that Drosophila telomeres form t-loops under normal circumstances. However, the possibility of Drosophila telomeres forming loops under extraordinary circumstances has not been excluded. When Drosophila telomeres are broken or truncated, a system known as terminal gene conversion is used to repair them (Biessmann and Mason 2003; Biessmann et al. 1992). The mechanisms responsible for terminal gene conversion are not completely understood, but at least two models have been proposed (Biessmann and Mason 2003). In one model, the 5’ end of the truncated telomere is shortened, leaving a 3’ overhang, which then invades the homologous telomere, and uses it as a template for extension in trans. In the second model, the 3’ overhang can loop back to invade a homologous sequence on the same chromosome, using it as a template for extension in cis. Recall that the formation of t-loops in both yeast and mammals involves the backward folding of a single-stranded 3’ end, which, at face value, resembles the type of loop formed in this second model of Drosophila telomere repair. Examination of DNA sequences from repaired chromosome ends suggests that both types of repair can and do occur in Drosophila (Biessmann and Mason 2003).
The latter model suggests that Drosophila telomeres are capable of forming triple-stranded t-loops in response to damage. The former model places emphasis on the pairing of telomere homologs to maintain telomere integrity. While telomere loops have not been observed directly in Drosophila, telomere pairing is frequently observed. Such paired telomeres often appear to be connected by strands of DNA that correspond to the sequences found at telomere ends (SIRIACO et al. 2002). This observation is interesting in that it provides at least one explanation of why homologous telomeres are paired. Pairing of telomere homologs could ensure quick access to the template needed to repair a broken chromosome end. Additionally, both models emphasize the need for tandemly repeated sequences to be located near the ends of chromosomes, so that such sequences can be used as templates to repair broken telomeres.

IVC Genetic mutations that influence telomere length maintenance.

The use of classical genetics has always been one of the principal advantages to working with Drosophila as a model organism. Indeed, with the possible exception of yeast, no model organism can offer a broader array of classical genetic tools than Drosophila. As a result, most of the studies conducted on Drosophila telomere length maintenance have involved the generation and study of genetic mutations that alter telomere length equilibrium, giving rise to longer or shorter telomeres. The observed change in length has usually been attributed to mutations that cause either an increase or a decrease in the rate of HeT-A or TART transposition, or an increase or decrease in the frequency of terminal gene conversion.
In the case of HeT-A and TART transpositions, the question is made more complicated by the fact that the rate of HeT-A and TART transposition is presumably determined by both the HeT-A and TART transcription rates, and the frequency with which the telomere end is made available for HeT-A and TART additions. The latter variable would require removal or alteration of the telomere cap in order to expose the chromosome end. Since the telomere capping proteins must be either removed or rearranged to allow new HeT-A or TART elements to be added, the rate of HeT-A and TART additions would depend upon both the number of HeT-A and TART transcripts available for addition, and the rate at which the telomere end is exposed, allowing them to be added (CenCI et al. 2005; Melnikova et al. 2005; Perrini et al. 2004; Savitsky et al. 2002). It is reasonable to assume that both of these processes must be carefully orchestrated in order to maintain Drosophila telomeres at an appropriate length.

Interestingly, however, only one of the proteins known to cap Drosophila telomeres, the chromodomain protein Heterochromatin Protein 1 (HP1), has been shown to cause both telomere elongations and telomere fusions when its gene is either mutated or removed. Mutations to other genes tend to either increase the frequency of telomere fusion, or alter the rate of telomere elongation, but not both. This suggests one of two things. Either the telomere elongation rate is less dependent upon the rate of end exposure than current models predict, or very few of the proteins that form the telomere cap in Drosophila have been identified thus far using mutations.
In cases where an increase in telomere elongation is observed in a mutant genetic background, the increase in length can be attributed either to an increase in HeT-A and TART additions, or an increase in terminal gene conversion. Furthermore, since two types of terminal gene conversion are known, one in which a downstream sequence from the same chromosome is copied, and another where a sequence from the homologous chromosome is copied, there are at least three different methods by which a receding telomere can be extended. It should be noted that experiments measuring rates of HeT-A and TART transposition are usually conducted on broken chromosome ends, rather than intact ones. This is a consequence of the fact that, while there is no simple method for measuring telomere length in Drosophila, a simple genetic assay is available for measuring HeT-A additions to the end of a broken chromosome (Danilevskaya et al. 1997). The simplicity of this assay has made it the method of choice for biologists studying HeT-A transposition rates, but the results of these experiments should be viewed with this experimental bias in mind.

The following is a summary of gene mutations that cause alterations to the telomere elongation rate via one of the three methods mentioned above. Mutations to the gene encoding HP1 cause both increased telomere fusions (Fanti et al. 2003; Fanti et al. 1998a; Fanti et al. 1998b), and ectopic telomere elongation (Savitsky et al. 2002). The observed increase in telomere length resulted mostly from increased transcription and transposition of HeT-A elements, rather than from increased terminal gene conversion (Savitsky et al. 2002), although increases in terminal gene conversion (from sequences on the same chromosome) were also observed. HeT-A elements were seen to transpose
to a truncated telomere end at a rate approximately one hundred times greater than normal when the HP1-encoding gene was mutated (Savitsky et al. 2002). Although the increase in telomere fusions in HP1 mutant backgrounds suggests that the HP1 protein is critical for protecting chromosome ends, the fact that increases in HeT-A transcription were also observed implies that the HP1 protein may also be involved in regulating HeT-A transcription. So far, mutations to the HP1-encoding gene are the only mutations known to cause changes in HeT-A element transcription.

In contrast to HP1 mutations, mutations removing either the Ku70 or Ku80 subunits of the double stranded DNA-binding Ku heterodimer caused increases in both HeT-A and TART transposition to broken chromosome ends, and increases in terminal gene conversion of both types (Melnikova et al. 2005). Unlike HP1 mutations, Ku mutations caused an increase in HeT-A transposition that was not accompanied by an increase in HeT-A transcription. The authors suggested, therefore, that the increase in attachment of HeT-A elements to the broken ends of chromosomes was the result of increased exposure of these broken ends, rather than by increased numbers of HeT-A elements being produced (Melnikova et al. 2005).

Two other spontaneous mutations, known as Telomere elongation (Tel) and Enhancer of terminal gene conversion (E(tc)) were isolated from wild populations, and laboratory populations of Drosophila, respectively. Although the proteins that these mutations effect are unknown, both Tel and E(tc) mutations increase telomere length (Melnikova and Georgiev 2002; Siriaco et al. 2002). In the case of E(tc), an increased rate of
terminal gene conversion, rather than an increase in HeT-A or TART transposition is known to be the reason (MElnikova and Georgiev 2002).

**IVD** The Drosophila telomere capping complex.

As indicated above, genes involved in Drosophila telomere length maintenance were identified functionally, by the observed changes in telomere length that followed their mutation. By the same token, proteins that cap Drosophila telomeres have usually been identified functionally by their presence at telomeres, as detected by immunofluorescent microscopy, and by a greater incidence of telomere fusions being observed when they are altered or removed. Since such studies involve extensive use of microscopy, they tend to be very labor intensive. Accordingly, progress in this area has been slow. Nevertheless, at least six proteins, or protein complexes have been demonstrated to be present at Drosophila telomeres, or to cause telomere fusions when mutated or removed. These proteins include the chromodomain protein HP1 (Cenci et al. 2005; Fanti et al. 2003; Fant et al. 1998b), an associated protein known as HOAP (Cenci et al. 2003; Shareef et al. 2001), the ubiquitin-conjugating enzyme UBCD1 (Cenci et al. 1997), the ATM kinase (Bi et al. 2004; Oikemus et al. 2004; Silva et al. 2004), and the RAD50/MRE11 complex (Bi et al. 2004; Ciapponi et al. 2004).

The HP1 protein is one of the founding members of a small group of proteins ubiquitously found in heterochromatin (Hiragami and Festenstein 2005), and is usually associated with the centromeres and telomeres of most organisms, including Drosophila. The HOAP protein associates with both HP1 and members of the Drosophila **Origin**
Recognition Complex (CENCI et al. 2003), as does HP1 itself (PAK et al. 1997; SHAREEF et al. 2001). Although mutations to members of the ORC do not cause telomere fusions, it was noted that they do suppress TPE (Chapter Two), indicating a possible role for the ORC in telomere functioning as well. Removal of HOAP and/or HP1 leads to telomere fusions, as does removal of the ubiquitin-conjugating enzyme UBCD1 (CENCI et al. 1997), although, unlike the former two proteins, UBCD1 has never been observed at Drosophila telomeres.

Finally, the removal of three proteins, known to be involved in DNA break repair, also cause telomere fusions. Mutations to the genes encoding the MRE11 and RAD50 proteins cause both telomere fusions, and a reduction of HOAP and HP1 localization to the telomere ends (Bi et al. 2004; CIAPONI et al. 2004), suggesting that these proteins may participate in the capping complex. Mutations to the gene encoding the ATM protein also cause increases in telomere fusions, as well as suppression of TPE, and alterations to the localization of HP1 at telomeres (OIKEMUS et al. 2004; SILVA et al. 2004).

**IVE TPE in Drosophila.**

In addition to length maintenance and end protection, TPE appears to be a common property of telomeres. TPE has been observed in a number of organisms, including Drosophila (BOIVIN and DURA 1998; CRYDERMAN et al. 1999a; DONALDSON et al. 2002; MASON et al. 2004; WALLRATH and ELGIN 1995), Saccharomyces cerevisiae
(Gottschling et al. 1990; Palladino and Gasser 1994; Tham et al. 2001; Tham and Zakian 2000; Tham and Zakian 2002), *Schizosaccharomyces pombe* (Nimmo et al. 1994), *Trypanosoma brucei* (Horn and Cross 1995), mouse embryonic stem cells (Pedram et al. 2006), and human cells (Baur et al. 2004; Baur et al. 2001; Koering et al. 2002). Yet, despite its potential universality, TPE has only been studied extensively in *S. cerevisiae*. Presumably, this is a consequence of the fact that yeast experimental protocols involving both classical and molecular genetics are well established, and easy to use. However, extensive studies of TPE in higher organisms have not yet been undertaken.

Next to yeast, however, Drosophila is probably the most easily manipulated model organism, and a number of well established genetic and biochemical experimental protocols are available for doing similar experiments in Drosophila. Indeed, although TPE has not yet been studied extensively in Drosophila, a number of other forms of position effect have been studied in Drosophila for years, including an analogous form of gene repression known as Position Effect Variegation (PEV; (Grigliatti 1991; Wakimoto 1998; Wakimoto and Hearn 1990; Wallrath 1998; Wallrath and Elgin 1995; Weiler and Wakimoto 1995). PEV-type gene repression is seen when a chromosome re-arrangement brings a euchromatic gene near to a large block of heterochromatin, such as a centromere, or to a heterochromatic breakpoint. Mutations that alleviate this form of gene repression are said to ‘suppress’ PEV, and are known as Suppressor of variegation, or Su(var) mutations. The generation and study of Su(var)
mutations has led to the identification of many chromatin-associated proteins, as well as proteins involved in epigenetic gene regulation in Drosophila.

Su(var) mutations are identified with the help of a repressed reporter gene that has been moved near to either a centromere, or another large block of heterochromatin. Genes that affect easily scored phenotypes, such as eye pigmentation, or bristle morphology, are usually used for the assay. Random mutations are then introduced into the Drosophila genome, and mutations that are able to alleviate repression of the reporter gene are said to suppress PEV, and are classified as Su(var) mutations. When such mutations are investigated in greater detail, they frequently turn out to encode proteins that are either components of chromatin, or proteins that regulate chromatin structure and function.

The generation and study of Su(var) mutations has been ongoing for many years, and a large number of Su(var) mutations have already been generated and characterized. Given that many of these mutations probably encode chromatin proteins, it is possible that libraries of Su(var) mutations may already contain mutations that suppress TPE as well as PEV, and can be used to study Drosophila telomere structure and function. Thus, a potential treasure trove of mutations and mutant phenotypes already exists in the field of Drosophila research, and it should be possible to tap some of this wealth for a study of TPE in Drosophila. This was my initial approach, and is described in greater detail in Chapter Two.
While only a few of the proteins associated with the Drosophila telomere have been identified thus far, those that have been identified are, for the most part, different from those found at yeast and mammalian telomeres. Furthermore, while the Drosophila telomere is believed to form triple stranded t-loops in response to damage, it is not known whether t-loops are formed under normal circumstances. Also, the acetylation state of the nucleosomes that comprise the Drosophila telomere has not been determined, and it is not known whether Drosophila telomeres are hypoacetylated. Finally, perhaps the most distinctive characteristics of the Drosophila telomere is that its length is not maintained by a telomerase complex. Drosophila telomere length is maintained instead through the sporadic transposition of retrotransposable elements, known as HeT-A and TART elements.
As mentioned above, prior to the onset of this study, no exhaustive and systematic search for mutations capable of suppressing TPE in Drosophila had been reported. However, a handful of TPE-suppressing mutations had been identified in various studies having to do with individual genes and TPE reporter gene systems. A brief survey of potential TPE-suppressing gene candidates resulted in the identification of two loci capable of suppressing TPE when mutated (CRYDERMAN et al. 1999a; CRYDERMAN et al. 1999b; WALLRATH and ELGIN 1995). Mutant alleles of the genes Suppressor 2 of zeste (Su(z)2), and Posterior sex combs (Psc) were found to suppress TPE (CRYDERMAN et al. 1999a). Three additional TPE-suppressing genes were subsequently reported after I had completed my study. The Su(var)3-9 locus, encoding a histone methyltransferase enzyme (DONALDSON et al. 2002), as well as the atm (OIKEMUS et al. 2004) and gpp (SHANOWER et al. 2005) loci have since been identified as TPE-suppressing loci.

In addition to these individual mutations, a systematic deficiency screen, designed to find suppressors of TPE, was also carried out recently. This study highlighted several regions of the genome that suppress TPE when made hemizygous, and thus these regions may contain TPE-suppressing loci (MASON et al. 2004). However, the authors did not extend their search to the study of individual genes. The present study expands on the search for suppressors of TPE in Drosophila using both individual mutations, and smaller deficiencies.
V Overview of thesis and objectives.

Given that TPE appears to be a property common to the telomeric regions of many organisms, and that the exploitation of TPE has been used successfully to identify components of the yeast telosome, I decided to take an analogous approach to identifying protein components of the Drosophila telomere. Chapter Two details an extensive and systematic search to find genetic mutations capable of suppressing TPE in Drosophila. Overall, 32 candidate genes were identified by their ability to suppress TPE when mutated or made hemizygous.

The 32 genes highlighted in Chapter Two were divided into subsets, with some being potential candidates for telomere capping, while others were candidates for other telomeric properties, such as telomere length maintenance, perinuclear localization of telomeres, and telomere position effects. However, since an investigation into all of these aspects of telomere biology was beyond the scope of this project, I selected a subset of these candidates for a more detailed study. Given that gene silencing at subtelomeric regions appears to be a characteristic that is common to many organisms, and not just Drosophila, I decided to investigate in greater detail a subset of the gene candidates that encode proteins already known to be involved in gene silencing in other areas of the genome.

Specifically, the histone deacetylase protein HDAC1, the histone methyltransferase protein SU(VAR)3-9, and two orthologs of the HP1 protein were selected for a more
detailed study in Chapter Three. Both HDAC1 and SU(VAR)3-9 are believed to negatively regulate gene activity by modifying the ‘histone code’ (COSGROVE et al. 2004; COSGROVE and WOLBERGER 2005; MARTIN and ZHANG 2005). The histone code hypothesis posits that certain post translational modifications of the histone proteins that comprise nucleosomes determines the transcriptional competence of the genes associated with them. Specifically, acetylation of histone tails is associated with gene activation, while histone deacetylation, followed by subsequent histone methylation is associated with gene silencing. The HP1 protein is also believed to be involved in the silencing process, and is thought to interact with methylated nucleosomes.

I began by asking whether or not these three proteins were actually associated with the subtelomeric regions of Drosophila telomeres at the chromatin level, and found that they were. I then asked whether or not the histone tail modifications believed to be mediated by these proteins were also present, and found that they were also. I then discuss the potential implications of these sorts of chromatin modifications at telomeres as a possible explanation of TPE, and discuss a potential biological role for TPE in telomere length maintenance in Drosophila in the Discussion chapter of this thesis (Chapter Four).
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Chapter Two

A Genetic Screen to Find Mutations that Suppress Telomere Position Effect in Drosophila.¹

¹ A version of this chapter will be submitted for publication. Doheny, J., Mottus, R., and Grigliatti, T. Telomere Position Effect in Drosophila Melanogaster.
I. INTRODUCTION

Telomeres comprise one of the two main constituents of heterochromatin in the nucleus, with centromeres being the other. When euchromatic genes are moved near to telomeres they become repressed and silenced, a phenomenon known as Telomere Position Effect (TPE). The observed gene repression is thought to be a property of telomeric heterochromatin, and mutations capable of alleviating (or ‘suppressing’) TPE are believed to encode proteins that constitute telomeric heterochromatin, or regulate telomere biology in other ways. Thus, one elegant method of identifying proteins that constitute telomeric heterochromatin is to search for genetic mutations that have the effect of suppressing TPE. This chapter describes a search for TPE-suppressing mutations as a prelude to identifying protein components of telomeric chromatin. I refer to TPE-suppressing mutations hereafter as Suppressor of Telomere Position Effect, or Su(TPE) mutations.

The generation and study of Su(TPE) mutations has already been used successfully to identify telomere components in yeast (SCHULZ and ZAKIAN 1994; SINGER and GOTTSCHLING 1994; SINGER et al. 1998), but this method of identifying components of telomeric chromatin has not yet lived up to its full potential in Drosophila. Prior to the onset of this study, only two Drosophila gene loci were known to suppress TPE when
mutated (Wallrath and Elgin 1995; Cryderman et al 1999). One reason for the paucity of known Su(TPE) mutations in Drosophila is that a convenient TPE-suppression reporter gene assay was not available until relatively recently (Wallrath and Elgin 1995). Fortunately, however, other forms of position effect had been studied more extensively in Drosophila, including a phenomenon analogous to TPE known as **Position Effect Variegation** (Grigliatti 1991; Wakimoto 1998; Wakimoto and Hearn 1990; Wallrath 1998; Wallrath and Elgin 1995; Weiler and Wakimoto 1995).

PEV is a form of heterochromatin-mediated gene repression that occurs when a euchromatic gene is juxtaposed to a heterochromatic breakpoint through a chromosome rearrangement, such as a pericentric inversion. Mutations capable of suppressing this form of gene silencing are known as **Suppressor of variegation**, or Su(var) mutations. The generation and study of Su(var) mutations has been used successfully by Drosophila researchers to identify a number of chromatin components, as well as chromatin regulatory proteins (Clegg et al. 1993; Grigliatti 1991; Henderson et al. 1987; Reuter and Szidonya 1983; Reuter and Wolff 1981; Sinclair et al. 1983; Wakimoto 1998; Wakimoto and Hearn 1990; Wallrath 1998; Wallrath and Elgin 1995; Weiler and Wakimoto 1995). Furthermore, since the study of PEV in Drosophila has been ongoing for nearly a century, an impressive number of Su(var) mutations has already been generated and collected by several Drosophila research laboratories, including our own. Therefore, it seemed only logical to test our library of Su(var) mutations for the ability to suppress TPE as the first step in a search for Su(TPE)
mutations, as these Su(var) mutations are already known to alleviate the heterochromatin-mediated gene repression associated with PEV.

Testing a previously generated library of Su(var) mutations for the ability to suppress TPE also raises an obvious question as to whether or not the type of gene silencing associated with PEV is a property common to all forms of heterochromatin, or if PEV and TPE are different phenomena. Most Su(var) mutations were initially identified by their ability to alleviate repression of a reporter gene located near pericentric heterochromatin. If a large percentage of these Su(var) mutations were also able to suppress TPE, it would suggest that pericentric heterochromatin and telomeric heterochromatin are similar, and that TPE is simply another example of PEV. If, on the other hand, very few Su(var) mutations were able to suppress TPE, it would suggest that pericentric heterochromatin and telomeric heterochromatin are fundamentally different, and that TPE constitutes a unique form of gene silencing. This question had never before been addressed in Drosophila, but this study provided the ideal opportunity to do so.

In the first part of this chapter I describe the testing of a large library of Su(var) mutations for the ability to suppress TPE. The second part of this chapter describes an expansion of this search to find additional Su(TPE) mutations using a ‘candidate gene’ approach. In the gene candidate search for Su(TPE) mutations, a large number of promising candidate loci were tested for the ability to suppress TPE. Candidate loci selection was based on a number of current models and theories about nuclear structure and function, and genomic position effects, and drew heavily on information derived from TPE studies carried out
with other model organisms, such as yeast. As such, in addition to a continued screen to find new Su(TPE) mutations, this part of my study can also be viewed as a test of a number of these models and theories.

Overall, the primary goal of this screen was to find as many Su(TPE) loci as possible, identify and define the proteins they encode as possible candidates for an involvement in Drosophila telomere structure and function, and select one or more of the more interesting candidates for study in greater detail. The primary method of identifying such loci was to demonstrate suppression of TPE when they were either mutated or made hemizygous. However, since the first stage of my search involved testing a library of Su(var) mutations for the ability to suppress TPE as well as PEV, I was also able to ask if TPE and PEV are the same phenomenon, and indirectly, if telomeric heterochromatin and centromeric heterochromatin are similar in form and function. Thus, the secondary goal of these experiments was to determine the degree of overlap between TPE and PEV by asking how many TPE-suppressing mutations are also able to suppress PEV and vice versa.

In total, 100 loci were tested for the ability to suppress TPE and PEV using two Drosophila eye pigment assays that were designed specifically for this purpose. Only about 13% of the Su(var) point mutations were able to suppress TPE as well as PEV, suggesting that TPE is a phenomenon largely distinct from PEV, and also that telomeric chromatin is probably different from pericentric chromatin in composition. Overall, I identified 30 novel candidate loci that are capable of suppressing TPE when either
mutated or made hemizygous. One of these loci was discovered in parallel by another lab while the screen was in progress, and thus, only 29 of these candidates can truly be considered unique to this study. Two additional candidate loci were known previously, and two others were reported after this study was complete, bringing the current total of known Su(TPE) loci in Drosophila to 34. These loci were divided into categories, based on the kinds of proteins they encode, and the candidate proteins from one of these categories was then selected for a more detailed study in the Chapter Three of this thesis.
II MATERIALS AND METHODS

IIA Assay used to test suppression of TPE.

I tested suppression of TPE using the $y^I w^{118}; P[w^+] 39C-5$ reporter stock (abbreviated 39C-5) as previously described (Cryderman et al. 1999; Wallrath and Elgin 1995). The 39C-5 strain carries a mini-white reporter transgene, driven by a 'leaky' hsp70 promoter in the left telomere of the Drosophila second chromosome (referred to hereafter as the 2L telomere; Figure 2.1). The mini-white gene is a cDNA version of the indigenous white ($w^+$) gene, which is responsible for deposition of the red eye pigment drosopterin in the eyes of adult wild-type flies. Wild-type flies have dark red eyes when the $w^+$ gene is located at its native position (region 2D) in the euchromatin of the X chromosome. The mini-white reporter gene construct is located in the subtelomeric Telomere-Associated Satellite-like repeats (TAS; Walter et al., 1995) of the 39C-5 reporter strain, and is repressed due to TPE (Wallrath and Elgin 1995). The eyes of 39C-5 flies have a uniform pale yellow eye phenotype, with less than 2% of the pigment observed in wild-type flies (see Results section).

This telomeric reporter gene was chosen because it is the most highly repressed of all the available telomeric reporter genes (Cryderman et al. 1999; Wallrath and Elgin 1995), and was therefore less likely to give false positive results due to a low threshold for suppression of TPE. The repressed eye phenotype associated with 39C-5 is due to the repressive effects of telomeric heterochromatin, since the same reporter gene construct inserted into euchromatin gives nearly fully pigmented eyes, with approximately 87% of the pigmentation observed in wild-type flies (data not shown). The drop in eye
The TPE reporter gene construct is comprised of an *hsp70* promoter fused to a *mini-white* reporter gene cloned into a transposable element designated *P*[hsp26-pt, hsp70-w] (A). The *mini-white* gene mediates red pigment (drosopterin) deposition in the Drosophila eye, and is a derivative of the indigenous *w}* gene with introns removed. The construct also carries a second reporter gene comprised of a barley gene driven by an *hsp26* promoter, but this reporter gene was not used in the present study.

This reporter gene construct was mobilized in the Drosophila genome, and successfully integrated into several locations, including the left telomere of the second chromosome (WALLRATH AND ELGIN 1995). In this stock, designated 39C-5, the reporter gene construct has integrated into the moderately repetitive subtelomeric TAS region at the 2L telomere (B), which is flanked on the distal side by two or more mobile genetic elements known as HeT-A elements, and on the proximal side by the *lethal (2) giant larvae (l(2)gl)* gene (WALLRATH AND ELGIN 1995; WALTER et al. 1995; CRYDERMAN et al. 1999). Direction of transcription for the various genes is indicated by arrows.

**Figure 2.1. TPE reporter gene construct.**
pigmentation associated with the repressed telomeric reporter gene is concomitant with 
an equivalent drop in transcription, and a reduction in accessibility of the reporter gene to 
restriction enzymes and nucleases (BOIVIN and DURA 1998; CRYDERMAN et al. 1999).

Various mutations and deficiencies were introduced into the 39C-5 strain, and any 
discernable suppression of TPE was recorded. Suppression of TPE was scored as strong 
(+++), moderate (++), weak (+), or no suppression (-) by visual inspection of fly eyes 
under a dissecting microscope. In order to confirm the validity of this scoring regime, 
pigment quantification assays were carried out (described below) on a selection of the 
TPE-suppressing mutations. Strong suppression of TPE corresponded to drosopterin 
levels approximately 300% greater, moderate suppression approximately 200% greater, 
and weak suppression approximately 100% greater than control pigment levels in a wild-
type genetic background. ‘Control pigment levels’ refers to the amount of pigmentation 
present in the eyes of a 39C-5 fly, carrying one copy of the telomeric reporter gene, in the 
absence of a suppressor mutation. The amount of eye pigment in such flies was found to 
be approximately 2% of wild-type (see Section IIE for details of the eye pigment 
quantification assay). Thus, the range of this particular assay is between 2% and 8% 
percent of maximum eye pigmentation, with 2% pigmentation corresponding to the 
amount of pigment present in the absence of a TPE-suppressing mutation. Although this 
range of pigmentation is narrow compared to the range available from the PEV assay 
described below), when test flies are scored next to internal control flies from the same 
mating (also described below) the differences in pigmentation are consistent and 
relatively easy to discern.
Figure 2.2. Generation of \( w^- \) stocks for second chromosome mutations and deficiencies.

Mutations and deficiencies were crossed into a \( w^- \) genetic background. For second chromosome mutations and deficiencies, construction of \( w^- \) stocks was carried out in three steps: 1) an individual male carrying the mutation was crossed to a white-eyed female carrying second chromosomes marked with the dominant markers \( Tft \) (Tufted), and \( CyO \) (Curly of Oster; \( In(2LR)O \), \( Cy \) \( dp^{bl} \) \( pr \) \( cn^2 \)). 2) an individual white-eyed male carrying the mutation and the \( Tft \) marker was then crossed to the same strain of female. 3) White-eyed progeny from this cross that carried the \( CyO \) marker but not the \( Tft \) marker were intercrossed to generate the stock.
Figure 2.3. Generation of \( w^- \) stocks for third chromosome mutations and deficiencies.

For third chromosome mutations and deficiencies, generation of \( w^- \) stocks was carried out in three steps: 1) an individual male carrying the mutation or deficiency was crossed to a white-eyed female carrying third chromosomes marked with the dominant markers \( Gl \) (Glued) and \( TM3 \) (\( In(3LR)TM3, y^+ ri p^p sep, Sb, Ser \)). 2) an individual white-eyed male offspring from this cross, carrying the \( Gl \) mutation but not the \( TM3 \) marker, was then crossed to the same strain of female. 3) white-eyed progeny from this cross that carried the \( TM3 \) marker but not the \( Gl \) marker were then intercrossed to generate the stock.
IIB Assay used to test suppression of PEV.

Suppression of PEV was tested using the inversion strain *In (1) wm4 (w^md)*, which has traditionally been used for this purpose (CLEGG et al. 1993; HENDERSON et al. 1987; REUTER and SZIDONYA 1983; REUTER and WOLFF 1981; SINCLAIR et al. 1983). The *w^md* stain carries a pericentric inversion of the X chromosome, which brings the endogenous *w^+* gene into close proximity to a broken fragment of X chromosome pericentric heterochromatin. As a result of its proximity to pericentric heterochromatin the *w^+* gene is subject to PEV, and lower levels of eye pigmentation are observed. Drosopeterin levels in the eyes of *w^md* strains are approximately 2 to 5% that of wild-type. Suppression of PEV by strong Su(var) mutations returns drosopeterin levels to approximately 100% of wild-type (see Results section).

Suppression of PEV was also scored as strong (+++), moderate (++), weak (+), or no suppression (-). Pigment assays on Su(var) mutations indicated that strong suppression of PEV corresponded to drosopeterin levels about fifty fold higher, moderate suppression 20 to 30 fold higher, and weak suppression 10 to 20 fold higher, than that observed in the *w^md* strain in the absence of Su(var) mutations (see Results section).

IIC Generation of w^-stocks for testing.

Because both the 39C-5 (TPE) and *w^md* (PEV) reporter strains use variations of the *w^+* gene as a reporter for position effects, it was necessary to first move all mutations and deficiencies to be tested into a w^- genetic background so that the indigenous *w^+* gene would not interfere with the eye pigment assays. This essentially involved moving the
chromosome bearing the mutation or deficiency to be tested into fly stocks which already had a complete deficiency for the $w^+$ gene. The procedure for moving the second chromosome is illustrated in Figure 2.2, and the procedure for moving the third chromosome is illustrated in Figure 2.3. These procedures had the added benefit of moving the mutations into a more common genetic background, and reducing the possibility of false positive results due to the presence of any un-recorded second site mutations that may have been present in the original stock. However, it should be noted that, while most of the chromosomes present in the original stock (as received from the stock centre) were replaced, the possibility of second site suppressor mutations being present on the chromosome carrying the mutation in question, and being responsible for any observed suppression cannot be ruled out.

IID Crosses used to score suppression of TPE and PEV included an internal wild-type standard.

After the various mutations and deficiencies were moved into a $w^-$ genetic background, these lines were crossed to the $39C-5$ and $w^{md}$ reporter stocks, and suppression of TPE and/or PEV was scored in the F1. Reciprocal crosses (male mutant crossed to female reporter, and vice versa) were carried out to test for maternal and paternal effects. Suppression of TPE was scored in the F1 by comparing the reporter gene (eye pigment) in a mutant background to both the reporter gene in a $w^-$ background (as a negative control), and to the reporter gene in balanced siblings who had received the balancer chromosome rather than the mutant chromosome (an internal wild type standard). This
protocol is illustrated in Figure 2.4. An analogous protocol, illustrated in Figure 2.5, was used to test for suppression of PEV.

Some mutations were homozygous viable and did not require the use of a balancer chromosome to maintain the mutation in a fly stock. Nonetheless, the final step in crossing these mutations into a $w^-$ background was to select for the mutation over a balancer chromosome, thus generating a mutant line that was temporarily heterozygous. These temporarily heterozygous lines were used for the eye pigment assays. In all cases, mutant stocks were tested for suppression of TPE and PEV immediately after the new strain was created to avoid any possible genetic drift, or accumulation of spontaneous second site Su(TPE) or Su(var) mutations in the genetic backgrounds of these stocks.

IIE Eye pigment assays.

Eye pigment assays were carried out as follows: Flies, aged two weeks, were placed in a glass screw cap tube, flash frozen in liquid nitrogen, and then decapitated by vortexing the tube. Ten heads were placed into a 1.7 ml eppendorf tube with 200 uL of 0.1% ammonium hydroxide. Heads were then homogenized with 20 strokes of a miniature Teflon pestle, sonicated with three five second pulses of a midi-tip sonicator set to 30% power output, and extracted against 100 uL of chloroform. Solid debris was removed by centrifugation, and the absorbance of the chloroform phase was measured in a spectrophotometer set to 485 nm, within the linear range of the machine. Samples were measured in quadruplicate.
Figure 2.4. Procedure for the TPE-suppression assay.

Flies hemizygous for a *mini-white* reporter gene embedded in the 2L telomere had pale yellow eyes. When a male homozygous for the reporter gene (top left) was crossed to a female carrying a ‘balanced’ mutation (top right), progeny receive either the balancer chromosome (in this case *CyO*; middle left) or the mutation (middle right), and are easily distinguished by wing phenotype. When TPE is suppressed by the mutation in question, flies have darker eyes than their balanced siblings (middle right and left). If the mutation fails to suppress TPE, both types of siblings have pale yellow eyes (bottom). Second chromosome mutations were balanced with the *CyO* balancer chromosome marked by a dominant ‘curly wing’ phenotype. Third chromosome mutations were balanced with the *TM3* balancer chromosome marked by a dominant ‘stubble bristle’ phenotype. (note: reciprocal crosses were also carried out, with females carrying the reporter genes.)
Figure 2.5. Procedure for the PEV-suppression assay.

Flies carrying the $w^{m4}$ inversion have ‘mottled’ eyes. When a male carrying a ‘balanced’ mutation is crossed to a female homozygous for the $w^{m4}$ reporter gene, progeny receive either the balancer chromosome (in this case $CyO$) or the mutant chromosome. These two types of offspring are easily distinguished by the difference in wing shape. When PEV is suppressed by the mutation, non-balanced offspring (middle right) have darker eyes than their balanced siblings (middle left). If the mutation fails to suppress PEV, both types of siblings have mottled eyes (bottom). Reciprocal crosses were also carried out, where fathers carried the $w^{m4}$ reporter gene and mothers carried the mutation. However, in this case only female offspring were scored since the male offspring did not receive the reporter gene carried on the X chromosome. Second chromosome mutations were balanced with the $CyO$ balancer chromosome, marked by a dominant ‘curly wing’ phenotype. Third chromosome mutations were balanced with the $TM3$ balancer chromosome, marked by a dominant ‘stumble bristle’ phenotype.
Although the PEV-suppression assay using the $w^{md}$ reporter construct gives fairly consistent results, I noted that the TPE-suppression assay using the $39C-5$ reporter construct showed a certain amount of variation from one experiment to another. However, the observed variation could be minimized by using $39C-5$ virgin females that were the same age, and were collected from the same cross. Comparing the eye pigmentation levels of flies that arose from different crosses, or using different ‘batches’ of virgin females is not recommended, and results in an unacceptably high degree of experimental variability.

### IIF  Fly crosses.

All crosses were performed at $22^\circ$C. Higher temperatures were avoided, as our lab has observed that increased temperatures tend to suppress PEV, even in the absence of a suppressor mutation (T. Grigliatti and R. Mottus, personal communications). Flies were reared on standard cornmeal/sucrose media supplemented with antibiotics, and 0.04% TegoSept$^\text{TM}$. TegoSept, rather than propionic acid was used as a fungal inhibitor because propionic acid also suppresses position effect, and leads to false positive results (data not shown). Crosses were carried out by crossing three groups of five virgin females (from the same virgin collection) to an equal number of males in three separate vials. Parents were transferred to fresh vials twice, at three day intervals, to avoid overcrowding of the offspring, as overcrowding tends to enhance position effect (data not shown).
Some mutant and deficiency stocks were generated in our lab, and others were gifts from other investigators, but most were obtained directly from the Bloomington Stock Centre. Bloomington stock numbers for mutant stocks are given in the tables. Deficiency stocks were chosen based on the cytological locations of the loci being tested, as reported by the Berkeley Drosophila Genome Project (BDGP) database (www.flybase.org). In all cases, the smallest possible deficiencies available were chosen, as larger deficiencies tend to produce false negative results (as discussed in the Results section). Wherever possible, several deficiencies for the same locus were tested.

IIIG  Protein homology searches.

In cases where putative fly homologs of proteins found in other organisms were tested, homologs were identified using amino acid sequences entered into the flybase Drosophila genome search engine (http://www.flybase.org/blast/), with all parameters set to ‘default.’

IIH  Lethal complementation analysis.

Complementation analysis is used to determine if two different mutations are actually alleles of the same gene, or if a gene is located within the region removed by a deficiency. This technique is particularly useful in cases where mutant alleles of the gene in question are not homozygous viable, as was the case for most of the mutant alleles and deficiencies tested in this study. Such mutations must be maintained over a multiply inverted homologous ‘balancer’ chromosome, such that at least one functional copy of the gene (supplied by the balancer chromosome) is present in the genome at all times.
Balancer chromosomes are marked with dominant phenotypic markers, and the presence of the balancer chromosome is easily determined from the phenotype of the fly. If two mutant fly stocks are crossed, and no non-balanced offspring appear, the two mutations are presumed to be mutations of the same gene. If, on the other hand, significant numbers of non-balanced offspring appear (greater than five from a total of 200 scored), the two mutations are said to ‘complement’ one another, and are presumed to be mutations of two different genes.

In addition to determining whether or not two different mutations are actually mutations to the same gene, this technique can also be used to determine whether or not a gene is located within a region that is removed by a deficiency. This technique is known as ‘deficiency mapping.’ In this case, a balanced homozygous-lethal mutation is crossed to a homozygous-lethal deficiency, and failure to complement is taken as evidence that the mutated gene maps within the region removed by the deficiency.

In the case of second chromosome mutations or deficiencies, stocks balanced over the $CyO$ balancer chromosome ($In(2LR)$, $Cy\ dp^{bl}\ pn\ cn^2$ ‘Curly of Oster’) were crossed, and the complete absence of straight-winged offspring was scored as a failure to complement (-). Complementation (+) was scored when straight-winged offspring appeared in the F1 of such a cross. The same technique was used for third chromosome mutations and deficiencies, except that the $TM3$ balancer chromosome ($In(3LR)TM3$, $y^+\ ri\ p^9\ sep\ Sb$ ‘Third Multiple’) was used instead of the $CyO$ balancer. A minimum of 100 offspring
were scored, and the complete absence of non-balanced offspring was taken as a failure to complement.
III RESULTS

The $w^+$ gene is located on the Drosophila X chromosome, and is responsible for deposition of the red eye pigment drosopterin into the eyes of adult flies, causing wild-type flies to have dark red eyes. The $w^+$ gene is used as a reporter gene in both the TPE and PEV assays described below. In the case of the TPE assay the $w^+$ gene is removed from its native position on the X chromosome, and an equivalent eye pigment gene is relocated to telomeric heterochromatin, where it is repressed as a result of TPE. In the case of the PEV assay the $w^+$ gene has been relocated near to pericentric heterochromatin where it is repressed as a result of PEV. In both cases, eye pigmentation levels are reduced to less than 20% wild-type flies. Mutations that cause eye pigmentation levels to increase again are collectively referred to as ‘suppressor’ mutations, because they suppress the mutant phenotype associated with either TPE or PEV.

Suppression of TPE was tested by introducing various mutations and deletions (also known as deficiencies) into the 39C-5 reporter strain which carries a repressed $w$ gene located in the 2L telomere. Gene expression is dramatically reduced, and the eyes of this strain have a uniform pale yellow appearance (Figure 2.6), containing less than 2% of the drosopterin seen in wild-type eyes (data not shown). Suppression of TPE by Su(TPE) mutations causes an increase in eye pigmentation, resulting in a light orange eye colour. Suppression of TPE by a previously identified Su(TPE) mutation is shown in Figure 2.6. Quantitative eye pigment assays were carried out on a selection of Su(TPE) mutations,
confirming that the introduction of Su(TPE) mutations into the genome of the 39C-5 reporter strain resulted in an increase in eye pigmentation (Figure 2.7).

Suppression of PEV was tested by introducing various mutations and deletions (also known as deficiencies) into the \( w^{md} \) reporter strain, which carries a repressed \( w \) gene located near the X chromosome centromere. As in the 39C-5 stock, the eyes of \( w^{md} \) flies contain only about 2-5% as much drosopetin as do wild-type eyes. However, gene repression by pericentric heterochromatin leads to a slightly different phenotype than gene repression by telomeric heterochromatin. Some eye facets are fully pigmented while others have no pigment at all, leading to a salt and pepper (or ‘mottled’) appearance. Examples of suppression of PEV by previously identified Su(var) mutations are shown in Figure 2.8. Quantitative eye pigment assays were also carried out on a selection of the Su(var) mutations, the results of which are illustrated in Figure 2.9.

Various mutations and deficiencies were introduced into these stocks, and suppression of TPE or PEV was scored as strong (+++), moderate (++), weak (+), or no suppression (-), and tabulated in tables 2.1 through 2.8. For purposes of organization and discussion, the mutations analyzed are placed into groups based on their known or presumed function, and each group is given a separate table. The relative suppression of either PEV or TPE is indicated in the two columns at the far right of the table. The type of gene mutation (point mutation or deficiency) is also indicated, along with the cytological location of the gene locus. In cases where the original mutant strain was obtained from the Bloomington
In the TPE reporter stock a mini-white reporter gene is embedded in the subtelomeric heterochromatin of the left telomere of chromosome two (Wallrath and Elgin 1995). In the absence of a TPE-suppressing mutation the reporter gene is highly repressed, and flies have pale yellow eyes (A, left). In the presence of a TPE-suppressing mutation, such as Su(2)5, the reporter gene is partially de-repressed, and flies have orange eyes (A, right). When a female homozygous for the reporter gene is crossed to a male carrying a balanced mutation (or vice versa) half of the offspring receive the mutation and the other half receive the balancer chromosome. Balanced siblings can then be used as an internal wild type standard for eye pigmentation. (A) Strong (+++) suppression of TPE by the Su(2)5 allele. w/Y; reporter gene/CyO (left), and a w/Y; reporter gene/ Su(2)5 sibling (right). (B) Weak (+) suppression of TPE by the Su(z)De26 allele. w/Y; reporter gene/ CyO (left), and a w/Y; reporter gene/ Su(z)De26 sibling (right). (C) non-suppression (-) of TPE by Su(var)2-5 (HP1a). w/Y; reporter gene/ CyO (left), and a w/Y; reporter gene/ Su(var)2-5 sibling (right) have identical pale yellow eyes.
Figure 2.7. Suppression of TPE by Su(TPE) mutations in the 39C-5 reporter strain.

Eye pigment assays were carried out for selected mutations to test for suppression of TPE by Su(TPE) mutations. In a wild-type genetic background, the TAS-embedded telomeric reporter gene is highly repressed, and the eyes of such flies have approximately 2% as much pigmentation as do wild-type flies carrying the \( w^+ \) mutation at its normal position in euchromatin. The 2% pigmentation level of the reporter stock in a wild-type genetic background was set as the background level of the assay (bottom bar), and all other values were normalized to it. Thus, the level of eye pigment found in flies carrying Su(TPE) mutations is reported as a fold increase above the 2% background level. (For illustrative purposes, the background level of the assay was set to zero.) Introduction of various mutant \( Su(z)2 \) and \( Psc \) alleles into the TPE reporter stock caused increases in eye pigmentation as shown, and all \( Su(z)2 \) and \( Psc \) mutants shown here had significantly higher levels of eye pigmentation ( \( p < 0.05 \) ) than 39C-5 reporter stock flies in the absence of a Su(TPE) mutation, or in the presence of a \( Su(var)2-1 \) mutation which did not suppress TPE. (n=4; error bars represent standard deviations.)
Figure 2.8. Suppression of PEV in the \( w^{md} \) reporter strain.

In the PEV reporter stock a pericentric inversion brings the \( w^+ \) gene near to the X-chromosome centromere, resulting in a repressed and “mottled” pattern of eye pigmentation (A, left). In the presence of a Su(var) mutation, eye pigmentation increases (A, right). When a female homozygous for the reporter gene is crossed to a male carrying a balanced Su(var) mutation, half of the offspring receive the mutation and half receive the balancer chromosome. Again, balanced siblings can be used as an internal wild type standard for eye pigmentation. (A) Strong (+++ suppression of PEV by a Su(var)2-5 (HP1a) mutations. \( w^{md}/w^-; +/CyO \) (left), and a \( w^{md}/w^-; +/Su(var)2-5 \) sibling (right). (B) Strong to moderate suppression by an Su(var)2-5 deficiency. \( w^{md}/Y; +/CyO \) (left), and a \( w^{md}/Y; +/Df (28E4-7; 29B2-C1) \) sibling (right). (C) Moderate (++) suppression by a putative Lamin B Receptor homolog \( (CG17952) \) deficiency. \( w^{md}/w^-; +/CyO \) (left), and a \( w^{md}/w^-; +/Df (57F2; 58A1) \) sibling (right).
Eye pigment assays were carried out for a selection of Su(VAR) mutations to test for suppression of PEV. In the mutant $w^m4$ fly strain, a pericentric inversion has brought the $w^+$ gene into close proximity to pericentric heterochromatin, and eye pigmentation levels are only about 2 to 5% of wild-type. Introduction of Su(var) mutations into the $w^m4$ stock are able to alleviate the gene repression associated with PEV, and eye pigment levels increase. The three mutant $Su(var)3-9$ alleles are able to return eye pigmentation to wild-type levels. Hemizygosity for the histone cluster (HIS-C) removes half of the histone genes from the genome, and causes a moderate suppression of PEV, resulting in eye pigment levels only slightly higher than $w^m4$ in a wild-type genetic background ($p < 0.05$). ($n=4$; error bars represent standard deviations.)
Drosophila Stock Centre, the stock number is also indicated in the table. However, as mentioned in the Materials and Methods section, no mutant stocks were tested for suppression of PEV or TPE in their original genetic background. Rather, mutations were moved into a more uniform genetic background before testing, to control for the possibility of second site mutations.

IIIA  Suppression of TPE by previously identified Su(var) mutations.

I began my search for Su(TPE) mutations by testing a library of previously identified Su(var) mutations for the ability to suppress TPE. Random mutagenesis screens, carried out in our lab as well as others, have resulted in the identification of over 30 dominant Su(var) mutations (Clegg et al. 1993; Henderson et al. 1987; Reuter and Szidonya 1983; Reuter and Wolff 1981; Sinclair et al. 1983). While only about ten of the genes associated with these mutations have been cloned thus far, most are known to encode basic components of chromatin, or proteins that regulate chromatin structure. I began by testing the Su(var)s that have been cloned and whose functions are known (Table 2.1), and then tested the Su(var)s which have not yet been cloned (Table 2.2).

IIIAi  Cloned Su(var)s (Table 2.1).

I began my analysis with 7 Su(var) genes that have been cloned and characterized at the molecular level. The Su(var)3-9 locus encodes the histone H3 lysine 9 specific methyltransferase (HMTase) SU(VAR)3-9, believed to be involved in gene silencing. I tested 11 alleles of Su(var)3-9 for the ability to suppress both PEV and TPE (Table
Table 2.1

Suppression of PEV and TPE by cloned Su(var) genes.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock(^a)</th>
<th>mutation</th>
<th>suppression of PEV</th>
<th>TPE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Su(var)3-9 alleles(^b)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-9(^{306})</td>
<td>88E6-8</td>
<td>na(^c)</td>
<td>null</td>
<td>+++</td>
<td>++</td>
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<td>na(^d)</td>
<td>D536N (SET domain)</td>
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<td>C462Y (preSET domain)</td>
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<td>“</td>
<td>“</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>3-9(^{324})</td>
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<td>“</td>
<td>C428Y (preSET domain)</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>3-9(^{311})</td>
<td>“</td>
<td>“</td>
<td>G521D (SET domain)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>3-9(^{318})</td>
<td>6209</td>
<td>“</td>
<td>R493Q (SET domain)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>3-9(^{325})</td>
<td>“</td>
<td>na(^d)</td>
<td>P582Q (SET domain)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>3-9(^{245})</td>
<td>“</td>
<td>“</td>
<td>S616L (postSET domain)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>3-9(^{21})</td>
<td>“</td>
<td>“</td>
<td>P element insertion into first exon</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><strong>B) Hdacl alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDAC1(^{326})</td>
<td>64B12</td>
<td>na(^e)</td>
<td>P204S</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>HDAC1(^{303})</td>
<td>“</td>
<td>“</td>
<td>C98Y</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>HDAC1(^{311})</td>
<td>“</td>
<td>“</td>
<td>R30C</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>C) other cloned Su(var)s</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var)3-7</td>
<td>87E3</td>
<td>na(^c)</td>
<td>small deficiency</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>“</td>
<td>“</td>
<td>3009</td>
<td>Df: 87E1; 87F12</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>HP1a</td>
<td>28F1-2</td>
<td>na(^f)</td>
<td>Point mutation (Su(var)2-5(^f))</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>“</td>
<td>“</td>
<td>“</td>
<td>(Su(var)2-5(^f))</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>“</td>
<td>“</td>
<td>179</td>
<td>Df: 28E4-7; 29B2-C1</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Su(var)2-10(^{2})</td>
<td>45A8</td>
<td>5526</td>
<td>point mutation</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Su(var)2-10</td>
<td>“</td>
<td>4966</td>
<td>Df: 45A6-7; 45E2-3</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>puc</td>
<td>84E12</td>
<td>na(^g)</td>
<td>P insert mutation</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>abo</td>
<td>32C1</td>
<td>2525</td>
<td>point mutation</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)stock= refers to the Bloomington Stock Centre identification number.

\(^b\)Su(var)3-9 alleles sequenced by P. Kalas (manuscript in preparation)

\(^c\)Su(var)3-9\(^{300}\) a gift from G. Reuter

\(^d\)Grigliatti lab constructs

\(^e\)Mottus et al. 2000

\(^f\)a gift from J. Mason

\(^g\)Toub, manuscript in preparation
2.1A). All $Su(var)3-9$ mutations were strong dominant suppressors of PEV, and 9 of the 11 alleles also suppressed TPE. As mentioned in the Materials and Methods section, the TPE-suppression assay has a very high threshold, and a relatively narrow range of suppression when compared to the PEV-suppression assay. Hence, the TPE-suppression assay is probably more likely to yield false negatives than the PEV-suppression assay, and it is possible that the remaining two $Su(var)3-9$ alleles suppressed TPE at a level that was below the threshold for this assay.

An alternative explanation for the observed allelic differences would be that some of the amino acid residues in the protein might be more important than others to the process of TPE in Drosophila. Nevertheless, I am reasonably confident that $Su(var)3-9$ mutations suppress TPE. Suppression of TPE by the $Su(var)3-9^{330}$ allele, which is known to be an extreme hypomorph (P. Kalas, manuscript in preparation) is shown in Figure 2.10. Quantitative eye pigment assays showing suppression of TPE by $Su(var)3-9$ mutations are illustrated in Figure 2.11.

Note that the strength of TPE suppression caused by the $Su(var)3-9^{064}$ null mutation is no stronger than that caused by the $Su(var)3-9$ point mutations, and is actually a weaker suppressor of TPE than the $Su(var)3-9^{330}$ point mutation. Furthermore, the $Su(var)3-9^{064}$ mutation is homozygous viable, while the point mutations are not. Interestingly, a similar situation was observed with a number of the $Hdac1$ mutations (Mottus et al. 2000), and suggests that such proteins may participate in large complexes, and that complete removal of the protein may results in a similar protein being substituted for the missing
protein in the same complex. Such a situation would be expected to produce a less severe mutant phenotype than point mutations which destroy the biological activity of the protein in question, while not preventing its assembly into the protein complex (Mottus et al. 2000).

The *Hdac1* locus encodes a histone deacetylase (HDAC) capable of removing acetyl groups from lysine residues in both histones H3 and H4. Our lab had previously generated 3 different missense mutations in this gene, each causing a different single amino acid substitution, all of which were strong Su(var)s (MOTTUS et al. 2000). I tested each of these 3 mutants for their effects on TPE. All alleles of *Hdac1* suppressed TPE, with some allelic differences again being observed (Table 2.1B).

The *Su(var)3-7* locus encodes a protein of unknown function, containing an unusual zinc finger motif (REUTER et al. 1990), that localizes primarily to centromeric heterochromatin (CLEARD et al. 1997). I tested a small deficiency that removes only *Su(var)3-7* and one adjacent gene; and found that it failed to suppress TPE (Table 2.1C). I also tested a larger deficiency that removes several genes, including *Su(var)3-7*, but noted only a weak suppression of TPE. Since the smaller deficiency did not suppress TPE, I believe that *Su(var)3-7* mutations do not suppress TPE.

The *Su(var)2-5* locus encodes the HP1 protein. HP1 is a widely conserved chromatin protein that is frequently found associated with centromeric heterochromatin. The protein has two signature domains, known as the chromodomain and the chromoshadow
domain, connected by a variable hinge region. The chromodomain derives its name from the fact that it is found in several proteins that are associated with chromatin, including the HP1 protein and the Polycomb (PC) protein (discussed in the next section). I tested two point mutations and a deficiency for \textit{Hp1}, but none of these suppressed TPE (Table 2.1C). The \textit{Su(var)2-5} \textsuperscript{5} allele is a complete null, while the \textit{Su(var)2-5} \textsuperscript{4} allele is a functional null, lacking a nuclear localization signal, so that it is unable to enter the nucleus (Eisenberg and Hartnett 1993; Eisenberg et al. 1992). None of these mutations suppressed TPE, and I believe, therefore, that HP1 mutations are not Su(TPE)s. These findings are in support of an earlier observation by Cryderman \textit{et al.} (1999), who showed that mutations of the HP1 protein did not suppress TPE.

This result was somewhat unexpected, given that HP1 is believed to be present at telomeres, and to participate in telomere capping. Using immunolocalization studies, other researchers have shown that HP1 localizes to both Drosophila and mammalian telomeres, and that mutations to the \textit{HP1} gene increase the frequency of telomere fusions, and also increase the transposition rates of the \textit{HeT-A} and TART elements in Drosophila (Fanti \textit{et al.} 1998; Savitsky \textit{et al.} 2002). It is interesting therefore that mutations of the HP1 gene do not suppress TPE, and suggests that the telomere capping and telomere length maintenance functions may be independent of whatever mechanism is responsible for TPE. It is also interesting that mutations to \textit{Su(var)3-9} suppress TPE, while mutations to the HP1-encoding locus do not, since it is widely believed that SU(VAR)3-9 and HP1 interact and cooperate to mediate heterochromain-associated gene silencing in Drosophila (Ebert \textit{et al.} 2006; Schotta \textit{et al.} 2003; Schotta \textit{et al.} 2002).
Figure 2.10. Suppression of TPE by *Su(var)3-9* mutations in the 39C-5 reporter strain.

Suppression of TPE by certain *Su(var)3-9* mutations (Table 2-1A) is at least as strong as suppression of TPE by individual *Psc* and *Su(z)2* mutations (Table 2-3). *w/Y; reporter gene/+; +/+* (top left), *w/Y; reporter gene/+; +/TM3* (top right), and a *w/Y; reporter gene/+; Su(var)3-9330/+* sibling (bottom).
Figure 2.11. Suppression of TPE in the 39C-5 reporter strain by various Su(var)3-9 mutant alleles.

Eye pigment assays performed on eight of the eleven Su(var)3-9 mutations tested (Table 2-1) indicated that all were suppressors of TPE, and caused eye pigment levels in the 39C-5 reporter strain to be significantly higher than that seen in either a wild-type genetic background, or a mutant Su(var)2-1 allele that was used as a negative control (p < 0.05). (n=4; error bars represent standard deviations.)

Mutations in the Su(var)2-10 locus have been shown to suppress PEV, and subsequent cytological analysis has indicated that the protein encoded by the Su(var)2-10 locus is
associated with Drosophila telomeres (Hari et al. 2001). Furthermore, Su(var)2-10 mutant nuclei show defects in telomere clustering, as well as altered telomere–nuclear lamina associations (Hari et al., 2001). Thus, the Su(var)2-10 locus was an ideal candidate for testing. I tested the only available point mutation of the Su(var)2-10 gene, and a deficiency for the Su(var)2-10 locus, and found that both were moderate suppressors of TPE (Table 2.1C).

The puckered (puc) gene encodes a protein phosphatase that has a known role in the JNK kinase pathway. I have found that mutations in puc are strong suppressors of PEV (Toub, manuscript in preparation). I tested one allele which is a strong Su(var), but it had no effect on TPE (Table 2.1C).

The abnormal oocyte (abo) gene encodes a protein that localizes to Drosophila histone genes, and is a negative regulator of histone transcription (Berloco et al. 2001). Mutations to abo are moderate suppressors of PEV, but had no effect on TPE (Table 2.1C).

III Aii Uncloned Su(var)s (Table 2.2).

The majority of Su(var) mutations have not been cloned, and thus remain uncharacterized at the molecular level. However, our lab had previously generated and positioned 21 uncloned dominant Su(var) mutations by recombination-mapping (unpublished results). These mutations are homozygous viable and had no morphologically distinct recessive phenotypes, and therefore it was not possible to place them into complementation groups.
However, recombination-mapping results indicated that the Su(var) phenotypes associated with these mutations clustered around eight distinct regions (indicated in Table 2.2). Since it is common for several different Su(var) genes to be found within a few map units of one another (CLEGG et al. 1993; SINCLAIR et al. 1983; SINCLAIR et al. 1992) it is possible that these mutations may represent as many as 21 different Su(var) genes, or as few as eight. All 21 mutations were strong suppressors of PEV, but none affected TPE.

In total therefore, a minimum of 15, and possibly as many as 28 Su(var) loci were tested for the ability to suppress TPE, but only three were able to do so, for an overlap of about 10 to 20%. This suggests that the mechanisms underlying PEV and TPE differ substantially, and investigating TPE as a separate and independent phenomenon from PEV is a valid undertaking. Furthermore, these results also suggest that pre-existing libraries of Su(var) mutations are unlikely to contain significant numbers of Su(TPE) mutations, and that screening other libraries of Su(var) mutations is unlikely to be helpful.

IIIB Previously identified Su(TPE) mutations.

IIIBi Previously known Su(TPE) loci do not suppress PEV (Table 2.3).

Prior to this study, only the Psc and Su(z)2 loci were reported to suppress TPE when
Table 2.2

Suppression of PEV and TPE by Su(var) mutations that have not been cloned.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEV</td>
</tr>
<tr>
<td><strong>Su(var)3-3</strong></td>
<td>(3-46.6)</td>
<td>+++</td>
</tr>
<tr>
<td>304</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>307</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>316</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>327</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)2-1</strong></td>
<td>(2-40.5)</td>
<td>+++</td>
</tr>
<tr>
<td>01</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>210</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>213</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>214</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td>215</td>
<td>“</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Other 3rd</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var)321</td>
<td>(3-47.6)</td>
<td>+++</td>
</tr>
<tr>
<td>Su(var)323</td>
<td>(3-47.3)</td>
<td>+++</td>
</tr>
<tr>
<td>Su(var)333</td>
<td>(3-49.8)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)301</strong></td>
<td>(3-55.5)</td>
<td>+++</td>
</tr>
<tr>
<td>Su(var)305</td>
<td>(3-56.8)</td>
<td>+++</td>
</tr>
<tr>
<td>Su(var)312</td>
<td>(3-56.2)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Other 2nd</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var)208</td>
<td>(2-5.7)</td>
<td>+++</td>
</tr>
<tr>
<td>Su(var)211</td>
<td>(2-6.2)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)204</strong></td>
<td>(2-33.8)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)209</strong></td>
<td>(2-35.4)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)206</strong></td>
<td>(2-51.3)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)203</strong></td>
<td>(2-65.7)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Su(var)21</strong></td>
<td>(2-64.2)</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Cytological location unknown. Position was determined by recombination mapping.

b Reuter et al 1986
c Sinclair et al 1992
d Hearn et al 1991
e Sinclair et al 1983
f Grigliatti lab, unpublished Su(var) mutants
mutated. Although not all existing alleles of these two genes had been tested, the $Su(z)2^5$ allele was found to be the strongest suppressor of TPE (Cryderman et al. 1999; Wallrath and Elgin 1995). To confirm and extend these results, as well as to continue the evaluation of the degree of overlap between PEV and TPE, I tested several alleles of these genes, and confirmed the ability of $Psc$ and $Su(z)2$ to suppress TPE (Table 2.3). However, none of these alleles suppressed PEV, reinforcing the findings of Section IIIA, that TPE is a distinct phenomenon from PEV.

IIIBii The $Su(z)2^5$ deficiency removes four independent Su(TPE) loci (Table 2.4).

The unusual strength of TPE suppression by the $Su(z)2^5$ allele was somewhat intriguing, and I decided to examine this mutation in greater detail by genetic dissection. $Su(z)2^5$ is actually a small deficiency that removes both the $Su(z)2$ and the $Psc$ loci (Wu and Howe 1995), each of which suppresses TPE as an individual mutation. Since single gene mutations in either $Su(z)2$ or $Psc$ suppress TPE, it was implicit that the simultaneous removal of both genes accounted for the unusually high TPE-suppressing strength of the $Su(z)2^5$ deficiency. However, since Su(var) loci are sometimes found in clusters, I decided to test the possibility that Su(TPE) mutations may also be clustered, and that the $Su(z)2^5$ deficiency may remove more than just two Su(TPE) loci. Accordingly, I analyzed the $Su(z)2^5$ deficiency in greater detail (Table 2.4).

I tested 21 single gene mutant alleles that had been mapped to the region in which $Su(z)2^5$ is found for both the ability to complement $Su(z)2^5$ and the ability to suppress TPE and/or PEV. Table 2.4 constitutes a test of all available mutant alleles mapped to the
The complementation analysis confirmed that both \textit{Psc} and \textit{Su(z)2} are removed by the \textit{Su(z)2}⁻ deficiency. Additionally, the analysis revealed two new \textit{Su(TPE)} mutations, \textit{Su(z)3} and \textit{Orc3} (alternatively known as \textit{latheo}). Thus, the \textit{Su(z)2}⁻ deficiency actually removes four independent \textit{TPE}-suppressing loci, which may account for its unusual strength. A lethal complementation analysis indicated that point mutations of \textit{Su(z)2}, \textit{Psc}, \textit{Su(z)3}, and \textit{Orc3} fail to complement one another (data not shown), confirming that they are separate genes.

\textbf{IIIC A candidate gene screening approach to finding additional \textit{Su(TPE)} loci.}

Having found three \textit{Su(TPE)} mutations in a large library of \textit{Su(var)s}, and two more from a detailed dissection of a small \textit{TPE}-suppressing deficiency, I continued my search for additional \textit{TPE}-suppressing loci using a candidate gene screening approach. This search used a ‘gene first’ strategy in which candidate loci were selected, based on information derived from similar experiments done with other organisms, and tested in the \textit{Drosophila} reporter assays. Candidate loci were also selected based on current theories about nuclear structure and telomere function. Thus, the purpose of this search was not only to find additional \textit{Su(TPE)} loci, but also to test the validity of several current theories about nuclear organization, and telomere structure and function, as all of these theories would predict that disruption of nuclear architecture should disrupt telomere localization, as well as \textit{TPE}, causing a suppression of \textit{TPE}.

As in the previous section, all gene candidates were tested in both the \textit{TPE}-suppressing and \textit{PEV}-suppressing assays. In cases where point mutations of genes of interest were
Table 2.3

Suppression of PEV and TPE by previously known Su(TPE)s.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock</th>
<th>type of mutation</th>
<th>suppression of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEV</td>
</tr>
<tr>
<td><strong>A) Psc alleles</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{sc}^{1.d20}$</td>
<td>49E6</td>
<td>5551</td>
<td>Point mutation</td>
<td>-</td>
</tr>
<tr>
<td>$P_{sc}^{b27}$</td>
<td>“</td>
<td>5547</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$P_{sc}^{e22}$</td>
<td>“</td>
<td>5546</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$P_{sc}^{1.d19}$</td>
<td>“ na$^a$</td>
<td>“</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$P_{sc}^{1}$</td>
<td>“ 4200</td>
<td>“</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td><strong>B) Su(z)2 alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Su(z)^{2.t}$</td>
<td>49E7</td>
<td>5573</td>
<td>Point mutation</td>
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</tr>
<tr>
<td>$Su(z)^{1.al}$</td>
<td>“</td>
<td>5549</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$Su(z)^{1.b7}$</td>
<td>“</td>
<td>5572</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$Su(z)^{2.De26}$</td>
<td>“ na$^a$</td>
<td>“</td>
<td>“</td>
<td>-</td>
</tr>
<tr>
<td>$Su(z)^{2}$</td>
<td>“ na$^a$</td>
<td>“</td>
<td>Small deficiency</td>
<td>-</td>
</tr>
</tbody>
</table>

$na^a$ = not applicable (stocks not obtained from stock centre)

$^a$ A gift from T. Wu
Table 2.4

Further analysis of region 49D-E.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>stock</th>
<th>suppression</th>
<th>complementation</th>
</tr>
</thead>
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<td>stock</td>
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<td>PEV</td>
<td>TPE</td>
</tr>
<tr>
<td>bic</td>
<td>1597</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aats-asp¹</td>
<td>5552</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l(2)49De³</td>
<td>5554</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Psc¹,a²</td>
<td>5551</td>
<td>-</td>
<td>+²</td>
</tr>
<tr>
<td>Su(z)²¹,a¹</td>
<td>5549</td>
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<td>+</td>
</tr>
<tr>
<td>Su(z)³¹</td>
<td>5550</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Su(z)³²os</td>
<td>5548</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>lat³</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>lat⁶</td>
<td>5571</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Dp⁴⁹FK-¹</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l(2)49Fj²</td>
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<tr>
<td>l(2)49Fj³</td>
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<td>-</td>
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<tr>
<td>l(2)49Fl¹</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l(2)49Fm³</td>
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<td>-</td>
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<td>sie³</td>
<td>5111</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>l(2)49Fb³</td>
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<td>-</td>
</tr>
<tr>
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</tr>
<tr>
<td>l(2)49Fg¹</td>
<td>5563</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l(2)49Fh¹</td>
<td>5564</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l(2)49Fp³²</td>
<td>5569</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cmn¹HK²</td>
<td>5039</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cpl¹cnbvs³⁸</td>
<td>5124</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
unavailable, the smallest available deficiencies were tested instead. Wherever possible, multiple, overlapping deficiencies of the same loci were tested.

IIICi Chromodomain proteins (Table 2.5).

The chromodomain is a common protein motif found in many chromatin-associated proteins, such as SU(VAR)3-9 and HP1. Such proteins are collectively referred to as chromodomain proteins, many of which are known to be involved in gene silencing (Brehm et al. 2004). Accordingly, I tested mutations to 13 chromodomain-protein encoding genes (Table 2.5), including the genes encoding the SU(VAR)3-9 and HP1 proteins mentioned above, both of which were originally identified as strong suppressors of PEV when mutated. As noted above, most mutant alleles of Su(var)3-9 were found to be dominant Su(TPE), while mutations in Hp1 had no effect on TPE.

In addition to the original HP1 protein, two orthologs of HP1 have recently been identified in Drosophila (Smothers and Henikoff 2001). These two HP1 orthologs were designated as HP1b and HP1c, necessitating the re-designation of the original HP1 protein as HP1a. Using cytological techniques, Smothers and Henikoff (2001) determined that, while HP1a localizes primarily to the centromeric compartment of the interphase nucleus as expected, the other two HP1 orthologs do not. HP1b and HP1c are instead found at a number of alternate locations, including telomeres. I examined the possibility that at least one of these orthologs may be involved in telomere function by testing whether or not a deficiency for HP1c suppressed TPE.
Table 2.5

Suppression of PEV and TPE by Chromodomain proteins.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock</th>
<th>type of mutation</th>
<th>suppression of PEV</th>
<th>suppression of TPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su(var)3-9&lt;sup&gt;330&lt;/sup&gt;</td>
<td>88E6-8</td>
<td>na</td>
<td>Point mutation</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>HP1a</td>
<td>28F1-2</td>
<td>na</td>
<td>Point mutation (Su(var)2-5&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot; (Su(var)2-5&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>179</td>
<td>Df: 28E4-7; 29B2-C1</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CG8120&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85D11</td>
<td>1931</td>
<td>Df: 85D8-12; 85E7-F1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1932</td>
<td>Df: 85D10-12; 85E1-3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HP1c&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94C4</td>
<td>1605</td>
<td>Df: 93E-F; 94C-D</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>CG15636&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25A1</td>
<td>693</td>
<td>Df: 24C2-8; 025C8-9</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Chro</td>
<td>79F5</td>
<td>4883</td>
<td>Df: 79E2+; 80; 70D1-2</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>msl-3&lt;sup&gt;l&lt;/sup&gt;</td>
<td>65E5</td>
<td>5872</td>
<td>Point mutation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MRG15</td>
<td>88E11</td>
<td>383</td>
<td>Df: 88E7-13; 89A1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>kis&lt;sup&gt;l&lt;/sup&gt;</td>
<td>21B4-6</td>
<td>431</td>
<td>Point mutation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>3637</td>
<td>Df: 21A1; 21B6-7</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>3636</td>
<td>Df: 21A1; 21B6-7</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Chd1</td>
<td>23C4</td>
<td>1567</td>
<td>Df: 23C1-2; 23E1-2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mi-2</td>
<td>76D3</td>
<td>5582</td>
<td>Df: 76B; 77A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CHD3</td>
<td>76B3</td>
<td>&quot;</td>
<td>&quot;</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Pc&lt;sup&gt;l&lt;/sup&gt;</td>
<td>78C6-7</td>
<td>1728</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

na= not applicable (stocks not obtained from stock centre)

<sup>a</sup>CG8120, HP1c, and CG15636 also have chromo shadow domains, and are therefore putative orthologs of HP1a

<sup>b</sup>also removes Kap-α1 (Table 2-7)
A deficiency for the *HP1c* locus suppressed TPE, but had no effect on PEV (Table 2.5). It is possible therefore, that *HP1c* may be a constituent of telomeric heterochromatin, while *HP1a* is a constituent of pericentric heterochromatin. Unfortunately, I was unable to test the effects of *HP1b* mutations because the *HP1b* locus is located on the X chromosome, and all of the available deficiencies for this locus carried a wild-type *w*\(^+\) gene that would have masked the expression of the reporter genes used here.

I also tested a number of putative chromodomain-containing proteins, including two additional putative HP1 orthologs. One of the signature features of *HP1a* is that it contains two well-defined domains, the chromodomain and the chromoshadow domain, separated by a non-conserved hinge region. A scan of the Drosophila genome database, using the Berkeley Drosophila Genome Project (BDGP) BLAST search engine (www.flybase.org) identified two additional proteins with both a putative chromodomain and a putative chromoshadow domain. Since these putative proteins contained both of the HP1 signature domains, I classified them as putative HP1 orthologs, and tested them for effects on TPE and PEV. The genes encoding these two putative orthologs, *CG8120* and *CG15636*, were tested using small deficiencies. A deficiency for *CG8120* suppressed TPE, but had no effect on PEV. Conversely, a deficiency for *CG15636* suppressed PEV but had no effect on TPE.

Using the same *in silico* hybridization method, I also identified seven more proteins in the Drosophila genome database that contained putative chromodomains, but no chromoshadow domains, and tested them for affect. Two of these proteins, encoded by
Chro and Chd1, suppressed PEV exclusively, while three others, encoded by MSL-3, MRG15, and kismet (kis), suppressed TPE exclusively. Unfortunately, the two remaining chromodomain-containing proteins, CHD3 and MI-2, were encoded by loci that were somewhat closely linked, and I was unable to find a deficiency that removed each of them. Consequently, I tested both loci with a single deficiency that was found to be a moderate suppressor of both PEV and TPE.

Interestingly, the only chromodomain protein tested that affected neither TPE nor PEV was POLYCOMB (PC). PC is the founding member of the Polycomb group (PcG) of proteins believed to be involved in maintaining the silenced state of the homeotic genes during development. The lab of H.W. Brock had previously tested a significant subset of the PcG of proteins for their effect on PEV and, with one exception, they did not influence PEV (SINCLAIR et al. 1998), suggesting that these proteins are not essential for establishing centromeric heterochromatin. In the present study I tested mutations in several PcG proteins for their effects on TPE, but this group of proteins did not appear to have any effect on TPE either (Table 2.6C).

Thus, mutations in all but two of the chromodomain proteins tested were found to suppress either TPE or PEV, but not both. The two exceptions being PC, which suppressed neither TPE nor PEV, and SU(VAR)3-9, which suppressed both TPE and PEV. As a result of these observations it is tempting to speculate that most chromodomain proteins are involved in creating a chromatin architecture that represses gene activity, albeit with different chromodomain proteins being specific to different
regions of the chromosome, such as telomeres and centromeres. SU(VAR)3-9 would be the only chromodomain protein to participate in both TPE and PEV. It is possible, therefore, that some of the gene silencing properties associated with TPE and PEV are the result of the HMTase activity of SU(VAR)3-9, but that the targeting of SU(VAR)3-9 to either telomeres or centromeres is a function of other chromodomain proteins, such as HP1c and HP1a, respectively.

IIICii  Chromatin-associated proteins (Table 2.6).

I also tested several other proteins known to be either primary constituents of chromatin, or proteins closely associated with chromatin in specific areas. Some of these proteins have been listed in other tables under different headings, but are repeated in Table 2.6 for comparative purposes.

IIICiia  The histone genes (Table 2.6A).

The histone proteins and the nucleosomes they comprise are the principal protein constituents of chromatin. The majority of the histone genes in Drosophila are clustered in a block of tandem repeats in the 39D-E region known as the histone cluster, abbreviated HIS-C (MOORE et al. 1979; MOORE et al. 1983; NER et al. 2002). Our lab had previously demonstrated that removal of one copy of the HIS-C, and hence, half of the histone genes, suppresses PEV in Drosophila (NER et al. 2002; SAMAL et al. 1981). I confirmed this result in the present study. Deletions of histone genes are also known to suppress TPE in budding yeast (KAUFMAN et al. 1998; NORRIS et al. 1988). Interestingly, however, haplo-deficiencies of the HIS-C had no effect on TPE in
Drosophila, suggesting that the structure and composition of Drosophila telomeres may be fundamentally different from both yeast telomeres, and Drosophila centromeres. This result also suggests that very little of what is known about TPE in budding yeast is transferable to Drosophila, and that specialized studies of TPE in Drosophila are warranted.

IIICiib  Histone variants (Table 2.6A).
In addition to the primary histone genes located at HIS-C, Drosophila also has histone variants, which are usually present as single copy genes located in other regions of the genome. The biological significance of these variants is not yet clear, but it is likely that they are involved in regulating transcription. I tested two histone variants, H3.3 and H2AvD, in the present study. A small intragenic null mutation in H2AvD (VAN DAAL et al. 1990) suppressed neither PEV nor TPE. A point mutation for H3.3 does not exist in the public databases, and therefore I tested the effects of a small deficiency that removes H3.3 (AHMAD and HENIKOFF 2002). Hemizygosity for H3.3 had a slight suppressing effect on TPE, but had no effect on PEV.

IIICiic  Origin Recognition Complex proteins (Table 2.6B).
As is the case with many higher organisms, replication of the Drosophila genome begins at certain predetermined origins, with the help of a large multiprotein complex known as the Origin Recognition Complexe (ORC). Mutations to certain subunits of the yeast ORC are known to suppress TPE in yeast (PRYDE and LOUIS 1999), and mutations to at
least one of the subunits of the Drosophila ORC have been shown to suppress PEV in
Drosophila (PAK et al. 1997). No members of the Drosophila ORC had been tested for
effects on TPE, however, and I therefore tested three members of the Drosophila ORC:
Orc2, Orc3 (also known as latheo) and Orc5 (Table 2.6B). Mutations in Orc2 were
previously reported to be weak suppressors of PEV (PAK et al. 1997), and I have
confirmed this result here, but found that Orc2 mutations had no effect on TPE. Point
mutations in Orc3 were weak to moderate Su(TPE), but had no effect on PEV. As noted
previously, I found that Orc3 was one of the genes removed by the Su(z)25 deficiency,
and probably contributes to the strong suppressor affect of this deficiency. Mutations in
Orc5 suppressed neither PEV nor TPE.

\textbf{IIICiid Polycomb group proteins (Table 2.6C).}

One of the first identified Su(TPE), Psc, is a member of the PcG of proteins. I confirmed
that mutations in Psc suppress TPE, and, as appears to be the case with other Su(TPE),
the observed effect was allele specific (Tables 2.3 and 2.6C). Of the four mutant alleles
tested, two were moderate Su(TPE)s, one was a weak Su(TPE), and one had no
discernable effect on TPE. I also tested mutations in six other PcG genes. With the
notable exception of the polyhomeotic-distal (ph-d) locus, none of these mutations had
any effect on TPE or PEV. The polyhomeotic (ph) gene complex appears to have arisen
through a duplication event that produced both a proximal and distal gene. These genes
are approximately 95% similar, but appear to have different functions (HODGSON et al.
1997). A mutation in the proximal gene, polyhomeotic- proximal (ph-p), had no effect on
TPE or PEV, but a mutation in distal gene, ph-d, was a strong suppressor of TPE.
Table 2.6

Suppression of PEV and TPE by chromatin-associated proteins.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock</th>
<th>type of mutation</th>
<th>suppression of PEV</th>
<th>TPE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Histones and histone variants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIS-C</td>
<td>39D3-E1</td>
<td>DS5</td>
<td>Df: 38C7-10; 39D3-E1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Df: 38F5; 39E7-F1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H3.3</td>
<td>25C1</td>
<td>1164</td>
<td>Df: 25A2-D5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H2AvD⁸₁₀</td>
<td>97D5</td>
<td>na</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>B) ORC proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orc2¹</td>
<td>88A3</td>
<td>na</td>
<td>Point mutation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Orc3 (lat¹)</td>
<td>49F7-8</td>
<td>5570</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Orc3 (lat²)</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Orc5²</td>
<td>34D6</td>
<td>3593</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>C) PcG proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ph-d⁴₀₁</td>
<td>2D2-3</td>
<td>na</td>
<td>Small Df of distal transcript</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>ph-p⁴₀₉</td>
<td>2D2-3</td>
<td>na</td>
<td>small Df of proximal transcript</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pc¹</td>
<td>78C6-7</td>
<td>1728</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Psc¹,d₂₀</td>
<td>49E6</td>
<td>5551</td>
<td>Point mutation</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Psc¹,h²₇</td>
<td></td>
<td>5547</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Psc²,e²²</td>
<td></td>
<td>5546</td>
<td></td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Psc¹,d₁₉</td>
<td></td>
<td>na</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Asx¹</td>
<td>51A4</td>
<td>4198</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Asx²</td>
<td></td>
<td>6041</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ez⁵₀</td>
<td>67E5</td>
<td>na</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>D) other chromatin-associated proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mt2</td>
<td>33C4</td>
<td>3079</td>
<td>Df: 32F1-33F2</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>CG6678</td>
<td>93F9</td>
<td>3013</td>
<td>Df: 93C3-6; 93F14-94A1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bj1</td>
<td>65A1</td>
<td>4503</td>
<td>Df: 64E1-13; 65C1-D6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mle⁹</td>
<td>42A2</td>
<td>5873</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>spt4</td>
<td>49B10</td>
<td>753</td>
<td>Df: 49A4-13; 49E7-F1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tou</td>
<td>48A2-3</td>
<td>3373</td>
<td>Df: 48A-B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dpa¹</td>
<td>43D1-3</td>
<td>4126</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mcm7</td>
<td>66E3</td>
<td>4500</td>
<td>Df: 66E1-6; 66F1-6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BEAF-32</td>
<td>51C2</td>
<td>1150</td>
<td>Df: 51B5-11; 51D7-E2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

na=not applicable (stocks not obtained from stock centre)

* Df(2L)DS5 (Moore et al. 1979).

¹ a gift from S. Elgin.

² a gift from R. Kelly.

³ a gift from J. Hodgson and H. Brock.

⁴ a gift from T. Wu.
IICii  Other chromatin-associated proteins (Table 2.6D).

I also tested twelve other chromatin-associated proteins thought to be involved in the modulation of chromatin structure. Hemizygosity for the DNA methyltransferase protein Mt2, and a deficiency removing the putative gene $CG6678$, which contains a Regulator of Chromatin Condensation (RCC1) domain suppressed TPE, but had no effect on PEV (Table 2.6D). The other candidate loci tested did not affect either phenomena.

IICiii  Proteins involved in nuclear structure (Table 2.7).

I also tested a number of loci that encode various structural components of the nucleus. The rationale for testing these loci was based on the ‘perinuclear tethering’ model of heterochromatin-mediated gene silencing. For most organisms, including Drosophila, heterochromatin is located at the nuclear periphery during interphase, a phenomenon known as perinuclear localization. Perinuclear localization of heterochromatin is believed to be a characteristic feature of higher eukaryotes. The perinuclear tethering model of gene regulation posits that the tethering of heterochromatin to the nuclear periphery is critical for heterochromatin-mediated gene silencing, and that heterochromatin formation and heterochromatin-mediated gene silencing are mediated by factors located at the nuclear periphery (FERREIRA et al. 1997; MARSHALL et al. 1996; SPANN et al. 2002; TADDEI and GASSER 2004; TADDEI et al. 2004a; TADDEI et al. 2004b; THAM et al. 2001).
Table 2.7

Suppression of PEV and TPE by proteins involved in nuclear structure.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock</th>
<th>type of mutation</th>
<th>suppression of PEV</th>
<th>TPE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Putative LBR homolog</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG17952</td>
<td>57F10-11</td>
<td>5764</td>
<td>Df: 57F2; 58A1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>“</td>
<td>“</td>
<td>5246</td>
<td>Df: 57D2-8; 58D1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><strong>B) Tubulins (mutations, deficiencies, and duplications)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αTub84B5</td>
<td>84B4</td>
<td>2412</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>αTub84B</td>
<td>84B4</td>
<td>3066</td>
<td>Dp: 84A- 85A</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>βTub85D0</td>
<td>85D15</td>
<td>2451</td>
<td>Point mutation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>βTub85D</td>
<td>85D15</td>
<td>294</td>
<td>Dp: 85D1-4; 87A5a</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>γTub23C</td>
<td>23C3-4</td>
<td>1567</td>
<td>Df: 23C1-2; 23E1-2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>C) Lamin and Lamin C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamin</td>
<td>25E6</td>
<td>25</td>
<td>Df: 25D4; 25F1-2</td>
<td>++b</td>
<td>-</td>
</tr>
<tr>
<td>“</td>
<td>“</td>
<td>3365</td>
<td>Df: 25E1-2; 26A7</td>
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<td>LamC</td>
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<td>Ote</td>
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<td>“</td>
<td>1547</td>
<td>Df: 55A; 55F</td>
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<tr>
<td><strong>D) Drosophila nuclear pore complex proteins</strong></td>
<td></td>
<td></td>
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<tr>
<td>mbo</td>
<td>87C5</td>
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<td>Df: 87C1-88C2</td>
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<td>Nup154</td>
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<td>11063</td>
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<td>Nup44A</td>
<td>44A2</td>
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<td>Df: 42E-44C</td>
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<td>Nup358</td>
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<td>4531</td>
<td>Df: 96A21-96B10</td>
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<td>gcl</td>
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<td>4940</td>
<td>Df: 95A5-7; 95C10-11</td>
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<tr>
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<td>“</td>
<td>2585</td>
<td>Df: 95A5-7; 95D6-11</td>
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</table>

a also includes a duplication of βTub85E.

b suppresses as a maternal effect only.
Table 2.7 cont.

<table>
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<th>Gene/allele</th>
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<th>stock</th>
<th>type of mutation</th>
<th>suppression of</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>PEV</td>
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<td><strong>E) Putative S. cerevisiae Nup145 homologs</strong></td>
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<tr>
<td>CG13560</td>
<td>59F7</td>
<td>3452 Df: 59E; 60A1</td>
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</tr>
<tr>
<td>CG14692</td>
<td>86C6</td>
<td>3128 Df: 86C1; 87B1-5</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CG9696</td>
<td>57D11-12</td>
<td>5246 Df: 57D2-8; 58D1</td>
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<td>-</td>
</tr>
<tr>
<td>CG5467</td>
<td>97B9-C1</td>
<td>1911 Df: 97B; 97E</td>
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<td>-</td>
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<td><strong>F) Putative S. cerevisiae Nup2 homologs</strong></td>
<td></td>
<td></td>
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<td></td>
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<td>CG31901</td>
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<td>179 Df: 28E4-7; 29B2-C1</td>
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<tr>
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<td>44B3</td>
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<td>86E16-17</td>
<td>3128 Df: 86C1; 87B1-5</td>
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<td>+</td>
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<td><strong>G) Drosophila nuclear import proteins</strong></td>
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<td></td>
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<td></td>
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<td>Fs(2)KetRX3</td>
<td>38E3</td>
<td>5314 Point mutation</td>
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<td>&quot;</td>
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<tr>
<td>Kap-α1</td>
<td>76C6</td>
<td>5582 Df: 76B; 77A</td>
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<td>+</td>
</tr>
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<td>Kap-α3</td>
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<td>1931 Df: 85D8-12; 85E7-F1</td>
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<td>Karyβ3</td>
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<td>lwr</td>
<td>21D1</td>
<td>3446 Df: 21C3; 21D4</td>
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<td>Ranbp9</td>
<td>86E7</td>
<td>3128 Df: 86C1; 87B1-5</td>
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<td>Ranbp11</td>
<td>52B5-C1</td>
<td>2468 Df: 51E3; 52C9-D10</td>
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<td>-</td>
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<td>&quot;</td>
<td>&quot;</td>
<td>3518 Df: 51D3-8; 52F5-9</td>
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<tr>
<td>Mtir (Bx34 and Tpr)</td>
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<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1145 Df: 48A3; 48C8</td>
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<td>-</td>
</tr>
<tr>
<td>Trn</td>
<td>65A8</td>
<td>4503 Df: 64F2; 65D3</td>
<td>-</td>
<td>-</td>
</tr>
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<td>CG10478</td>
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<td>&quot;</td>
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<td>CG8219</td>
<td>65A8</td>
<td>&quot;</td>
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</tr>
</tbody>
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---

\(^a\) Also removes the putative chromodomain protein CG8120.

\(^b\) also removes Mi-2 and CHD3 (Table 2-5).
Interactions between heterochromatic regions of the genome and components of the nuclear lamina, such as the Lamin B Receptor (LBR) and/or nuclear pore complex (NPC) proteins may be necessary for tethering, perinuclear localization, and gene silencing (Kourmouli et al. 2001; Kourmouli et al. 2000; Polioudaki et al. 2001; Ye et al. 1998; Ye et al. 1997; Ye and Worman 1996). Indeed, not only has the mammalian LBR been shown to anchor proteins of the nuclear lamina to the inner nuclear membrane, but it is also known to have affinity for histones, HP1, and even DNA (Holmer and Worman 2001). All of these observations are in support of the tethering model. It has also been demonstrated that such interactions can be disrupted by soluble forms of tubulin during cell division (Kourmouli et al. 2001), implying that the tethering process is reversible, and predicting that it should be possible to suppress TPE by overexpressing tubulin proteins in the cell. A prediction that was tested here, by the introduction of various tubulin gene duplications into the TPE and PEV reporter strains.

I examined 33 loci whose products have been implicated in tethering heterochromatin to either the nuclear periphery or the nuclear matrix. I have subdivided these genes into five categories based on the types of proteins they encode: a) putative lamin receptors (Table 2.7A), b) tubulins (Table 2.7B), c) lamin and other structural components of the nuclear envelope (Table 2.7C), d) Drosophila nuclear pore complex proteins and putative Drosophila homologs of the yeast nuclear pore complex proteins, NUP145 and NUP2 (Tables 2.7D, E and F), and e) nuclear import proteins that are believed to associate with nuclear pores (Table 2.7G).
III CIiiia A putative Drosophila lamin receptor (Table 2.7A).

Since a LBR has not yet been identified in Drosophila, I conducted a homology search of the Drosophila genome and identified one putative Drosophila homolog of the human and murine LBRs. The protein encoded by the putative gene CG17952 shares 25% and 24% identity with the human and murine LBRs respectively. I found that deficiencies of this locus suppressed PEV, but not TPE (Table 2.7A). While this result is interesting, and lends tentative support to the idea that centromeres may be tethered to the nuclear periphery through an association with a putative Drosophila LBR, it does not support the idea that telomeres are tethered to the nuclear periphery through such an association.

III CIiib Tubulin proteins (Table 2.7B).

As noted above, one model of perinuclear tethering posits that heterochromatin is tethered to the nuclear periphery through an association with an LBR-like protein, and that this association can be disrupted by soluble forms of tubulin. If true, this model predicts that it should be possible to partially alleviate this form of gene repression through overproduction of tubulin proteins. As a test of this model, I examined several duplications and point mutations of the tubulin genes for effects on PEV and TPE. Duplications of the αTub84B, βTub85D, and βTub85E loci did indeed suppress TPE in support of the model. In contrast to the duplication results, point mutations in these genes did not suppress TPE or PEV. These finding also supported the model, which predicts that overproduction of tubulin proteins should interfere with the ability of telomeres or centromeres to interact with the nuclear envelope, while mutations of the
tubulin-encoding genes should not (Ye et al. 1997; Ye and Worman 1996). I noted, however, that a deficiency containing the γTub23C locus had a mild suppressing effect on PEV (Table 2.7B). This observation did not support the model, which predicts that lowering the amount of tubulin in the cell should enhance PEV and/or TPE, since the interaction between telomeres and/or centromeres and the nuclear envelope is thought to be disrupted by tubulin proteins.

III.Ciiic Lamin, lamin C, and Otefin (Table 2.7C).

I tested three lamin-related proteins for effects on TPE and PEV. The lamin proteins are an integral part of the nuclear envelope and nuclear matrix, and are therefore potential candidates for interactions with telomeres, and the perinuclear localization of telomeres. Deficiencies for the Lamin C locus affected neither TPE nor PEV. Two deficiencies of the lamin locus were also tested (Table 2.7C), only one of which suppressed PEV. The reason for this discrepancy is not known. It is possible that the breakpoints of the deficiency that did not suppress PEV (Df: 25E-26A, Table 7C) were incorrectly recorded, or alternatively, the locus could be distal to the left breakpoint of this deletion. However, no point mutation was available for the Lamin gene, and I was unable to test this with a complementation analysis. In either case, however, TPE was not affected. The Otefin (Ote) locus encodes a protein that is also a constituent of the nuclear envelope and the inner nuclear membrane, but hemizygosity for Ote had no effect on either phenomenon.
Mutations in certain NPC proteins and NPC-associated proteins cause suppression of TPE in yeast (Feuerbach et al. 2002; Galy et al. 2000), suggesting that telomeres and/or centromeres may be tethered to the nuclear periphery through an association with such proteins. Accordingly, I tested mutations of a number of known Drosophila NPC proteins for the ability to suppress TPE and PEV in Drosophila (Table 2.7D). I found that haplo-deficiencies for the mbo locus had a mild suppressing effect on both PEV and TPE, and deficiencies for the Nup154 or Nup44A loci had mild suppressing effects on TPE, while having no effect on PEV. Removal of one copy of the Nup358 locus had no effect on either phenomenon. Finally, a deficiency for the Nup98 locus acted as a moderate suppressor of both PEV and TPE, but a point mutation of the Nup98 gene failed to suppress both types of position effect (Table 2.7D), suggesting that removal the of Nup98 was probably not responsible for the observed effect.

In addition to the known Drosophila NPC proteins listed above, I also tested putative Drosophila homologs of yeast NPC proteins. While NPC proteins have been characterized extensively in S. cerevisiae, far less information is available about the constituents of Drosophila NPC proteins. Nevertheless, I decided to continue the investigation into the effects of NPC protein mutations on TPE and PEV in Drosophila by searching the Drosophila genome for putative homologs of yeast NPC proteins that are known to affect TPE in yeast. Accordingly, I canvassed the Drosophila genome for putative homologs of these proteins, and tested deficiencies of the loci that encode them (Table 2.7, sections E and F).
Four loci with homology to the yeast NUP145 protein, \textit{CG13560}, \textit{CG14692}, \textit{CG9696}, and \textit{CG5467}, were tested (Table 2.7E). Interestingly, haplo-deficiencies of all four of these loci suppressed PEV, and in the case of \textit{CG13560}, TPE was also suppressed. While not helpful in the search to find Su(TPE) loci, this rather high rate of ‘hits’ for putative Drosophila NUP145 homologs and their ability to suppress PEV was impressive, and suggests that Drosophila NPC proteins warrant further investigation by those interested in both Drosophila NPCs, and possible interactions between NPC proteins and centromeres.

I also tested three putative Drosophila genes with similarity to yeast Nup2 (Table 2-7F). A deficiency for \textit{CG14712} strongly suppressed PEV, and was a weak suppressor of TPE. A deficiency for \textit{CG31901} suppressed PEV only, and a deficiency of \textit{CG2158} had no affect on either phenomenon (Table 2.7F).

\textbf{IIIciiie Nuclear import proteins (Table 2.7G).}

I tested thirteen proteins believed to be involved in the nuclear import process in Drosophila (Table 2.7G). This class of proteins is associated with nuclear pore complexes (ISHII \textit{et al.} 2002), and has been implicated in both gene silencing and perinuclear tethering in yeast and flies. Both a point mutation in, and a haplo-deficiency (deletion of one of the two genes) of the gene encoding the nuclear import protein FS(2)KET suppressed PEV but not TPE (Table 2.7G). Deficiencies removing one copy of the loci encoding the nuclear import proteins KAP\textalpha{}1 and RANBP9 suppressed both TPE and PEV, and removal of one copy of the \textit{Kap\textalpha{3}} locus suppressed TPE (Table
Removal of other loci encoding nuclear import proteins had no effect on TPE or PEV.

IIICiv Telomere capping and telomere-associated proteins (Table 2.8).

I also investigated the effects of twelve proteins known to be associated with telomeres, or involved in the telomere capping function. As mentioned above, HP1α is present at telomeres and appears to be involved in telomere capping (Cenci et al. 2005; Cenci et al. 1997; Fantì et al. 1998). However, mutations of the HP1α-encoding locus Su(var)2-5 do not suppress TPE (Cryderman et al. 1999), a result I confirmed in section IIIAi with both point mutations and a deficiency of the Su(var)2-5 locus. This result suggested that the telomere-silencing mechanism is probably distinct from telomere-capping mechanism. Nevertheless, I decided to test some of the other proteins known to be associated with Drosophila telomeres for effects on TPE.

I tested eleven proteins that are known to be either present at telomeres, or are believed to be involved in telomere capping. These included the known and putative SAP domain-containing proteins (Table 2.8A), which are thought to be involved in various mechanisms of chromosome organization (Aravind and Koonin 2000). The Su(var)2-10 gene encodes a SAP domain-containing protein which localizes to Drosophila telomeres (Hari et al. 2001). Both point mutations within, and a deficiency containing the Su(var)2-10 locus suppressed both TPE and PEV (Tables 2-1C and 2-8A). I also tested three other putative SAP domain encoding loci, identified by “in silico hybridization” (genome database homology search) as described above, for possible
effects on TPE and PEV. A haplo-deficiency of the \textit{CG8149} locus had a moderate effect on TPE, but no effect on PEV, while haplo-deficiencies for both \textit{CG30122} and \textit{CG6995} had no effect on either phenomenon. Thus, it appears that only two of the four SAP domain-encoding genes tested had any effect on TPE.

In yeast and other organisms, the yKU70/yKU80 heterodimer is present at telomeres, and participates in telomere capping, as well as the repair of double stranded breaks in DNA (BERTUCH and LUNDBLAD 2003; GALLEGÓ \textit{et al.} 2003; JACKSON 2002; SALDANHA \textit{et al.} 2003). It is also involved in the perinuclear localization of telomeres in yeast (HEDIGER \textit{et al.} 2002; LAROCHE \textit{et al.} 1998; MARTIN \textit{et al.} 1999). Based on the results from yeast experiments I investigated five similar proteins in Drosophila (Table 2.8B). These genes were originally discovered and classified according to a mutagen-sensitive phenotype that resulted when they were mutated. As a result, they were named 'mutagen sensitive' or 'mus' mutations (HENDERSON \textit{et al.} 1987), and the proteins they encode are known to be involved in DNA repair (HENDERSON 1999; HENDERSON \textit{et al.} 1994). Many of the proteins that these genes encode have similarity to the yKU70/yKU80 proteins, mutations in which cause a similar mutagen-sensitive phenotype in yeast. I found that a deficiency that removes two of these loci (\textit{mus309} and \textit{Irbp}) suppresses both TPE and PEV in Drosophila (Table 2.8B). Mutations in, or deficiencies for the other three loci tested had no effect on either phenomenon.

Finally, the ubiquitin conjugating enzyme UBCD1 (also known as EFFETE) is present at telomeres, and is essential for telomere capping and proper separation of telomeres at
metaphase (Cenci et al. 1997; Cenci et al. 2003). I tested the effects of a loss of function mutation to \textit{UbcD1}, as well as deficiencies to a related protein, UBCD6, but none of these had any effect on TPE or PEV (Table 2.8C). The observation that both UBCD1 and HP1a are essential to the telomere capping function in Drosophila, but neither is able to suppress TPE when altered or removed reinforces the idea that telomere capping and telomere-mediated gene silencing are two unrelated phenomena in Drosophila.
Table 2.8

Suppression of PEV and TPE by telomere-associated proteins and telomere capping proteins.

<table>
<thead>
<tr>
<th>Gene/allele</th>
<th>location</th>
<th>stock</th>
<th>type of mutation</th>
<th>suppression of PEV</th>
<th>TPE</th>
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<tr>
<td>Su(var)2-10&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>5526</td>
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<td>++</td>
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<td>Su(var)2-10</td>
<td>45A8</td>
<td>4966</td>
<td>Df: 45A6-7; 45E2-3</td>
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<td>++</td>
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<td>CG8149</td>
<td>85D24-25</td>
<td>1932</td>
<td>Df: 85D10-12; 85E1-3</td>
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<td>+</td>
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<tr>
<td>CG30122</td>
<td>55E3</td>
<td>5426</td>
<td>Df: 54F2; 56A1</td>
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<td>Df: 55A; 55F</td>
<td>-</td>
<td>-</td>
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<td>CG6995</td>
<td>96A23</td>
<td>4531</td>
<td>Df: 96A21; 96C2</td>
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<td>-</td>
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<td><strong>B) yKu70/80 homologs</strong></td>
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<tr>
<td>mus 309</td>
<td>86F4-7</td>
<td>3128</td>
<td>Df: 86C1; 87B1-5</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Irbp</td>
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<td></td>
<td>+++</td>
<td>+</td>
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<td>Df: 51D3-8; 52F5-9</td>
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<td>Df: 51E3; 52C9-D1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>C) Other telomere capping proteins</strong></td>
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<td>HP1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28F1-2</td>
<td>na</td>
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<td>+++</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>“ (Su(var)2-5&lt;sup&gt;5&lt;/sup&gt;)”</td>
<td>+++</td>
<td>-</td>
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<td></td>
<td></td>
<td></td>
<td>179</td>
<td>Df: 28E4-7; 29B2-C1</td>
<td>++</td>
</tr>
<tr>
<td>UbcD1 (eff&lt;sup&gt;mer4&lt;/sup&gt;)</td>
<td>88D2</td>
<td>6401</td>
<td>Point mutation</td>
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<td>-</td>
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<td>Df: 82C4; 82F3-7</td>
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<td>Df: 82D3-8; 82F3-6</td>
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</table>
IV DISCUSSION

IVA TPE as a separate phenomenon from PEV.

To summarize the results of the genetic screen, 100 loci were tested in total; using 90 point mutant alleles to test 45 loci, and 85 haplo-deficiencies to test an additional 55 loci. Since point mutant alleles constitute mutations to individual genes, while even the smallest deficiencies often remove several loci at once, the point mutation results should obviously be given more credence. The single gene mutation data are summarized in Table 2.9. Of the 45 loci tested with point mutations, 24 were able to suppress PEV, and 10 were able to suppress TPE. Of the 24 Su(var) mutations, only three were able to suppress TPE, for an overlap of approximately 13%. The fact that only a limited number of Su(var) mutations were able to suppress TPE strongly supports the idea that telomere-mediated gene silencing relies on a different mechanism than does centromere-mediated gene silencing, and also suggests that the composition of telomeric heterochromatin is different from that of centromeric heterochromatin.

Since point mutations were not available for every gene of interest, I tested small deficiencies in several cases. Genes tested with deficiencies are summarized in Table 2.10. Of the 55 loci tested with deficiencies, 24 were able to suppress PEV, 22 were able to suppress TPE, and of these, 12 were able to suppress both; for an overlap of approximately 50-55%. Obviously deficiency data must be viewed with greater skepticism, but, taken at face value, and combined with the point mutation data, the cumulative results show suppression of PEV by 48 loci, suppression of TPE by 32 loci,
and, of these, 15 loci were able to suppress both phenomenon. This translates roughly to 31% of the Su(var) loci being able to suppress TPE, and 47% of the Su(TPE) loci being able to suppress PEV. These results are fairly close to one another, and although the combined results are not as convincing as the point mutation data alone, they still suggest that TPE is a phenomenon that is largely distinct from PEV.

IVB Summary and analysis of TPE-suppressing loci.

A comprehensive summary of all known Drosophila TPE-suppressing gene candidates is given in Table 2.11. This table includes the results of the screen, as well as the results of others (as indicated). Before this study was undertaken, two loci, Psc and Su(z)2 had been identified as Su(TPE) loci (Cryderman et al. 1999). The Su(var)3-9 locus was reported to be a Su(TPE) during the course of the study (Donaldson et al. 2002), and atm (Oikemus et al. 2004) and gpp (Shanower et al. 2005) were identified as Su(TPE) loci shortly after this study was completed. Thus, of the 34 loci listed in Table 2.11, only 29 can be said to be truly unique to this study. Nonetheless, this screen has increased the number of Su(TPE) loci from five to 34, and added a number of new candidate loci to the list of genes that are likely to play a role in telomere biology. Su(TPE) loci are grouped according to the classes of proteins they encode, and also by the kind of mutation (point mutation, deficiency, or duplication) tested in the experiment.

Several classes of proteins stand out in Table 2.11, while others are conspicuously absent. The chromodomain proteins, for example, represent nearly one fifth of the new TPE-suppressing candidates identified. More importantly, 12 of the 13 chromodomain
Table 2.9

Summary of PEV and TPE suppression by single gene mutations.

<table>
<thead>
<tr>
<th>Suppression of PEV only</th>
<th>Suppression of TPE only</th>
<th>Suppression of Both PEV and TPE</th>
<th>Suppression of neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su(var)3-7</td>
<td>Su(z)2</td>
<td>Su(var)3-9</td>
<td>H2AvD</td>
</tr>
<tr>
<td>Su(var)2-1</td>
<td>Psc</td>
<td>HDAC1</td>
<td>mle</td>
</tr>
<tr>
<td>HP1a</td>
<td>Su(z)3</td>
<td>Su(var)2-10</td>
<td>ph-p</td>
</tr>
<tr>
<td>Orc2</td>
<td>Orc3 (lat)</td>
<td></td>
<td>dpa</td>
</tr>
<tr>
<td>Abo</td>
<td>ph-d</td>
<td></td>
<td>β-Tub85D</td>
</tr>
<tr>
<td>Fs(2)Ket</td>
<td>msl-3</td>
<td></td>
<td>α-Tub84B</td>
</tr>
<tr>
<td>puc</td>
<td>kis</td>
<td></td>
<td>mus306</td>
</tr>
<tr>
<td>Su(var)3-3</td>
<td></td>
<td></td>
<td>mus307</td>
</tr>
<tr>
<td>Su(var) 321</td>
<td></td>
<td></td>
<td>UbcD1</td>
</tr>
<tr>
<td>Su(var) 323</td>
<td></td>
<td></td>
<td>Orc5</td>
</tr>
<tr>
<td>Su(var) 333</td>
<td></td>
<td></td>
<td>Nup98</td>
</tr>
<tr>
<td>Su(var) 301</td>
<td></td>
<td></td>
<td>Pc</td>
</tr>
<tr>
<td>Su(var) 305</td>
<td></td>
<td></td>
<td>Asx</td>
</tr>
<tr>
<td>Su(var) 312</td>
<td></td>
<td></td>
<td>Ez</td>
</tr>
<tr>
<td>Su(var) 208</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 211</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 204</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 206</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 203</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var) 212</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total=21</td>
<td>Total=7</td>
<td>Total=3</td>
<td>Total=14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total tested=45</td>
</tr>
</tbody>
</table>
Table 2.10
Summary of PEV and TPE suppression by loci tested with deficiencies.

<table>
<thead>
<tr>
<th>Suppression of PEV</th>
<th>Suppression of TPE</th>
<th>Suppression of Both PEV and TPE</th>
<th>Suppression of neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chd1</td>
<td>H3.3</td>
<td>Irbp</td>
<td>Bj1</td>
</tr>
<tr>
<td>His-C</td>
<td>HP1c</td>
<td>Kap-α1\textsuperscript{a}</td>
<td>Spt4</td>
</tr>
<tr>
<td>Chro</td>
<td>Kap-α3</td>
<td>Mi-2\textsuperscript{a}</td>
<td>tou</td>
</tr>
<tr>
<td>His-C</td>
<td>MRG15</td>
<td>CHD3\textsuperscript{a}</td>
<td>Mcm7</td>
</tr>
<tr>
<td>γTub23C</td>
<td>Mt2</td>
<td>mbo</td>
<td>lamin</td>
</tr>
<tr>
<td>CG5467</td>
<td>Nup44A</td>
<td>mus309</td>
<td>lamC</td>
</tr>
<tr>
<td>CG9696</td>
<td>Nup154</td>
<td>Ranbp9</td>
<td>Ote</td>
</tr>
<tr>
<td>CG10712</td>
<td>CG8120</td>
<td>CG13560</td>
<td>Nup358</td>
</tr>
<tr>
<td>CG14692</td>
<td>CG8149</td>
<td>CG14712</td>
<td>gel</td>
</tr>
<tr>
<td>CG17952</td>
<td>CG6678</td>
<td>Mt2</td>
<td>CG2158</td>
</tr>
<tr>
<td>CG31901</td>
<td>CG6678</td>
<td>CHD3</td>
<td>Karyβ3</td>
</tr>
<tr>
<td>CG15636</td>
<td></td>
<td></td>
<td>Iwr</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Ranbp11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mtor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trn\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CG10478\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CG8219\textsuperscript{b}</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>BEAF-32</td>
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<td></td>
<td></td>
<td></td>
<td>CG30122</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CG6995</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mus210</td>
</tr>
<tr>
<td>Total=12</td>
<td>Total=10</td>
<td>Total=12</td>
<td>Total=21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total tested=55</td>
</tr>
</tbody>
</table>

\textsuperscript{a} removed by the same deficiency.
\textsuperscript{b} removed by the same deficiency.
### Table 2.11

Summary of TPE-suppressing loci.

<table>
<thead>
<tr>
<th>Function</th>
<th>Point mutation</th>
<th>Deficiency</th>
<th>Duplication</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histone modification</strong></td>
<td>Su(var)3-9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>H3.3</td>
</tr>
<tr>
<td></td>
<td>Hdac1</td>
<td></td>
<td>HP1c</td>
</tr>
<tr>
<td></td>
<td>gpp&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>MRG15</td>
</tr>
<tr>
<td><strong>Histone variants</strong></td>
<td></td>
<td></td>
<td>CG8120</td>
</tr>
<tr>
<td><strong>Chromodomain</strong></td>
<td></td>
<td></td>
<td>Mi-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chd3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CG8149</td>
</tr>
<tr>
<td><strong>SAP domain</strong></td>
<td>Su(VAR)2-10</td>
<td></td>
<td>CG6678</td>
</tr>
<tr>
<td><strong>Orc proteins</strong></td>
<td>Orc3 (lat)</td>
<td></td>
<td>Mt2</td>
</tr>
<tr>
<td><strong>PcG and Su(z)</strong></td>
<td>ph-d</td>
<td></td>
<td>mus309</td>
</tr>
<tr>
<td></td>
<td>Psc&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>Irbp</td>
</tr>
<tr>
<td></td>
<td>Su(z)2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Su(z)3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chromatin associated</strong></td>
<td>Tefu (atm&lt;sup&gt;a&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>yKu70/80 paralogs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tubulins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear pore</strong></td>
<td></td>
<td></td>
<td>αTub84B</td>
</tr>
<tr>
<td></td>
<td>mbo</td>
<td></td>
<td>βTub85D</td>
</tr>
<tr>
<td></td>
<td>Nup44A</td>
<td></td>
<td>βTub85E</td>
</tr>
<tr>
<td></td>
<td>Nup154</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG13560</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG14712</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear import</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kap-α1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kap-α3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ranbp9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> previously identified as TPE suppressors
candidates suppressed either TPE or PEV. This was an unusually high success rate compared to the other categories of proteins tested, suggesting that chromodomain proteins play an important role in heterochromatin-mediated gene silencing, and deserve greater attention in future studies. The role of most of these chromodomain proteins appears to be location-specific, with one set of chromodomain proteins being specific to telomeres, while the other is specific to centromeres. Only one of the candidate chromodomain proteins, SU(VAR)3-9, was unambiguous in its ability to suppress both TPE and PEV as a point mutation, and is worthy of special consideration, which it is given in the next chapter. As noted previously, the SU(VAR)3-9 protein is known to have histone H3K9 methyltransferase activity. Thus, methylation of H3K9 may be a form of gene silencing that is common to both centromeres and telomeres. This issue is also worthy of further investigation, and is revisited in the next chapter.

The other category of proteins heavily represented in Table 2.11 are the nuclear pore complex proteins, which also comprise nearly one fifth of the candidates. Indeed, when both TPE and PEV are considered, I noted that 11 of the 13 NUP loci tested suppressed either one or both of these phenomena, and again comprise an unusually high rate of successful ‘hits’ on candidate loci. Furthermore, nuclear import proteins are often considered to be associated with nuclear pore complexes, and, when the two categories are combined, they comprise nearly one third of the Su(TPE) candidates shown in Table 2.11. I believe that this category of proteins also deserves further attention in future studies.
Certain other categories of proteins are unexpectedly absent from Table 2.11. Haplo-deficiencies of the nuclear lamin proteins, for example, had no detectable affects on TPE, despite several theories that suggest they should (Burke and Stewart 2002; Holmer and Worman 2001). Another interesting observation is that neither UbcD1 nor HP1a mutations had any effect on TPE. However, mutations in both of these genes are known to influence telomere capping and increase rates of telomere fusions. Taken together, these data suggest that the telomere-capping and telomeric gene-silencing functions of telomeres are largely separate phenomena, mediated by separate sets of proteins.

IVBi  Deficiency studies of TPE in Drosophila.

Overall, 19 of the 29 novel candidate loci highlighted in the present study were identified using deficiency mutations only. Since only one other extensive search for Su(TPE) loci in Drosophila has been reported thus far (Mason et al. 2004), and this study was carried out exclusively with deficiencies, it might be useful to compare these results with those of Mason et al (2004), including both the deficiency and point mutation data from this study in the comparison. Mason et al. (2004) carried out a systematic deficiency screen to find regions of the genome that suppress TPE when removed. The screen made use of a ‘deficiency kit,’ comprised of large deficiencies that allowed the investigators to scan approximately 70% of the genome for the presence of dosage-sensitive Su(TPE) loci. Mason et al. tested for suppression of TPE with a slightly different telomeric reporter gene than the one used in this screen (Golubovsky et al. 2001; Mason et al. 2004). The telomeric reporter gene used by Mason et al. (2004) was located between the
terminal HeT-A array and the TAS region of the 2L telomere, rather than inside the TAS, and had a lower threshold for suppression than the one created by Wallrath and Elgin (1995).

Approximately one third of the larger deficiencies tested by Mason et al. suppressed TPE. However, they discounted the majority of these as false positives based on one of four possible reasons. Positive results were discounted if: a) the deficiency in question was only a weak suppressor of TPE, b) overlapping deficiencies of the same region gave conflicting results, c) the chromosome bearing the deficiency was also found to have a truncated 2L telomere, something believed to suppress TPE in and of itself (Golubovskiy et al. 2001), or d) the TPE-suppressing loci did not map to the same region as the deficiency, indicating a second-site suppressor mutation was responsible for the effect. Having taken this conservative approach to the interpretation of their results, and discounting the majority of their candidate regions, Mason et al. were left with 18 regions that they felt contained bona fide Su(TPE) loci.

Comparing my candidates to the results of Mason et al., I found that eight of the candidates localized to TPE-suppressing regions scored as positives by Mason et al. These included Psc, Su(z)2, Su(z)3, Orc3, Kap-1, CG8120, CG8149, and the histone variant H3.3. Three of the candidates Su(var)3-9, ph-d and Nup154, were in regions that Mason et al. did not test. Three more, αTub84B and βTub85D and γTub85E are not comparable because they suppressed TPE as duplications. One candidate, MRG15, is located in a region that Mason et al. discounted because it was only a weak suppressor of
TPE. Nine candidates, Nup44A, Su(var)2-10, Mi-2, Chd3, Kap-α3, Mus 309, Mbo, CG6678, and HP1c fell into regions that suppressed TPE, but were discounted by Mason et al. because multiple, overlapping deficiencies of the same region gave conflicting results. Two of the candidates, Mt2 and kis fell into a regions that suppressed TPE, but were discounted because the chromosome bearing the deficiencies also had a truncated 2L telomere. Finally, five of the candidates, Hdac1, msl-3, Irbp, Kap-1, and Ranbp9 were located in regions that Mason et al. identified as non-suppressing regions, and thus, probably constitute the greatest contradiction between these results and those of Mason et al.. Additionally, gpp (SHANOWER et al. 2005) and atm (OIKEMUS et al. 2004), which were identified as Su(TPE) loci by other investigators, are located in regions classified as non-suppressors by Mason et al..

Although the comparison between the results of Mason et al. and those reported here is interesting, the value of such a comparison is limited. The Mason et al. study was rightly intended to take a very conservative approach to the identification of TPE-suppressing loci, and is more likely to yield false negative results than was ours. It is possible that larger deficiencies, such as those used in a deficiency kit, may remove both suppressors as well as enhancers of TPE, leading to a net suppression effect of zero. To examine this possibility, I tested three larger deficiencies that removed the entire 49D-E region, based on cytology (Bloomington stock numbers 754, 752, and 434). Theoretically, if the cytology is accurate, these deficiencies removed all four of Psc, Su(z)2, Su(z)3 and Orc 3. However, none of these large deletions suppressed TPE (data not shown), despite the
observation that point mutations in these genes, and the smaller $Su(z)2^{5}$ deficiency did suppress TPE.

I then asked whether these larger deletions actually removed all three of $Su(z)2$, $Su(z)3$, and $Psc$, as the cytology would suggest. A complementation analysis of these three larger deficiencies indicated that, in reality, only deficiency stock 752 removed all three loci. Deficiency stock 754 complemented all three of $Su(z)2$, $Su(z)3$ and $Psc$, indicating that the deficiency did not remove these three genes, and deficiency stock 434 complemented $Su(z)2$ and $Su(z)3$, but not $Psc$, indicating that the $Psc$ locus was not removed by the deficiency (data not shown). The most likely explanation for the latter two results is that the deficiencies in question are not, in fact, single large deficiencies. It is more likely that they are compound deficiencies, which remove several smaller regions, including the bands used to classify the deficiency, while leaving other, intervening regions intact.

This is probably quite common for many of the larger deficiencies in the Bloomington Stock Centre deficiency collection, and may also explain why several of the deficiencies tested by Mason et al. gave conflicting results. However, this would not explain why stock 752 failed to suppress TPE, since this deficiency did indeed remove all three of $Su(z)2$, $Su(z)3$ and $Psc$. In this case, the more likely explanation is that, while removal of $Su(z)2$, $Su(z)3$, and $Psc$ should suppress TPE, there were other loci removed by this deficiency that enhanced TPE, for a net effect of zero. Hence, it is possible that many of the larger deficiencies used by Mason et al. gave false negative results. Taken together,
these observations suggest that screening for Su(TPE) loci by point mutation is preferable to screening by deficiency, and should be used wherever possible.

**IVBii Point mutation studies of TPE in Drosophila.**

Considering the point mutation results separately, eight candidates, *Hdac1, Su(var)3-9, Su(z)3, Orc3, msl-3, ph-d, Su(var)2-10*, and *kis* were identified in the present study, in addition to *Psc* and *Su(z)2*, which were known prior to its onset (Cryderman *et al.* 1999). Of these, *Su(z)3* and *Orc3* were in regions Mason *et al.* classified as positives, while *Hdac1* and *msl-3* were in regions Mason *et al.* classified as negatives. Four others, *Su(var)3-9, ph-d, Su(var)2-10* and *kis* were in regions Mason *et al.* either did not test, or classified as false positives. The region containing the *ph-d* locus was not tested, while the region containing *Su(var)2-10* was classified as a false positive due to the fact that multiple, overlapping deficiencies of the same region gave conflicting results. The region containing *kis* was also classified as a potential false positive due to the fact that the chromosome containing the deficiency was found to contain a truncated 2L telomere.

As mentioned previously, I believe that many of the larger deficiencies obtained directly from the Bloomington Stock Centre may contain compound breakpoints, and may not remove the entire region that is reported to be removed. Hence, testing for suppression of TPE with large deficiencies may lead to false negatives, and, therefore, I cannot discount the possibility that *Su(var)2-10* may be a *bona fide* Su(TPE). In addition, Dr. Mason was kind enough to supply us with the two stocks used by Mason *et al.* to determine whether or not the 2L telomere was truncated in the deficiency stock being tested (MASON *et al.*
I tested all of the point mutation stocks which gave us a positive result, and found that all, with the exception of the $Su(z)2^5$ allele, complemented these stocks, and hence, probably did not have a truncated 2L telomere (data not shown).

Given that deficiencies can give either conflicting or false negative results, and given that the TPE-suppressing point mutations tested by us did not appear to have truncated 2L telomeres, I believe that $Su(var)3$-9, $Hdac1$, $mls$-3, $Su(var)2$-10, $Orc3$, $ph-d$, and $Su(z)3$ are still promising $Su(TPE)$ candidate loci. The case of $Su(var)3$-9 is particularly interesting, given that multiple $Su(var)3$-9 alleles suppressed TPE. Furthermore, mutations to the mammalian version of $Su(var)3$-9 have already been shown to cause rearrangements to the histone methylation patterns of mammalian telomeres and centromeres, as well as causing increases in telomere length through an as yet unknown mechanism (GARCIA-CAO et al. 2004). Also, since histone deacetylation is a necessary prerequisite to histone methylation, histone deacetylases and histone methyltransferases can be viewed as two different sides of the same gene-silencing coin. Thus, the suppression of TPE by both mutations of $Su(var)3$-9 and $Hdac1$ was particularly intriguing, and the proteins encoded by these two genes may constitute an essential part of the gene-silencing mechanism at telomeres. HDAC1 and SU(VAR)3-9 were selected for a more detailed study of telomeres at the molecular level, as described in the next chapter of this thesis.
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Chapter Three

An Examination of the Chromatin Composition of the Drosophila 2L Telomere.²

² A version of this chapter will be submitted for publication. Doheny, J. and Grigliatti, T. A Molecular Analysis of the 2L Telomere of Drosophila Melanogaster.
I INTRODUCTION

Three of the proteins identified in the previous chapter as possible constituents of telomeric heterochromatin were HDAC1, SU(VAR)3-9 and HP1c. These proteins were selected as candidates for further study based on the observation that repression of a telomeric reporter gene is partially alleviated when the genes encoding them are mutated. I now ask if these three proteins are actually present at the telomeric reporter gene used to make this assessment. If present, it would suggest that they constitute components of telomeric heterochromatin, and participate in TPE. If not, it would suggest that the observed suppression of TPE was an indirect effect. Accordingly, I looked for a direct association between HDAC1, SU(VAR)3-9, and HP1c and the telomeric reporter gene using a Chromatin ImmunoPrecipitation (ChIP) assay designed to detect DNA-protein interactions in vivo. I also ask if a fourth protein, HP1a, is present at the telomeric reporter gene. Since mutations of HP1a failed to suppress TPE, I would predict that it is not. Finally, having addressed the question of whether these four proteins are present at a repressed reporter gene located in the 2L telomere, I address the question of whether they are also present at the native 2L telomere in the absence of the reporter gene.

Although all four of HDAC1, SU(VAR)3-9, HP1c, and HP1a are known to be chromatin-associated, their specific association with the Drosophila telomere has never been examined at the molecular level. Moreover, results from the TPE and PEV assays described in Chapter Two suggest that some of these proteins may play alternative roles in the various forms of gene silencing associated with different kinds of heterochromatin.
Mutations in HDAC1 and SU(VAR)3-9 suppress both PEV and TPE, while mutations in Hp1c suppress TPE only, and mutations in HP1a suppress PEV only. Since HP1a and HP1c have similar structures, but opposing actions with respect to PEV and TPE, their presence or absence at telomeres and various other locations is of great interest. Also, since all three of HP1a, HP1c, and SU(VAR)3-9 are chromodomain proteins, and chromodomain proteins featured prominently in the previous chapter as possible modulators of PEV and TPE, I decided to analyze the potential role of these proteins in modulating TPE in greater detail.

Recall that suppression of TPE was evaluated using a reporter gene embedded in the subtelomeric TAS region of the 2L telomere. The first part of this chapter (Section IIIA) is devoted to asking whether or not HDAC1, SU(VAR)3-9, HP1c and HP1a are directly associated with this reporter gene when it is located in the 2L TAS. The ChIP results from the telomeric reporter gene are compared to results from a Drosophila strain carrying an identical reporter gene in euchromatin, where it is fully expressed and not subject to TPE. Since the DNA sequences of the reporter genes are identical in both strains, any differences in the proteins associated with them would presumably be the result of their different chromosomal positions.

In addition to the two identical reporter genes located in the two different chromosomal positions, a second gene, Actin 42A, was tested as an internal control for these experiments. The Actin 42A gene is constitutively expressed, and is located at the same chromosomal position in both strains. As such, it is not subject to position effects the
way the two reporter genes are, and the chromatin composition of this gene should be similar in both strains, allowing it to be used as an internal standard.

Our results indicated that only minimum amounts of HDAC1, SU(VAR)3-9, and HP1c were present at the euchromatic reporter gene and the two *Actin 42A* control genes, while significantly higher levels of these proteins were found at the telomeric reporter gene. This suggests that HDAC1, SU(VAR)3-9, and HP1c are components of telomeric heterochromatin, but are not components of active genes located in euchromatin. The HP1a protein, by contrast, did not show a preferential association with the telomeric reporter gene, a result that suggests HP1a is not a component of telomeric heterochromatin. These results were consistent with the suppression of TPE results described in Chapter Two, where mutations in the genes encoding HDAC1, SU(VAR)3-9 and HP1c suppressed TPE, while mutations in the gene encoding HP1a did not, and tended to validate the method used to identify these proteins as potential components of telomeric heterochromatin. These results also suggested that the phenomenon of TPE is mediated, at least in part, by these three proteins.

Having determined that HDAC1, SU(VAR)3-9, and HP1c were associated with a telomere-embedded reporter gene, I then tested to see if this protein profile was also typical of an unmodified telomere in the absence of a reporter gene (Section IIIB). Six unique regions of the native, unadulterated 2L telomere were examined by ChIP, including the TAS region into which the reporter gene used to test TPE had been inserted, the terminal HeT-A array on the distal (telomere) side of the TAS, and three regions of
single copy (unique) DNA on the proximal (centromere) side of the TAS. These results indicated that these four proteins were present at the 2L telomere, but were not evenly distributed across it. Rather, HDAC1, SU(VAR)3-9, and HP1c were preferentially associated with the TAS region. Regions on either side of the TAS were associated with relatively small amounts of HDAC1, SU(VAR)3-9, and HP1c when compared to the TAS. Thus, the protein profile observed for the telomeric reporter gene was similar to the TAS region into which it had been inserted, but was not typical of the entire 2L telomere.

Finally, the question of how HDAC1, SU(VAR)3-9 and HP1c are able to mediate TPE is addressed. Since both the HDAC1 and SU(VAR)3-9 proteins have previously been identified as mediators of the ‘histone code’ model of transcriptional regulation, I also examined specific post translational modifications of the histone proteins that comprised the nucleosomes associated with the telomeric reporter gene, as well as the native 2L telomere (Sections IIIC and IIID, respectively). The histone code model of transcriptional regulation (reviewed by Cosgrove and Wolberger 2005) posits that certain post translational modifications of the histone proteins that constitute nucleosomes are able to confer transcriptional competence to the chromatin regions they comprise. Lysine nine of histone H3 (H3K9) is believed to be particularly important to this process, and acetylation of H3K9 (H3K9ac) is generally associated with transcriptional competence. In contrast, removal of acetate groups from H3K9ac by histone deacetylases such as HDAC1 is associated with gene silencing (Mottus et al. 2000). Furthermore, methylation of H3K9 (H3K9me) by histone methyltransferases such
as the SU(VAR)3-9 protein is also associated with gene silencing (SCHOTTA et al. 2003a; SCHOTTA et al. 2002; SCHOTTA et al. 2003b; SCHOTTA and REUTER 2000). Finally, it is also believed that methylation of H3K9 creates a binding site for the HP1a protein, which is ubiquitously associated with heterochromatin and gene silencing near centromeres (STEWART et al. 2005). Accordingly, I examined the distribution patterns of H3K9ac and H3K9me at the reporter gene, and across the native 2L telomere by ChIP, and addressed any observed correlation between the distribution patterns of H3K9me and HP1a at the telomere. I also compared the distribution pattern of the HP1a ortholog HP1c to the distribution pattern of H3K9me.

Our results indicated that H3K9ac levels were high in areas where HDAC1 levels were low, and vice versa, a result consistent with the idea of HDAC1 removing acetate groups from H3K9ac as part of a gene silencing process. Furthermore, higher levels of H3K9me were observed in areas where SU(VAR)3-9 was more abundant, suggesting that SU(VAR)3-9, rather than some other HMTase, was the source of the observed H3K9me. Finally, these results indicated that HDAC1, SU(VAR)3-9, and H3K9me levels were highest at the TAS region, suggesting that transcription from the TAS-embedded reporter gene analyzed in Chapter Two had been repressed by histone deacetylation and methylation, and that these modifications may constitute part of the mechanism responsible for TPE in Drosophila. These findings also suggest that TPE may be a property localized to the TAS region, and raises the possibility of TAS regions playing a specific and important role in telomere biology.
II MATERIALS AND METHODS

IIA Generation of 39C-X/Su(var)3-9GFP and 39C-5/Su(var)3-9GFP flies.

The reporter gene construct used to assay TPE is located in the TAS region of the 2L telomere of the 39C-5 fly strain (WALLRATH and ELGIN 1995). Another fly strain, designated 39C-X carries the identical reporter gene near its native position, cytological band 2D of the X chromosome, where it is not repressed or otherwise subject to TPE (WALLRATH and ELGIN 1995). Both strains were a generous gift from S. Elgin and L. Wallrath.

The 39C-5 and 39C-X Drosophila strains were used to evaluate the distribution patterns of HDAC1, SU(VAR)3-9, HP1a, HP1c, H3K9ac, and H3K9me for the reporter gene located in telomeric heterochromatin versus the identical reporter gene located in euchromatin. While the distribution patterns of HDAC1, HP1a, HP1c, H3K9ac, and H3K9me were determined using antibodies directed specifically against these proteins and protein modification, the distribution pattern of SU(VAR)3-9 was evaluated using a chimeric protein comprised by SU(VAR)3-9 and the Green Fluorescent Protein (GFP), designated as SU(VAR)3-9GFP (Schotta and Reuter 2000). Flies carrying the Su(var)3-9GFP gene were a generous gift from G. Reuter. In order to determine the SU(VAR)3-9GFP distribution pattern it was first necessary to cross the Su(var)3-9GFP fusion gene into the 39C-5 and 39C-X Drosophila strains. ChIP assays were then carried out using α-GFP antibodies on the hybrid strains carrying the reporter gene, and also expressing the SU(VAR)3-9GFP fusion protein.
Approximately 100 virgin females of the 39C-X and 39C-5 strain were collected from bottle cultures and mated to an equivalent number of males carrying the Su(var)3-9GFP chimera. Since the reporter gene is carried on the X chromosome in the 39C-X stock, this crossing scheme ensured that both male and female embryos would carry one copy of the reporter gene. (Note that, if the reciprocal crossing scheme had been used, female embryos of the 39C-X stock would have carried two copies of the reporter gene, while males would have carried only one. Recall also that the TPE-suppression assay described in Chapter Two uses a hemizygous reporter gene. TPE and suppression of TPE show different characteristics when two reporter genes are used, as will be discussed in Chapter Four.)

Flies were then allowed to lay eggs on standard apple juice/agar egg plates, supplemented with yeast paste. Egg-laying was allowed to proceed for six hours at 22°C, and then flies were transferred to fresh plates. The original plates were then left for an additional two hours at 22°C to ensure that all embryos were aged between two and eight hours. Since this study involved heterochromatin-mediated gene silencing, it was necessary to consider the time point at which heterochromatin is believed to first form in the developing embryo. Heterochromatin does not form during the first two hours of development, and thus, it would have been meaningless to study position effects in embryos that had not been aged for at least two hours.
IIB  ChIP Assays.

After collection, embryos were processed in accordance with an empirically derived ChIP protocol that was loosely based on the protocol supplied by Upstate™ Biotechnology Inc. for use with cell-lines, and which was then optimized for use with Drosophila embryos (data not shown). Briefly, the staged embryos were dechorionated in 1% bleach, rinsed in a 100 mM NaCl, and then fixed for one minute at the interface of a two phase system containing 10 mL of 1% formaldehyde and 10 mL of n-heptane. 100 embryos were then placed into 1.7 mL eppendorf tubes containing 1 mL of sonication buffer (10 mM Tris 8.1/ 1 mM EDTA/ 0.5 mM EGTA/ 10% w/v glass beads), and sonicated with three ten second pulses of a Cole Parmer™micro-tip ultrasonic processor, with power output set to 30% of maximum. Care was taken to avoid foaming, and tubes were kept on ice for one minute between pulses. 0.7 mL of 6M urea was then added to each tube, and the tubes were agitated at room temperature for one hour. Samples were then transferred to dialysis tubing, and dialyzed at 4°C against ChIP binding buffer (10 mM Tris 8.1/ 1 mM EDTA/ 0.5 mM EGTA/ 10% glycerol/ 1% Triton-X 100/ 0.1% deoxycholate).

ChIP extracts were then transferred back to 2 mL eppendorf tubes, and pre-cleared by the addition of 100 uL of Protein G Sepharose (PGS; Sigma™ P-3296) matrix. ChIP extracts were then centrifuged to remove solid debris and PGS, and transferred to fresh tubes. The concentration of chromatin in each sample was then equalized by diluting all samples to an O.D. of 0.2ₐ₂₆₀ units, using ChIP binding buffer, as recommended by Spencer et al. (2003). 5 ug of antibody was then added to 1.7 mL of ChIP extract, and allowed to bind for one hour at room temperature with gentle agitation. 100 uL of PGS
matrix was then added to bind antibodies, and left at room temperature for two hours with gentle agitation. This process was repeated with another 50 uL of PGS matrix, and the matrices were then pooled. Matrices were washed twice with 100 uL of a low salt wash (0.1% SDS/ 1% Triton-X 100/ 2 mM EDTA/ 20 mM Tris 8.1/ 150 mM NaCl), twice with a high salt wash (as above with 500 mM NaCl), twice with a lithium chloride, non-ionic detergent wash (0.25 mM LiCl/ 1% NP40/ 1% deoxycholate/ 1 mM EDTA/ 10 mM Tris 8.1), and rinsed twice with TE buffer. Matrices were then re-suspended in 200 uL of 1% SDS/ 0.1 M NaHCO₃, and heated to 65°C for four hours to reverse the formaldehyde DNA-protein crosslinks. Samples were then treated with 10 ug/mL Proteinase K, and the PGS matrix removed. The resulting supernatant, containing the ChIP DNA was then phenol/chloroform extracted to remove proteins, and the organic phase back extracted to recover as much DNA as possible. The DNA was then precipitated with ethanol, and re-suspended in 100 uL of HPLC-grade, de-ionized dH₂O.

Antibodies used were α-GFP (Molecular Probes™ A-11122, rabbit IgG polyclonal), α-dimethylated H3 K9 (Upstate™ 07-212, rabbit IgG polyclonal), α-acetylated H3 K9 (Upstate™ 07-352, rabbit IgG polyclonal), α-HP1a (C1A9 rabbit IgG monoclonal; a gift from S. Elgin), α-HDAC1 (Abcam™ ab1767-100, rabbit IgG polyclonal), α-HP1c (rabbit polyclonal antisera; a gift from S. Henikoff), and α-bacteriophage T₇-Tag (Novagen 69522-3; rabbit IgG monoclonal). Note that these experiments were carried out with antibodies specific for di-methylated H3K9 before tri-methylated H3K9 was discovered. Upstate™ subsequently tested their H3K9me2-specific antibody with tri-methylated H3K9 and observed a minor cross-reaction with H3K9me3. Accordingly, the
H3K9me results presented below should be considered as a combination of H3K9me2 and H3K9me3.

### IIC Quantitative PCR (qPCR) of Reporter Gene, *Actin 42A*, and various regions of the 2L telomere.

A standard three step PCR was carried out according to the following conditions. Step 1: 94°C for 1 minute (initial denaturation for hot start). Step 2: 92°C for thirty seconds (denaturation). Step 3: 55°C for thirty seconds (primer annealing). Step 4: 75°C for twenty seconds (extension). Step 5: 75°C for five minutes (final extension). The reaction was ‘hot started,’ and Taq polymerase was not added until the template had reached 94°C. 100 uL of silicon oil was then overlaid on the 100 uL reaction mixture and the reaction was allowed to proceed. An annealing temperature of 55°C, and a final Mg^{++} concentration of 2.5 mM were found to be satisfactory for all primer sets used. The number of times steps two through four were repeated (the number of cycles) was determined empirically for each primer set in order to ensure that the reaction was terminated well before the plateau phase of the reaction was reached, so that the final amount of PCR product would be roughly proportional to the amount of input template.

The amplification kinetics of each primer set were determined empirically, by adding excess template to the reaction, and then quantifying the amount of PCR product after 10, 15, 20, 22, 25, 27, 29, 30, 32, 34, 36, 38, 40, and 45 cycles, respectively. The amount of PCR product was quantified by pre-staining with SybrGreen™ stain according to
manufacturer’s specification. The primers used for each region, and the number of cycles at which the reaction was terminated was listed in Table 3.1.

PCR was carried out by adding 2.5 U of Taq polymerase to a 100 uL reaction mixture containing the ChIP template, 0.5 uM of each primer, 0.2 mM of each nucleotide, 2.5 mM MgCl₂, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). After the reaction was terminated by the addition of EDTA and storage on ice, the 100 uL reaction mixture was divided in half, and half was stained with EtBr for use in the figures, while the other half was prestained with SybrGreen™, run through a 2% agarose gel, and quantified using a STORM™ Imager, and ImageQuant™ software.

A graphical representation of the reporter gene located in the 2L telomere is given in Figure 3.1, showing the approximate position of the reporter gene primers. Multiplex qPCR was used to analyze the telomeric and euchromatic reporter genes, where the reporter gene and the Actin 42A gene from each stock were amplified simultaneously in the same reaction tubes. Primers sets for the reporter gene and the Actin 42A gene had identical amplification kinetics, and did not interfere with each other (data not shown). Multiplex PCR products stained with EtBr for the reporter genes and internal control Actin 42A genes are shown in Figure 3.2. The six regions amplified for the native, unadulterated telomere are illustrated graphically in Figure 3.3A, and EtBr-stained PCR products are shown in Figure 3.3B.
### Table 3.1

Primers sets used for ChIP analysis.

<table>
<thead>
<tr>
<th>Region amplified</th>
<th>Primer sequences</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter gene</td>
<td>5W3: 5’-AGT GAA CAC GTC GCT AAG CGA AAG 3W2: 5’-GGG ATT TTT GTG GGT CGC AGT TCT</td>
<td>30</td>
</tr>
<tr>
<td>Actin 42A</td>
<td>5A4: 5’-TGT CTG TGC GGT CAT TAT TAT TCC 3A12: 5’-GAT CTT CTC CAT GTC GTC CCA GTT</td>
<td>30</td>
</tr>
<tr>
<td>Region 1</td>
<td>HeT-1: 5’-CTG TCT CCG TAC CTC CAC CAG C HeT-G1: 5’-GGC GAA GTT TCC CTA GGG TGA AAG</td>
<td>25</td>
</tr>
<tr>
<td>Region 2</td>
<td>HeT-1A: 5’-GCT GGT GGA GGT ACG GAG ACA G 2Lsat-A: 5’-GGT GGC GGA TGA ACG AGA TG</td>
<td>38</td>
</tr>
<tr>
<td>Region 3</td>
<td>3TP2: 5’-GGT CAA TCT ATT CAC AGA AAT ACA CAG 5TP7: 5’-GCT CGA ACA TAG AAG ATA GGC TTG AAC</td>
<td>38</td>
</tr>
<tr>
<td>Region 4</td>
<td>5C23L: 5’-GCG AAT AGG GAC GTA TTA ATT GCC 3C23L: 5’-TCT CCT CCG TTA CTC CGT TAC TCG</td>
<td>38</td>
</tr>
<tr>
<td>Region 5</td>
<td>5L21: 5’-AAC CAC GGC CTC GCG AGA ATT GAA 3L21: 5’-GAC GCT ACA GTC GAG TTA CCC ATT</td>
<td>38</td>
</tr>
<tr>
<td>Region 6</td>
<td>5L2P: 5’-CGT GTG GTG GAC GGA ATT GCT AGT 3L2P: 5’-CCT TAC GAT GTA GCG TTG TCT GTC</td>
<td>38</td>
</tr>
</tbody>
</table>
Figure 3.1. Structure of the 2L telomere, and location of the telomeric reporter gene.

The Drosophila 2L telomere (A) is characterized by the presence of approximately 13 kb of moderately repetitive Telomere Associated Sequences (TAS) located proximal to a tandem array of HeT-A elements (WALTER et al. 1995). A reporter gene construct, mediating eye pigment deposition, was inserted into the TAS region (WALLRATH AND ELGIN 1995; B). In this subtelomeric location, the reporter gene is subject to TPE, and expression of the reporter gene is highly repressed (Chapter Two). The reporter gene consisted of a mini-white gene being driven by an hsp70 promoter (C). ChIP primers were designed to straddle the boundary between the hsp70 promoter and the mini-white coding sequence, as shown. This primer set was specific for the hsp70-mini-white fusion gene, and primed neither the native hsp70 gene, nor the native w+ gene (data not shown).
ChIP extracts were prepared from staged embryos containing the reporter gene located in either the 2L telomere (top gel) or euchromatin (bottom gel). Three separate ChIP extracts were prepared in all cases (n=3). Multiplex PCR was carried out on ChIP extract DNA using primers for the reporter gene promoter (529 bp amplicon) and the *Actin 42A* gene promoter (409 bp amplicon). The two primer sets had similar amplification kinetics, and did not interfere with one another (data not shown). PCR was terminated while amplification was in the exponential amplification phase (linear range) of the primers. PCR products were then quantified using SybrGreen™. Antibodies used for the ChIP protocol are indicated above the gels. A monoclonal antibody raised against the bacteriophage T7 protein was used as a negative control to establish a baseline for the ChIP protocol, and the amount of DNA isolated by other antibodies is reported as either a fold increase above this baseline, or as a proportion normalized to the internal *Actin 42A* gene. The molecular weight (mw) marker at the far left corresponds to a DNA fragment approximately 550 bp in size.
Figure 3.3. Quantitative PCR of the Drosophila 2L telomere.

PCR primers were designed to prime six selected regions of the native Drosophila 2L telomere, as shown (A). These regions included the HeT-A element promoters (1), the distal TAS boundary (2), the proximal TAS boundary (3), and three regions 3.5kb, 7kb, and 14kb on the proximal side of the TAS region (4, 5, and 6, respectively). Region 6 is the promoter region for the l(2)gl gene. Amplification kinetics experiments were carried out for each primer set to determine the exponential amplification phase (linear range) for each, and reactions were stopped within this range to ensure an approximate linear relationship between the amount of initial template in the reaction, and the final amount of PCR product. The ChIP protocol was carried out on staged embryos with antibodies for the proteins indicated (B), and PCR products were quantified as described above. As before, an α-T7 antibody was used to determine the baseline for each region. ChIP extracts were prepared in triplicate, as before (n=3).
III RESULTS

IIIA The HDAC1, SU(VAR)3-9, and HP1c proteins are present and enriched at the reporter gene when it is located in the telomere.

The ChIP assay was used to determine whether HDAC1, SU(VAR)3-9, HP1c, and HP1a were present at, and directly associated with the telomeric reporter gene (Figure 3.1), relative to the same reporter gene located at its native position in euchromatin. Briefly, the ChIP assay uses in situ formaldehyde cross linking of chromatin in the nuclei of staged embryos to reversibly cross link genomic DNA sequences to the proteins closely associated with them (SPENCER et al. 2003). After cross linking of DNA and proteins, the chromatin is sheared into fragments approximately 0.5 to 1.0 kb in length, and antibodies directed against specific proteins are used to purify chromatin fragments that contain these proteins. DNA fragments are then isolated by reversal of the protein-DNA cross links, and quantified using qPCR (Figures 3.2 and 3.3). Thus, the number of times that a specific protein is found associated with a specific DNA sequence can be estimated by quantifying the abundance of specific DNA fragments that were isolated by antibodies specific to the protein in question. The relative abundance of HDAC1, SU(VAR)3-9, HP1c, and HP1a at the telomeric reporter gene was estimated this way, normalized to the Actin 42A internal control, and reported as a fold increase above background. Background levels for the assay were determined using a non-specific T7 antibody.

In the telomeric location, the reporter gene is subject to TPE, is known to be transcriptionally repressed, and inaccessible to nucleases and other factors in Drosophila larvae (Boivin and Dura 1998; Frydrychova et al. 2007), and is also highly repressed in
The euchromatic reporter gene is not repressed, and was used as an example of active chromatin for comparison to the repressed telomeric reporter gene. Results obtained from the telomeric reporter gene were compared to results obtained from the euchromatic reporter gene after normalization of each to the Actin 42A internal controls.

### IIIA i  The HDAC1 protein is preferentially associated with the telomeric reporter gene.

ChIP results indicated that the HDAC1 protein was approximately nine times more abundant at the telomeric reporter gene promoter than at the promoter of an identical reporter gene located in euchromatin (Figure 3.4). Since the HDAC1 protein is normally believed to be associated with gene silencing, this result was consistent with the observation that the telomeric reporter gene is highly repressed in adult flies, and that repression can be partially alleviated by mutations in the gene encoding the HDAC1 protein. By contrast, the amount of HDAC1 associated with the euchromatic reporter gene was minimal.

### IIA ii  A SU(VAR)3-9GFP chimera is preferentially associated with the telomeric reporter gene.

To assess whether the SU(VAR)3-9 protein was physically associated with the telomeric reporter gene or not, a chimeric protein consisting of the SU(VAR)3-9 protein fused to the GFP was introduced into the fly stocks carrying the reporter genes. Antibodies
The ChIP protocol was carried out on an identical reporter gene, located in two different positions in two different fly strains, using antibodies specific to the HDAC1 protein. Results indicated that the relative concentration of HDAC1 was approximately nine times greater at a reporter gene located in the 2L telomere TAS region than at an identical reporter gene located in euchromatin (p < 0.001). Results from the two reporter genes were normalized to the Actin 42A gene, which is located at the same position in both strains. Three ChIP extracts were prepared (n=3; error bars represent standard deviations), and for each extract the amount of PCR product for both the reporter gene and the Actin 42A internal control was determined. The amount of PCR product for the reporter gene was then divided by the amount of PCR product for the Actin 42A gene from the same extract, and reported as a ‘Relative Fold Increase,’ relative to the Actin 42A gene.
directed against the GFP were then used to isolate DNA fragments that were closely associated with the SU(VAR)3-9GFP chimera. The SU(VAR)3-9GFP fusion protein has a chromosomal distribution pattern identical to the native SU(VAR)3-9 protein (Schotta et al. 2003; Schotta et al. 2002; and Schotta and Reuter 2000) and can be used to analyze the distribution of SU(VAR)3-9. Furthermore, this protein has HMTase activity, and can substitute for the native SU(VAR)3-9 protein in the PEV assay (R. Mottus and P. Kalas, unpublished results).

ChIP results indicated that approximately four times more SU(VAR)3-9GFP was associated with the telomeric reporter gene than with an identical reporter gene located in euchromatin (Figure 3.5). Since SU(VAR)3-9 is normally believed to be associated with gene silencing, these results were consistent with the observation that the telomeric reporter gene is highly repressed in adult flies, but that the repression can be partially alleviated by mutations of the Su(var)3-9 gene, as illustrated in Chapter Two. These results also suggest that SU(VAR)3-9 may be a component of telomeric chromatin, and one of the proteins responsible for TPE in Drosophila.

IIIAiii The HP1c protein is preferentially associated with the telomeric reporter gene.

ChIP results indicated that the HP1c protein was approximately eight times more abundant at the promoter of the telomeric reporter gene than at the promoter of an identical reporter gene located in euchromatin (Figure 3.6). These results suggest that
Figure 3.5. A SU(VAR)3-9GFP chimera preferentially associates with the telomeric reporter gene.

A fusion protein consisting of the SU(VAR)3-9 protein and the GFP is known to have the same distribution pattern as the native SU(VAR)3-9 protein (SCHOTTA et al. 2003; SCHOTTA et al. 2002; AND SCHOTTA AND REUTER 2000), and was used to measure the relative abundance of SU(VAR)3-9 at the telomeric and euchromatic reporter genes. Results indicated that approximately four times more SU(VAR)3-9GFP was associated with the telomeric reporter gene than with an identical reporter gene located at its native position in euchromatin (p < 0.05). (n=3; error bars represent standard deviations.)
Figure 3.6. HP1c is preferentially associated with the reporter gene when it is located in the telomere, relative to the same reporter gene located in euchromatin.

The HP1c protein was approximately eight times more abundant at a reporter gene located in the 2L telomere than at an identical reporter gene located in its native position in euchromatin (p < 0.05). (n=3; error bars represent standard deviations.)
HP1c may be a component of telomeric chromatin, and may be one of the proteins responsible for TPE in Drosophila.

**IIIa.ii** The **HP1a protein is not preferentially associated with the telomeric reporter gene.**

Mutations in the gene encoding the HP1a protein did not suppress TPE. I asked whether the HP1a protein was associated with the reporter gene or not. ChIP results indicated that the amount of HP1a that was associated with the telomeric reporter gene was not significantly different from the amount associated with the euchromatic reporter gene (Figure 3.7).

In summary, therefore, the three proteins that caused suppression of TPE when either mutated or made hemizygous were preferentially associated with the telomeric reporter gene relative to the same reporter gene located in its native position in euchromatin. By contrast, a protein that did not suppress TPE when either mutated or made hemizygous was not preferentially associated with the telomeric reporter gene. These results suggest that HDAC1, SU(VAR)3-9, and HP1c are integral components of telomeric heterochromatin, as well as potential mediators of TPE in Drosophila. These results also validate the use of the TPE-suppression assay as a means of identifying components of telomeric heterochromatin.
Figure 3.7. HP1a is not enriched at the TAS-embedded reporter gene.

The HP1a protein was not preferentially associated with the telomeric reporter gene, and concentrations of HP1a were approximately equal for reporter genes located in either the 2L telomere, or the native position in euchromatin. (n=3; error bars represent standard deviations.)
HDAC1, SU(VAR)3-9, HP1c are present at the native 2L telomere, but are preferentially associated with the TAS region.

The reporter gene used to assay TPE was embedded in the subtelomeric TAS region of the 2L telomere, as illustrated in Figure 3.8. I found that three proteins that suppressed TPE when mutated were directly associated with, and enriched at this reporter gene when located in the TAS region. The next logical question to ask was whether or not these proteins were also present and enriched at the native 2L telomere in the absence of a reporter gene, or if their presence at the telomeric reporter gene was merely an artifact that resulted from the insertion of the reporter gene into the telomere. Accordingly, I repeated the ChIP assay with a 2L telomere that did not carry a reporter gene.

Six different regions of the 2L telomere were analyzed, as illustrated in Figure 3.8. The distal end of the 2L telomere is populated by a tandem array of class I transposable elements known as HeT-A elements (Walter et al. 1995), which maintain telomere length in Drosophila (Danilevskaya et al. 1997). The direction of HeT-A transcription is indicated by the arrows (Figure 3.8). The promoter regions of these HeT-A elements were analyzed (Region 1). It was not possible to analyze the promoter region of any individual HeT-A element, since they are reiterated copies of identical sequences, and the results from Region 1 should be considered an average of all the HeT-A elements in the genome, the vast majority of which are believed to reside at telomeres.
Figure 3.8. Structure of the 2L telomere with and without the reporter gene.

The reporter gene used to assay TPE is inserted into the 13 kb subtelomeric TAS region (A). The ChIP assay was carried out on the reporter gene promoter from this fly strain (Section IIIA). The ChIP assay was also carried out on a native, unadulterated 2L telomere (B; Section IIIB). Six independent regions were analyzed, including the HeT-A element promoter regions located in the 3’ UTRs of each element (1), the TAS region distal boundary (2) which abuts the HeT-A elements, the TAS region proximal boundary (3), and three regions which are 3.5 kb, 7 kb, and 14 kb proximal to the TAS region (4, 5, and 6, respectively). Region 4 does not contain any confirmed genes. Region 5 is located in the first intron of the essential \(l(2)gl\) gene, and Region 6 is the \(l(2)gl\) gene promoter.
(Biessmann et al. 1990; Biessmann et al. 1992; Biessmann et al. 1994; Biessmann et al. 1993; Biessmann et al. 1998; Biessmann and Mason 1992; Biessmann and Mason 1994; Biessmann and Mason 1997; Biessmann and Mason 2003; Biessmann et al. 2005; Walter et al. 1995).

The distal and proximal boundaries of the TAS were also analyzed (Regions 2 and 3, respectively). Due to the repetitive nature of the TAS, it was not possible to carry out a ChIP assay on internal TAS sequences. It was only possible to carry out the assay on the TAS regions that border on single copy DNA, allowing one of the PCR primers in each pair to hybridize to a region of non-repetitive DNA.

Three regions of unique, single copy DNA sequences, located approximately 3.5, 7, and 14 kb proximal to the TAS (Regions 4, 5, and 6, respectively) were also analyzed. Region 4 does not contain any known genes. Region 5 is located in the first intron of the Lethal (2) giant larvae (l(2)gl) gene, and Region 6 is the l(2)gl gene promoter. The l(2)gl gene is active in developing embryos (Klaembt and Schmidt 1986), with transcription in the direction indicated by the arrow (Figure 3.8). Since the l(2)gl gene is known to be active and transcriptionally competent in larvae, its promoter can be used as an example of transcriptionally competent chromatin. Regions 4 and 5, by contrast, were used as examples of relatively ‘neutral’ chromatin.
IIIBi The HDAC1 protein is preferentially associated with the TAS region of the Drosophila telomere.

ChIP results indicated that the distribution of HDAC1 was not uniform across the entire 2L telomere, and that significantly more HDAC1 was associated with the TAS boundaries (Regions 2 and 3) than with any other region of the telomere analyzed (Figure 3.9). Recall that significantly more HDAC1 was also found at a reporter gene when located in the TAS region (Section IIIA).

IIIBii SU(VAR)3-9GFP is preferentially associated with the TAS and HeT-A regions.

Distribution of the SU(VAR)3-9 protein across the native 2L telomere was assessed using the SU(VAR)3-9GFP chimera, as described previously. Results indicated that, like HDAC1, the SU(VAR)3-9GFP chimera was not uniformly distributed across the telomere, but was instead predominantly associated with the TAS region (Figure 3.10). Approximately six and three times more SU(VAR)3-9GFP was seen at the distal and proximal TAS boundaries, respectively, than at the three regions proximal to the TAS region. Furthermore, significantly greater amounts of SU(VAR)3-9GFP were also associated with the HeT-A elements, than with the three regions located downstream of the TAS.

As with the results for HDAC1, the results for SU(VAR)3-9GFP suggest that the telomeric reporter gene had taken on a chromatin composition that was more like the
Figure 3.9. Distribution of HDAC1 across the 2L telomere.

The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific for the HDAC1 protein. Results (B) indicated that significantly more HDAC1 was associated with the TAS proximal and distal boundaries (Regions 2 and 3; p < 0.05) than with the other areas examined. No significant difference was found between the amount of HDAC1 associated with Regions 1, 4, 5, and 6. (n=3; error bars represent standard deviations.)
Figure 3.10. Distribution of SU(VAR)3-9GFP across the 2L telomere.

The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific to the GFP portion of the SU(VAR)3-9GFP chimera. Results (B) indicated that the SU(VAR)3-9GFP chimera was found in greater abundance at the distal TAS boundary (Region 2) than at any other area tested (p< 0.05). Enrichment for SU(VAR)3-9GFP was also seen at the HeT-A region, and the proximal TAS boundary (Regions 1 and 3, respectively), with these two regions showing a relative abundance of SU(VAR)3-9GFP more than three times greater than at the three regions on the proximal side of the TAS (p < 0.05). (n=3; error bars represent standard deviations.)
TAS region into which it had been inserted, than any other region of the telomere. It should be noted, however, that the amount of SU(VAR)3-9GFP associated with the telomeric reporter gene (Figure 3.5) was significantly lower than the amount of SU(VAR)3-9GFP associated with the unadulterated TAS region and HeT-A region, and thus, the telomeric reporter is not a completely accurate model of the TAS region of the telomere.

III Biii HP1c is moderately enriched at the TAS region.

ChIP results indicated that the HP1c protein was moderately enriched at the TAS boundaries relative to regions 2 and 3 (Figure 3.11). In addition to the moderate increase of HP1c at the TAS region, HP1c was also slightly more abundant at Region 4 than at Regions 1, 5, and 6. The amount of HP1c seen at the TAS region was, again, similar to the amount of HP1c seen at the telomeric reporter gene (Figure 3.6), indicating that the reporter gene had taken on a chromatin composition similar to the TAS region into which it had been inserted. The amount of HP1c seen at the active l(2)gl promoter was minimal, and similar to that seen at the euchromatic reporter gene (Figure 3.6).

III Biv The HP1a protein is not enriched at the 2L telomere.

ChIP results indicated only a slight increase in the amount of HP1a associated with the TAS proximal boundary (Region 3) and Region 4, relative to the other areas examined (Figure 3.12). Regions 1, 2, and 5 also showed a slight increase in the amount of HP1a
Figure 3.11. Distribution of HP1c across the 2L telomere.

The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific for the HP1c protein. Results (B) indicated that HP1c was slightly more abundant at Regions 2, 3, and 4 (p <0.05) than at Regions 1, 5, and 6. (n=3; error bars represent standard deviations.)
Figure 3.12. Distribution of HP1a across the 2L telomere.

The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific for the HP1a protein. Results (B) indicated a small, but significantly greater abundance of HP1a at the proximal TAS boundary (Region 3) and the region 3.5 kb proximal to this boundary (Region 4) than at regions 1, 2, and 5 (p< 0.05). Regions 1, 2, and 5 also showed HP1a in slightly greater abundance than did the l(2)gl promoter (Region 6; p < 0.05). (n=3; error bars represent standard deviations.)
relative to the active \( l(2)gl \) promoter (Region 6). However, when these results are compared to the euchromatic reporter gene (Figure 3.7), no significant increase for HP1a is seen at any of the telomeric regions examined in this study, suggesting that HP1a is not a constituent of telomeric or subtelomeric chromatin.

IIIC Distribution of histone modifications at the telomeric reporter gene.

The results listed in Section IIIA suggest that the HDAC1 and SU(VAR)3-9 proteins are associated with the telomeric reporter gene used to test TPE in Chapter Two. Furthermore, the results listed in Section IIIB suggest that these proteins are preferentially associated with the TAS region of the native Drosophila telomere, and that the TAS region may be critical to the phenomenon of TPE due to its putative affinity for these proteins. Since HDAC1 is known to remove acetate groups from H3K9, mediating gene silencing in other regions of the genome (MOTTUS et al. 2000), and since SU(VAR)3-9 is known to methylate H3K9, also mediating gene silencing in other regions of the genome (SCHOTTA et al. 2002), I decided to analyze the distribution patterns of H3K9ac and H3K9me at the reporter genes (Section IIIC), as well as across the native 2L telomere (Section IIID).
IIICi  The telomeric reporter gene promoter shows enrichment for methylated H3K9, a post translational modification associated with the HMTase activity of SU(VAR)3-9, and with gene silencing.

Mutations in the Su(var)3-9 gene suppressed TPE, and a SU(VAR)3-9GFP chimera was found at the telomeric reporter gene used to assay TPE, suggesting that the HMTase activity of SU(VAR)3-9 may be one of the mechanisms responsible for TPE in Drosophila. Accordingly, I assayed for the presence of methylated H3K9 at the telomeric reporter gene promoter (Figure 3.13). Results indicated that approximately 14 times more H3K9me was associated with the reporter gene when it is located in the TAS region of the telomere than when it is located in euchromatin. The increase in the amount of H3K9me seen at the telomeric reporter gene paralleled the increase in SU(VAR)3-9GFP seen at the telomeric reporter gene (Figure 3.5), suggesting that SU(VAR)3-9 was responsible for the observed increase in H3K9me, and that methylation of H3K9 by SU(VAR)3-9 may be one of the mechanisms responsible for TPE in Drosophila.

IIICii  Despite the presence of HDAC1, the telomeric reporter gene promoter showed increased acetylation of H3K9, a post translational modification normally associated with gene activation.

Despite the greater abundance of the histone deacetylase HDAC1 seen at the telomeric reporter gene (Figure 3.4), a larger amount of acetylated H3K9 was also seen at the telomeric reporter gene (Figure 3.14). Approximately four times more H3K9ac was found at the telomeric reporter gene than the euchromatic reporter gene.
Figure 3.13. Enrichment of H3K9me at the telomeric reporter gene.

The concentration of methylated H3K9 was approximately 14 times greater for a reporter gene located in the 2L telomere, than for an identical reporter gene located at its native position in euchromatin (p < 0.05). The increase in the amount of methylated H3K9 observed at the telomeric reporter gene was consistent with the increased amount of SU(VAR)3-9GFP observed at the telomeric reporter gene. (n=3; error bars represent standard deviations.)
Figure 3.14. The telomeric reporter gene shows enrichment for acetylated H3K9.

Despite an increase in the amount of HDAC1 associated with the telomeric reporter gene (Figure 3.4), the reporter gene was associated with approximately four times more H3K9ac when located in the 2L telomere than when located at its normal position in euchromatin (p < 0.05). The observation that the telomeric reporter gene was associated with higher levels of H3K9ac than the euchromatic reporter gene was unexpected, given that H3K9ac is thought to be associated with gene activity, and that the telomeric reporter gene is strongly repressed. (n=3; error bars represent standard deviations.)
This result was surprising for at least three reasons. Firstly, the telomeric reporter gene is highly repressed in both larvae and adult flies, yet, according to the histone code model of gene regulation, acetylated H3K9 is a modified histone normally associated with the nucleosomes of active genes, rather than silenced ones. Secondly, larger amounts of HDAC1 were seen at the telomeric reporter gene. Since HDAC1 is known to remove acetate groups from H3K9, an increase in the amount of HDAC1 present would predict that less H3K9ac should be present in the same area. Finally, since acetylation of H3K9 and methylation of H3K9 are mutually exclusive modifications to the same histone protein residue, the observed increase in the amount of H3K9me seen at the telomeric reporter gene would have predicted that less H3K9ac should be seen in the same location. This pattern of post-translational modifications was perplexing, but the same pattern was also observed at the native 2L TAS region, suggesting that it was not simply an artifact associated with the insertion of a gene into the telomere. This pattern was also observed for the native telomere (see below), and possible explanations for it are addressed in the discussion section of this chapter.

IIID Distribution of histone modifications across the native Drosophila 2L telomere.

The distribution patterns of H3K9ac and H3K9me were also evaluated for the native 2L telomere in the absence of a reporter gene. As with HDAC1 and SU(VAR)3-9GFP, H3K9ac and H3K9me were not found to be evenly distributed across the 2L telomere.
However, the distribution patterns of H3K9ac and H3K9me were roughly consistent with the distribution patterns of HDAC1 and SU(VAR)3-9GFP, with H3K9ac levels being high where HDAC1 levels were low, and H3K9me levels being high where SU(VAR)3-9 levels were high. Results from the TAS region, however, were an exception. Like the TAS-embedded reporter gene, the histone modifications associated with the TAS region were unexpected, with the TAS boundaries showing increased levels of both H3K9ac and H3K9me, an apparent contradiction to the histone code.

**HIDi** The HeT-A region of the telomere is highly enriched for acetylated H3K9ac, a histone modification normally associated with transcriptionally-competent chromatin.

ChIP results indicated that the HeT-A region was highly acetylated at H3K9, a modification that would tend to suggest these elements were transcriptionally competent (Figure 3.15). These results are consistent with results from other studies which showed that HeT-A elements are transcriptionally active in developing and proliferating cells (WALTER and BIESSMANN 2004). The promoter of the l(2)gl gene, which is active in Drosophila larvae, was also relatively highly acetylated when compared to the two regions of neutral chromatin (Regions 4 and 5). Interestingly, however, the distal and proximal TAS boundaries showed a level of H3K9 acetylation that was at least as high as the active l(2)gl promoter. This observation was again unexpected, given that a reporter gene inserted into the TAS is highly repressed, and is known to be both transcriptionally repressed and inaccessible to nucleases and transcription factors (FRYDRYCHOVA et al. 2007; BOIVIN and DURA 1998).
The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific to acetylated H3K9. Results indicated that H3K9ac was considerably more abundant in the HeT-A region than in all other areas (p < 0.001), an observation that would be consistent with the HeT-A elements being transcriptionally active. The distal and proximal TAS boundaries were also enriched for H3K9ac relative to Regions 4 and 5 (p < 0.05). (n=3; error bars represent standard deviations.)
Superimposition of the H3K9ac and HDAC1 distribution patterns indicates that, with the possible exception of the TAS region, the distribution pattern for H3K9ac is essentially the inverse of the HDAC1 distribution pattern, as one would expect (Figure 3.16). In the HeT-A region and the l(2)gl promoter, the level of H3K9 acetylation was relatively high, while HDAC1 was reduced. In the two regions of neutral chromatin (Regions 4 and 5) levels of both H3K9ac and HDAC1 were relatively low. By contrast, the TAS boundaries were characterized by high levels of both H3K9ac and HDAC1, an apparent contradiction. Contradiction or not, these results were in agreement with those obtained from the TAS-embedded telomeric reporter gene, which was also associated with relatively high levels of both H3K9ac (Figure 3.14) and HDAC1 (Figure 3.4).

IIIDii The distal HeT-A/TAS boundary is highly enriched for methylated H3K9, a modified histone normally associated with the HMTase activity of SU(VAR)3-9.

ChIP results indicated that the distal TAS boundary was associated with significantly greater amounts of methylated H3K9 than the other regions examined (Figure 3.17). Superimposition of the H3K9me distribution pattern with the SU(VAR)3-9GFP pattern indicates that the pattern of H3K9me distribution is roughly the same as that of SU(VAR)3-9GFP (Figure 3.18), as one might predict. This would tend to suggest that the HMTase activity of SU(VAR)3-9 was responsible for the observed pattern of H3K9 methylation.
Figure 3.16. The distribution pattern of H3K9ac is roughly the inverse of the distribution pattern for HDAC1.

In areas of active gene transcription, such as the HeT-A and \(l(2)gl\) promoters (Regions 1 and 6, respectively), the abundance of histone H3 acetylation is relatively high, while the corresponding abundance of HDAC1 is relatively low. The TAS region, represented here by Regions 2 and 3, shows levels of H3K9 acetylation that are approximately equivalent to that of the active \(l(2)gl\) promoter. This observation was interesting, given that a reporter gene inserted into the TAS region is highly repressed. However, the TAS region also showed levels of HDAC1 that were higher than the surrounding regions, which may account for the observed silencing of the reporter gene.
Figure 3.17. Distribution of H3K9me across the 2L telomere.

The ChIP protocol was carried out for six different regions of the Drosophila 2L telomere (A) using antibodies specific for H3K9me. Results (B) indicated that H3K9me was present in the greatest abundance at the distal TAS boundary (Region 2), relative to other areas (p < 0.05). Regions 1, 3, 5, and 6 were not significantly different from one another. (n=3; error bars represent standard deviations.)
Figure 3.18. The distribution pattern of SU(VAR)3-9GFP is similar to the distribution pattern of H3K9me.

A comparison of the distribution patterns of SU(VAR)3-9GFP and H3K9me across the 2L telomere indicated that the greatest concentrations of both SU(VAR)3-9GFP and H3K9me were found at the distal TAS boundary, straddling the HeT-A and TAS regions.
IV DISCUSSION

IVA TPE and the chromatin composition of TAS regions.

Suppression of TPE was used as a starting point in a search designed to find protein constituents of telomeric heterochromatin. Three of the TPE-suppressing candidate proteins identified in Chapter Two were directly associated with the telomeric reporter gene used to assay TPE, while a fourth, non-TPE suppressing protein was not. These results tend to validate this approach to finding telomere-specific proteins in Drosophila, and suggests that the other protein candidates highlighted in Chapter Two are worthy of further investigation in future studies of Drosophila telomeres.

The reporter gene used for these experiments was associated with greater amounts of HDAC1, SU(VAR)3-9, and HP1c when it was located in the TAS region of the 2L telomere than when it was located at its native position in euchromatin. Indeed, the HDAC1, SU(VAR)3-9, and HP1c levels associated with the reporter gene in its euchromatic location were close to the background level of the ChIP assay, and may have been absent altogether. Since the DNA sequence of the reporter gene was identical in both cases, the difference in chromatin composition seen at the telomere could only have been a result of the reporter gene’s new location in the telomere, suggesting that HDAC1, SU(VAR)3-9, and HP1c are components of telomeric heterochromatin.
A broader examination of the native 2L telomere indicated that HDAC1, SU(VAR)3-9, and HP1c were preferentially associated with the TAS region into which the telomeric reporter gene had been inserted. Thus, the telomeric reporter gene had taken on a chromatin composition similar to the region into which it had been inserted, but which was not typical of the entire telomere. The terminal HeT-A array on the distal side of the TAS, and the two regions of neutral chromatin and the l(2)gl promoter on the proximal side of the TAS were associated with lesser amounts of these proteins.

Since HDAC1 and SU(VAR)3-9 are implicated in the histone code model of gene regulation, I also examined post-translational histone modifications known to be associated with these two proteins. Acetylation of histone H3K9 is normally associated with transcriptionally competent chromatin, and the observation that greater amounts of H3K9ac were seen at the HeT-A promoters is consistent with the idea of these elements being transcriptionally active during development (WALTER and BIESSMANN 2004). Deacetylation of H3K9ac, by contrast, is associated with gene silencing, and HDAC1 is known to deacetylate histone H3K9. Thus, the observation that the TAS was associated with greater amounts of HDAC1 was consistent with the observation that a reporter gene inserted into it is highly repressed.

Methylation of H3K9 by HMTases such as SU(VAR)3-9 is also associated with gene silencing, and the observed enrichment for SU(VAR)3-9 and H3K9me at the TAS is also consistent with the TAS-embedded reporter gene being highly repressed. Thus, these results suggest that HDAC1 and SU(VAR)3-9 are associated with the TAS, and that the
histone deacetylation and histone methylation functions of these proteins are components of the mechanism responsible for TPE in Drosophila. This also suggests that the TAS region is the locus of TPE in Drosophila.

**IVB The HP1a protein is not a constituent of subtelomeric chromatin.**

The observation that HP1a did not preferentially associate with the telomere regions examined here was somewhat unexpected, given that HP1a is seen at telomeres in cytological studies of chromosomes, is believed to form part of the telomere capping complex, and is generally found associated with heterochromatin and methylated H3K9 at centromeres. Indeed, current theories about the nature of HP1a posit that it has an affinity for di and tri-methylated H3K9, and that methylation of H3K9 by SU(VAR)3-9 creates a binding site for the HP1a protein (LACHNER et al. 2001), thus presumably explaining why they are often found together in pericentric heterochromatin.

While a superimposition of the SU(VAR)3-9GFP and H3K9me distribution patterns (Figure 3.18) suggests that SU(VAR)3-9 is the source of methylated H3K9 at telomeres, a similar superimposition of the H3K9me and HP1a distribution patterns reveals no such similarity (Figure 3.19). Superimposition of the HP1c and H3K9me distribution patterns reveals some similarity in pattern (Figure 3.19), but neither the distribution pattern of HP1c nor the distribution pattern of HP1a is a particularly good match for the distribution pattern of H3K9me. As a result of these and other results, it is possible that the proposed relationship between SU(VAR)3-9, H3K9me, and HP1a in the formation of
Figure 3.19. There is no obvious correlation between the distribution patterns of H3K9me and HP1a at the 2L telomere.

The distribution pattern of methylated H3K9 at the 2L telomere is not a match for either the distribution pattern of the HP1a protein or the distribution pattern of the HP1c protein.
heterochromatin will have to be re-evaluated. Indeed, there is already evidence that the
distribution patterns of HP1a and HP1c are not always correlated with the distribution
pattern of SU(VAR)3-9 throughout the genome (Greil et al. 2003), and the results
presented in this study suggest that this is also true of HP1a, HP1c and SU(VAR)3-9, at
least at the 2L telomere.

Furthermore, while various lines of evidence suggest that the HP1a protein may form part
of the telomere capping complex (Cenci et al. 2005; Cenci et al. 2003; Fantì et al.
2003; Fantì et al. 1998a; Fantì et al. 1998b), there is no direct evidence to suggest that
it is a component of subtelomeric chromatin in Drosophila. The results presented here
suggest that it is not. Indeed, if the telomere capping function is separate from the
telomere silencing (TPE) function, it is likely that the HP1a seen at telomeres in
cytological studies of chromosomes was part of the telomere capping complex, but was
not necessarily associated with the subtelomeric regions, including the TAS. The genetic
evidence presented in Chapter Two, and the ChIP evidence presented here suggest that
HP1c may be a more important constituent of subtelomeric chromatin than is HP1a.
HP1a, by contrast, may form part of the telomere capping complex, while being
essentially absent from subtelomeric regions.
IVC  The nucleosomes that make up the TAS region show enrichment for both acetylated H3K9 and methylated H3K9.

A fairly striking contradiction to the histone code model of gene regulation was also observed at the telomeric regions examined here. The observation that the repressed TAS-embedded reporter gene was both highly acetylated and highly methylated at H3K9 was somewhat perplexing. The presence of methylated H3K9 residues at the repressed reporter gene was entirely consistent with gene repression, according to the histone code. Acetylation of H3K9, by contrast, is a modification normally associated with actively transcribing genes, as witnessed by the high levels of acetylation seen at the euchromatic reporter gene, the l(2)gl promoter, and the two Actin 42A promoters. The finding that the telomeric reporter gene promoter was associated more acetylated H3K9 than any of the active genes analyzed was unexpected, and inconsistent with the observation that the telomeric reporter gene is highly repressed.

There are several possible explanations for this, two of which are fairly trivial. One possible explanation is that the TAS region is transcriptionally active early in development, and silenced later, or vice versa. Thus, the ChIP extracts used here could have contained chromatin fragments derived from both early and late embryos, and hence, a mixture of both types of chromatin. Since the embryos used here were staged within a six hour time frame, this would require a fairly dramatic reversal of acetylation and methylation in a fairly short period of time.
A second possible explanation is that the chromatin fragments used here, which were between 0.5 and 1.0 kb in length, contained some nucleosomes that were acetylated at H3K9 while other nucleosomes were methylated at H3K9. Fragments of this size could easily accommodate several nucleosomes. Thus, both the native TAS region, and the repressed reporter gene may have contained at least two kinds of nucleosomes at the same time, some of which were acetylated at H3K9 while others were methylated at H3K9.

In either case, two populations of nucleosomes would appear to be associated with the TAS. One population is methylated at histone H3K9, while the other is acetylated at histone H3K9. The question as to whether both methylated and acetylated nucleosomes are present on the same fragments of DNA could theoretically be resolved by a sequential ChIP experiment, or a ChIP experiment followed by a western blot. In such an experiment, ChIP fragments could first be precipitated with antibodies specific for the acetylated form of H3K9. These fragments could then be re-probed with antibodies specific for the methylated form of H3K9. If the fragments responded to the second antibody, it would suggest that both methylated and acetylated nucleosomes were resident on the same chromatin fragments. A converse experiment could also be carried out which used antibodies specific to methylated H3K9 first.

The idea of both acetylated and methylated nucleosomes being associated with a repressed reporter gene raises an interesting third possibility. Since the TAS-embedded reporter gene is highly repressed, this would imply that the presence of both methylated
H3K9 and acetylated H3K9 residues in a particular region of chromatin leads to silencing by default, and that the gene silencing mechanisms associated with methylation of H3K9 are epistatic to the gene activation mechanisms associated with acetylation of H3K9, and take precedence over them.

Figure 3.20 is a compilation of the ChIP profiles obtained from the HeT-A region of the telomere, the distal TAS boundary, the telomeric reporter gene, and four other genes that are known to be transcriptionally active, including the euchromatic reporter gene, the \( l(2)gl \) gene, and the two \( \text{Actin 42A} \) genes used as internal controls. HDAC1, SU(VAR)3-9, acetylated H3K9, and methylated H3K9 are shown. (In this case, none of the results are normalized to the \( \text{Actin 42A} \) genes, and fold increases above the baseline, as determined by the T\(_7\) antibody are shown instead.) Note that the four active genes (shown on the right) have very similar chromatin profiles. All four are characterized by a relatively high level of H3K9 acetylation, and minimal levels of HDAC1, SU(VAR)3-9GFP and H3K9me. While the level of H3K9 acetylation is much higher at both the TAS-embedded reporter gene and distal TAS boundary, so are the levels of SU(VAR)3-9GFP and H3K9me.

It is possible that a certain level of histone methylation imposes gene silencing by default, regardless of the level of histone acetylation present in the same region. This would be an example of a ‘threshold effect’ for histone H3K9 methylation. If a threshold level of histone methylation were indeed responsible for gene silencing, this would suggest a ‘digital’ method of transcriptional control. The presence of a certain threshold level of
H3K9me would lead to gene silencing by default, regardless of how much H3K9ac may be present in the same region.

On the other hand, it may also be possible that control of transcription is ‘analog’ rather than ‘digital,’ and that it is the ratio of acetylation to methylation of H3K9 in a particular region that determines transcriptional competence. Figure 3.21 shows the ratio of histone acetylation to histone methylation for the same regions shown in Figure 3.20. If the ratio of histone acetylation to histone methylation is able to determine whether or not a gene is on or off, then a ratio of approximately six or higher could mean that a gene is transcribed, since all four of the active genes have an acetylation to methylation ratio that is greater than six. The repressed telomeric reporter gene, by contrast, has an acetylation to methylation ratio of less than three, and the distal TAS boundary has an acetylation to methylation ratio of less than one. Given that the telomeric reporter gene is highly repressed, a ratio between three and six may represent the threshold for the off versus on state.

**IVD**  
The Drosophila telomere appears to be a region of intense competition between chromatin modifications that facilitate transcription, and chromatin modifications that inhibit transcription.

Our results indicate that the TAS region of the Drosophila telomere may be a locus for TPE, through its association with HDAC1 and SU(VAR)3-9, and the histone deacetylation and histone methylation activities associated with them. Both of these
activities are thought to mediate gene silencing according to the histone code model of gene regulation. The presence of elevated levels of acetylated H3K9 in the TAS region were inconsistent with this model, but I have proposed both a putative digital and a putative analog model as an adjunct to the histone code model to explain the observed silencing of the TAS-embedded reporter gene.

Regardless of whether a digital or an analog model of the histone code is invoked, the Drosophila telomere appears to be an area of intense competition between chromatin modifications that infer transcriptional competence to chromatin, such as acetylation of H3K9, and chromatin modifications that inhibit transcription, such as deacetylation and subsequent methylation of H3K9 by HDAC1 and SU(VAR)3-9, respectively. The subtelomeric TAS region appears to be the region where this competition is most intense, showing elevated levels of both acetylation and methylation of histone H3K9.

The HeT-A region, which is responsible for maintaining telomere length in Drosophila, does not appear to be exempt from this competition either, since the observed levels of both SU(VAR)3-9GFP and H3K9me were found to be higher in this area than they were for the active genes analyzed. The relatively high levels of histone acetylation seen at the HeT-A elements, alone, may not confer transcriptional competence to this region given that histone methylation levels are also higher in this area.

Moreover, since the HeT-A elements are responsible for maintaining telomere length in Drosophila, the regulation of transcription from these elements is of considerable interest.
Figure 3.20. Relative abundance of H3K9ac, HDAC1, SU(VAR)3-9GFP, and H3K9me at the HeT-A region, the distal TAS boundary, the telomeric reporter gene, and the promoters of four active genes.

The chromatin compositions of the HeT-A region (A), the distal TAS boundary (B), and the TAS-embedded telomeric reporter gene (C) are compared to the chromatin compositions of the promoter regions of four active genes. The active genes shown include the euchromatic reporter gene (D), the \textit{l(2)gl} gene (E), and the \textit{Actin 42A} genes from the two fly stocks carrying the reporter genes (F and G). The promoters of actively transcribing genes were characterized by relatively high levels of acetylated H3K9ac, and minimal levels of HDAC1, SU(VAR)3-9GFP, and H3K9me. The repressed, telomeric reporter gene was characterized by even higher levels of acetylated H3K9, but also by high levels of both SU(VAR)3-9GFP and methylated H3K9.
Figure 3.21. Ratios of acetylated H3K9 to methylated H3K9 for a repressed telomeric reporter gene, and four active genes.

The relative abundance of acetylated H3K9 was divided by the relative abundance of methylated H3K9 to give a ratio of H3K9ac to H3K9me for the repressed, telomeric reporter gene (C), and four active genes (D through G). The active gene promoters were characterized by acetylation to methylation ratios of six or greater. The repressed, telomeric reporter gene had an acetylation to methylation ratio of less than three. The distal TAS boundary, which was associated with very high levels of H3K9me, had an acetylation to methylation ratio of less than one. The four active gene promoters, and the HeT-A region had acetylation to methylation ratios that were significantly greater than the repressed, telomeric reporter gene, and the distal TAS boundary (p < 0.05).
The results presented here suggest that both the HeT-A region, and TAS region in particular, are regions of intense competition between forces that confer transcriptional competence to chromatin, and forces that inhibit transcription, at least as far as the histone code model of gene regulation is concerned. The method by which transcription of the HeT-A elements is controlled is not known, but the results presented here suggest that deacetylation of H3K9 by HDAC1, and methylation of H3K9 by SU(VAR)3-9 may be involved in the regulation of HeT-A element activity, as well as the phenomenon of TPE. Perhaps more importantly, these results also suggest that the phenomenon of TPE, and the mechanisms responsible for it, may be involved in the regulation of HeT-A activity and telomere length maintenance in Drosophila.

The idea that TPE may be related to a telomere length control mechanism is not a new one, and has already been suggested by other investigators (Golubovsky et al. 2001; Kurenova et al. 1998; Mason et al. 2000; Mason et al. 2003a; Mason et al. 2003b). This model for TPE, known as the ‘HeT-A activation model,’ asserts that the phenomenon of TPE in Drosophila is the result of a competition between forces attempting to activate transcription of the HeT-A elements, and forces attempting to silence their transcription, and that this competition is part of the telomere length control mechanism. Moreover, the authors speculate that the TAS region may be involved in this process because of the observed ability of the TAS region to block enhancer activity in an enhancer-blocking assay (Kurenova et al. 1998). The authors, however, did not propose a mechanism by which the TAS region may participate in this competition, or how it may block enhancer activity.
The results presented here suggest that the histone code may play a role in this competition, and that it is the close association of the TAS with HDAC1 and SU(VAR)3-9 which gives this region special properties and functions in this regard. The method by which the TAS region may participate in the regulation of HeT-A elements is discussed in the next chapter.
V REFERENCES


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Chapter Four

CONCLUDING CHAPTER

Composition of Telomeric Chromatin, and its Relationship to Telomere Position Effect and Telomere Length Maintenance in Drosophila: a general discussion with supplementary results.
I TPE as a telomere length-control mechanism in Drosophila.

IA What is the function of TPE in Drosophila, if any?

Our results suggest that the Drosophila telomere is a region of intense competition between chromatin modifications that confer transcriptional competence to chromatin, such as acetylation of H3K9, and chromatin modifications that inhibit transcription, such as deacetylation and methylation of H3K9. Interestingly, these findings are in agreement with a previously published model of TPE in Drosophila known as the ‘HeT-A Activation Model’ (GOLUBOVSKY et al. 2001; MASON et al. 2000; MASON et al. 2003a; MASON et al. 2003b; WALTER and BIESSMANN 2004). This model proposes that TPE and suppression of TPE are manifestations of a competition between factors attempting to activate HeT-A element transcription, and factors attempting to silence it. Thus, TPE is part of a telomere length control mechanism that ensures transcription of HeT-A elements is repressed until they are needed to lengthen receding telomeres. Genetic mutations that suppress TPE would result in increased levels of HeT-A transcription, while mutations that enhance TPE would result in decreased levels of HeT-A transcription. While the authors of this model did not propose a specific mechanism for this form of HeT-A element transcriptional repression, the results of this study suggest that histone H3K9 deacetylation by HDAC1 and histone methylation by SU(VAR)3-9 may play a role in this mechanism, and hence, in telomere length control.

IB What is the function of the TAS region of the telomere?

The authors of the HeT-A Activation Model have also suggested that the TAS region may be of particular importance to this telomere length control mechanism, due to the
observation that TAS repeats appear to have insulating properties, and are able to block enhancer activity in an enhancer-blocking assay (KURENOVA et al. 1998). Although the ability to block enhancers in cis is not necessarily analogous to TPE, many believe the two phenomena are related. The authors also suggest that the TAS region may function as a boundary element, and that its enhancer-blocking properties are intended to protect genes on the proximal side of the TAS from the powerful enhancers present in the HeT-A elements that reside distal to them (KURENOVA et al. 1998). A boundary element of this sort would be particularly important when dealing with HeT-A elements, since HeT-A elements are known to work in tandem, with one HeT-A element stimulating transcription of the HeT-A element centromere-proximal of it, such that longer arrays of elements lead to higher levels of transcription in the area (DANILEVSKAYA et al. 1997; GOLUBOVSKY et al. 2001).

Indeed, run-through transcription of reporter genes located centromere-proximal to the terminal HeT-A array has already been reported (FRYDRYCHOVA et al. 2007), suggesting that, if not for the presence of the TAS, the l(2)gl gene might suffer the same fate. This would be a particularly serious problem for the l(2)gl gene, since transcription from the l(2)gl promoter runs in the opposite direction from the HeT-A elements, and read-through transcription from HeT-A elements would lead to antisense l(2)gl transcripts being produced. However, the authors of the model were unable to suggest a specific mechanism by which the TAS is able to block enhancer activity, or regulate HeT-A transcription. The results of this study suggest that the TAS region may function as a ‘damper’ or enhancer block to run-through transcription as a result of its association with
the HDAC1 and SU(VAR)3-9 proteins, and the histone deacetylation and methylation activities associated with them. By converting nucleosomes associated with the TAS region from the transcription-competent acetylated form to the transcription-inhibiting methylated form, run-through transcription would be terminated once the TAS was reached.

IC What is the mechanism by which the TAS region is able to act as a boundary element, or a negative regulator of HeT-A transcription?

The results described in Chapter Three were consistent with the idea that the TAS region functions as a boundary element to protect TAS-proximal genes, such as the l(2)gl gene, from HeT-A run-through transcription. Analysis of the chromatin in the telomere region indicated that the HeT-A promoters were highly acetylated at histone H3K9, with little HDAC1 being present in this area. Both of these features are believed to be epigenetic marks of transcriptionally competent chromatin, and suggest that the HeT-A elements are transcriptionally active, or at least transcription-competent. The TAS region, on the other hand, was characterized by an abundance of HDAC1, SU(VAR)3-9 and methylated H3K9, suggesting that this area was hostile to transcription, and may prevent run-through transcription by acting as a ‘buffer zone’ between the HeT-A promoters and any genes downstream of them.

The observation that histone H3K9 acetylation levels dropped sharply on the centromere-proximal side of the TAS was consistent with the idea of the TAS functioning as a buffer zone or insulator. Furthermore, given that HDAC1, SU(VAR)3-9 and methylated histone
H3K9 were found to be integral components of the chromatin associated with the TAS, these results suggest that H3K9 methylation may be part of the mechanism responsible for any potential insulating activity of the TAS. Thus, chromatin is converted from a transcriptionally competent form to a less competent form at the TAS boundary, as a result of H3K9 deacetylation and subsequent methylation. The association of HDACs and HMTases with the TAS region would also explain the TPE observed in this area. Any genes ectopically relocated in or near the TAS region would become transcriptionally repressed due to histone deacetylation and methylation.

However, the hypothesis given above does not necessarily explain how the HDAC1 and SU(VAR)3-9 proteins associated with the TAS are able to negatively regulate transcription of HeT-A elements located upstream of the TAS region. While HDAC1 and SU(VAR)3-9 are able to deacetylate and methylate the nucleosomes directly associated with them, just how far their activity is able to extend on either side is not known. However, since the \textit{l(2)gl} gene promoter is located seven kilobases from the TAS region, is known to be active in developing embryos, and was associated with only minor amounts of H3K9me, one can assume that the ability of HDAC1 and SU(VAR)3-9 to modify nucleosomes is limited to a distance of less than seven kb. This is approximately the size of one HeT-A element. How then could the TAS region repress or regulate transcription from a tandem array consisting of several HeT-A elements, most of which are located many kilobases from the TAS? I propose a pairing-dependent model of how proteins associated with the TAS could down-regulate the transcription of nearby HeT-A elements.
A pairing-dependent model of how the TAS region is able to negatively regulate tandem arrays of HeT-A elements.

Recall that the TAS boundary was associated with SU(VAR)3-9 and H3K9me levels approximately 40 fold and 25 fold above background (as determined by the T7 antibody), respectively, for a SU(VAR)3-9 to H3K9me ratio of approximately 1.6. The TAS-embedded reporter gene, by contrast, had SU(VAR)3-9 and H3K9me levels of five and 10 fold above background, respectively, for a ratio of only about 0.5. Thus, the TAS-embedded reporter gene construct was associated with a smaller amount of SU(VAR)3-9 than one might predict, given the level of H3K9me observed in the area, and the ratio of SU(VAR)3-9 to H3K9me seen in some other areas. What then was the source of the additional H3K9 methylation? Assuming that the reporter gene had not been methylated by a different HMTase enzyme, I propose that it had been further methylated by SU(VAR)3-9 proteins associated with another part of the chromosome, which had been brought into close proximity to it through a pairing and/or looping mechanism that is also a function of the TAS region.

I propose that the telomeric reporter gene was methylated by SU(VAR)3-9 proteins associated with other regions of the telomere, and offer two different but related models of how this might occur. The first model proposes that the telomeric reporter gene was methylated in trans by SU(VAR)3-9 proteins associated with the uninterrupted TAS region of the paired telomere homolog. The second model proposes that the telomeric reporter gene was methylated in cis by SU(VAR)3-9 proteins associated with TAS
repeats on the same telomere, through a process in which the telomere loops back upon itself. Both models rely on the pairing of homologous regions of TAS as a mechanism for the observed histone methylation.

IIA A paired-sliding model of telomere length-control in Drosophila.

It has long been established that homologous regions of DNA are often found paired in interphase nuclei, at least in Drosophila. Furthermore, repetitive regions can pair not only with homologous regions on homologous chromosomes, but also with homologous regions on the same chromosome to form loops. Formation of such loops sometimes results in the creation of chromosomal deletions or duplications. Pairing of homologous telomeres has been observed in Drosophila (SIRIACO et al. 2002). I propose that the pairing of homologous telomeres is part of a telomere length control mechanism whereby the HDAC1 and SU(VAR)3-9 proteins associated with the TAS region of one telomere are able to deacetylate and methylate the HeT-A elements of the paired telomere, as illustrated in Figure 4.1.

Since both the HeT-A and TAS elements are repeated several times in tandem, the most stable pairing alignment between homologous telomeres would be to have each HeT-A element and each TAS repeat paired with the one on the opposite telomere, as shown in Figure 4.1A. When the paired telomeres are perfectly aligned (Figure 4.1A) the HDAC1 and SU(VAR)3-9 proteins are able to deacetylate and methylate the H3K9 residues on the opposite TAS region, as well as those on the same telomere. However, transient incorrect pairing of the TAS repeats would lead to deacetylation and methylation of the
HeT-A elements on the paired telomere by the HDAC and SU(VAR)3-9 proteins associated with the TAS region (Figure 4.1B), or to acetylation of the distal TAS boundary by HATs associated with the HeT-A elements of the paired telomere (Figure 4.1C), or both. Incorrect pairing could occur in either direction, leading to a sort of ‘sliding’ back and forth of the paired telomere homologs. Such sliding could explain the presence of both acetylated and methylated H3K9 at and near the distal TAS boundary, as well as at the HeT-A elements upstream of the TAS region.

This model predicts that the HeT-A elements (as well as the distal TAS boundary) would always be subjected to a certain degree of histone acetylation, as well as histone deacetylation and methylation. Whether this would lead to a complete shut down of HeT-A transcription or merely a reduction in transcription would depend on a number of factors, as discussed in the digital and analog models of transcription presented in Chapter Three. In either case, the only time when this inhibitory force would be removed would be during gametogenesis, when haploid cells are formed, and telomere pairing is impossible. This model would predict, therefore, that HeT-A elements should be most active in germline cells. Indeed, elevated levels of HeT-A transcription are observed in germline tissues (WALTER and BIESSMANN 2004). This model also predicts that mutations which disrupt pairing of homologous chromosomes should also suppress TPE, and increase transcription of HeT-A elements. This prediction is also testable, but has not yet been investigated.
When telomeres are closely paired and perfectly aligned, HATs associated with the HeT-A region of one telomere are able to acetylate the H3K9 residues in the HeT-A region of the paired homolog \textit{in trans} (A). Similarly, HDAC1 and SU(VAR)3-9 proteins associated with the TAS region are able to deacetylate and methylate H3K9 residues of the paired TAS region. However, transient misalignment of the telomeres can lead to both deacetylation and methylation of the HeT-A region (B), and acetylation of the TAS distal boundary (C). Whether this leads to repression of HeT-A transcription or not depends on a number of other variables, including whether methylation-mediated silencing is epistatic to acetylation-mediated activation or not. (Note that a typical Drosophila 2L telomere would have 28 or more TAS repeats, two or more HeT-A elements, and possibility TART elements as well (Walter \textit{et al.} 1995), but only three HeT-A elements and three TAS repeats are shown here, for simplicity.)
IIB  A looping model of telomere length-control in Drosophila.

As noted in the introductory chapter, t-loop formation appears to be a common feature of the telomeres of other organisms. While t-loop formation has never been directly observed in Drosophila, sequence analysis of repaired telomeres suggests that Drosophila telomeres may form loops under certain circumstances, such as the repair of truncated telomeres (Biessmann and Mason 2003; Biessmann et al. 1992). If Drosophila telomeres also form loops under normal circumstances, as the result of homologous pairing of TAS repeats, this could also form the basis of a telomere length control mechanism, as shown in Figure 4.2. Loop formation caused by perfect pairing and alignment of the TAS repeats with other TAS repeats on the same chromosome would cause deacetylation and methylation of the TAS region only (Figure 4.2A). On the other hand, misalignment of the loop in one direction would lead to deacetylation and methylation of the HeT-A elements, as well as acetylation of the TAS region (Figure 4.2B). Misalignment of the loop in the opposite direction (Figure 4.2C), would lead to deacetylation and methylation of the region between the proximal TAS boundary, and the \( l(2)gl \) gene.

The looping model predicts that the proximal TAS boundary should be acetylated more often than the distal TAS boundary. It would also predict that the area between the proximal TAS boundary and the \( l(2)gl \) gene should be highly methylated. Since the results of this study did not show either of these things, I tend to favor the sliding model.
In the looping model of telomere length-control, perfect pairing of TAS repeats leads to a bend or loop in the TAS region. As in the paired-sliding model, HDAC1 and SU(VAR)3-9 proteins are able to deacetylate and methylate not only the TAS repeats that they are associated with, but also those opposite (A). Sliding of the loop in one direction or the other leads to either the HeT-A region (B), or the region on the proximal side of the TAS region (C). The same would also be true for HATs associated with the HeT-A region.

Figure 4.2. A looping model of telomere length-control in Drosophila.

*Figure 4.2.* A looping model of telomere length-control in Drosophila.
IIC The sliding model of telomere length-maintenance is consistent with other characteristics of TPE in Drosophila.

One of the most interesting characteristics of TPE in Drosophila is that repression of the reporter gene construct is much stronger when only one of the telomeres carries the reporter gene, as opposed to both telomeres carrying a reporter gene. This property is unique to TPE, and is not seen when the reporter gene is located in other areas. When the reporter gene construct is located near its normal position in euchromatin, it is not subjected to position effects. Flies carrying two copies of the euchromatic reporter gene have approximately the same level of eye pigmentation as wild-type flies (Figure 4.3). When the euchromatic reporter gene is made hemizygous (one copy), the amount of eye pigmentation drops to approximately 87% of wild-type. Thus, only a minor drop in eye pigmentation observed seen when a euchromatic reporter gene construct is made hemizygous.

This is not the case when a telomeric reporter gene is made homozygous. When both telomeres carry the reporter gene construct, eye pigmentation levels are only about 39% of that seen in wild-type flies, presumably due to TPE-related gene repression. However, when the telomeric reporter gene is made hemizygous, and only one of the telomeres carries a reporter gene, eye pigmentation levels drop to less than 3% of normal, an approximate ten fold drop in eye pigmentation compared to that seen with two reporter genes are present (Figure 4.3).
Figure 4.3. The telomeric reporter gene is more strongly repressed when it is paired with an intact TAS region.

Eye pigmentation assays were carried out on female flies carrying both homozygous and hemizygous reporter genes located in euchromatin, as well as homozygous and hemizygous reporter genes located in the TAS region of the 2L telomere. Bars represent eye pigmentation levels as a percentage of wild-type flies, carrying the native \( w^+ \) gene. Error bars represent standard deviations (n=4). Flies carrying two reporter genes located in euchromatin had eye pigmentation levels approximately equal to that of wild-type flies. When only one euchromatic reporter gene is present, there is a slight but significant drop in eye pigmentation levels ( \( p < 0.05 \) ). Homozygous reporter genes located in the TAS region of the 2L telomere are moderately repressed, with eye pigmentation levels being approximately 39% of that seen in wild-type flies. A ten fold drop in eye pigmentation levels is seen when the telomeric reporter gene is made hemizygous, with eye pigmentation levels being only 3% of that seen in wild-type flies. (Eye pigmentation assays were carried out as described in Chapter Two.)
The dramatic increase in the strength of TPE-associated repression seen when only one of the telomeres carries a reporter gene is easily explained by the paired-sliding model (Figure 4.4). When only one of the telomeres carries a reporter gene, the reporter gene is paired directly with the uninterrupted TAS region of the other telomere, and is deacetylated and methylated by the HDAC1 and SU(VAR)3-9 proteins associated with the paired homolog (Figure 4.4A). By contrast, when both telomeres carry a reporter gene, the reporter genes are usually paired with one another, and do not find themselves opposite TAS-associated HDAC1 and SU(VAR)3-9 proteins as often (Figure 4.4B). While the sliding mechanism may still result in sporadic deacetylation and methylation of the reporter genes, repression would not be as strong as if the reporter gene were permanently paired with a TAS region. This looping model does not offer an explanation of this phenomenon.

Another interesting feature of TPE in Drosophila is that TPE is suppressed when a TAS-embedded reporter gene is paired with either an abnormally long, or an abnormally short telomere. Pairing of a TAS-embedded reporter gene with a truncated 2L telomere, which does not carry a TAS region, results in suppression of TPE (Figure 4.5). Again, this observation is easily explained by the paired-sliding model of TPE, since the reporter gene is not paired with an adjacent TAS region (Figure 4.6A). Likewise, pairing of a TAS-embedded reporter gene with an abnormally long telomere, featuring an unusually long array of HeT-A and TART elements (Siriaco et al. 2002) results in suppression of TPE (Figure 4.5). This is also explained by the paired-sliding model. While correct alignment of the two telomeres is favored (Figure 4.6B), misalignment can still occur. In
Figure 4.4. TAS-mediated repression of a reporter gene by an intact versus interrupted TAS region.

Repression of the telomeric reporter gene is stronger when it is paired with an intact TAS region than it is when it is paired with another TAS-embedded reporter gene. The drop in eye pigmentation levels associated with the hemizygous reporter gene is stronger than what one would predict based on gene dosage alone (Figure 4-3). However, this phenomenon can be easily explained by the paired-sliding model, where a hemizygous telomeric reporter gene would be subjected to a greater degree of histone deacetylation and methylation as a result of being paired with an intact TAS region (A), than homozygous telomeric reporter genes, that are paired with one another (B).
Suppression of TPE was measured, as described in Chapter Two, using a hemizygous telomeric reporter gene. The $Su(z)2^5$ mutation was used as a positive control for suppression of TPE, and the $Su(var)2-5^4$ ($HP1a$) mutation were used as a negative control for suppression of TPE. Suppression of TPE was also observed when the reporter gene was paired with either an abnormally long telomere (Gaiano strain), or an abnormally short, truncated telomere, which lacked a TAS region. Eye pigmentation levels for long and short telomeres were significantly higher than the negative control and wild-type flies ($p < 0.05$). (Long and short telomeres were generous gifts from J. Mason.)

Figure 4.5. Suppression of TPE by abnormally short and abnormally long telomeres.
Figure 4.6. Suppression of TPE by long and short telomeres.

According to the paired-sliding model of TPE, suppression of TPE by a truncated telomere is explained by the fact that the telomeric reporter gene has no TAS to pair with, and is less likely to be deacetylated and methylated in trans (A), although some deacetylation and methylation may still occur as a result of the HDAC1 and SU(VAR)3-9 proteins located on either side of the reporter gene. Suppression of TPE by an abnormally long telomere, carrying a longer array of HeT-A elements (B) is explained by the frequent mispairing of HeT-A elements, causing intermittent pairing of the reporter gene with the HeT-A region, and HATs associated with it (C).
this case, however, since there are many more HeT-A elements present on the long telomere, there are many more chances for the HeT-A elements on the reporter gene-carrying telomere to pair with the wrong HeT-A element on the telomere opposite (Figure 4.6C). This would cause the TAS-embedded reporter gene to be found opposite HeT-A elements more often than TAS repeats, leading to a net increase in acetylation, and a net decrease in deacetylation and methylation of the reporter gene. Again, the looping model offers no explanation for this observation, suggesting that the sliding model is probably the better of the two.

III Suggestions for future research.

Many aspects of the models of TPE and telomere length-maintenance described here are testable, and represent interesting avenues for future research. Furthermore, many of the results presented in chapters two and three point to obvious areas of future research which could either confirm or negate some of the hypothetical explanations given for these results in the discussion sections of these chapters. The purpose of this section is to list some of the more obvious avenues of future research, and also to summarize some of the preliminary work that I have already done in these areas, which either gave negative results or were inconclusive. Thus, future research strategies can be targeted and honed with these preliminary results in mind.

Firstly, the concept that the TAS region has insulator properties, and acts as a boundary element is an interesting one. The results presented here indicate that the TAS region is closely associated with the HDAC1 and SU(VAR)3-9 proteins, and I have proposed that
the histone deacetylation and methylation functions associated with these proteins may play a role in any potential insulating ability of the TAS. To this end, it would be interesting to see if the TAS region retains its enhancer-blocking abilities in *Su(var)3-9* and *Hdac1* mutant backgrounds. Moreover, it would be interesting to see if the histone H3K9 modifications associated with the TAS region are lost or changed in *Su(var)3-9* or *Hdac1* mutant backgrounds. I have conducted preliminary ChIP experiments with *Su(var)3-9*<sup>O6A</sup> mutant flies, and found that overall levels of H3K9ac increase in a *Su(var)3-9*<sup>O6A</sup> mutant background, while H3K9 methylation is greatly reduced. (Preliminary results are presented in Appendix One). The latter result is particularly interesting, since it again suggests that SU(VAR)3-9, rather than some other HMTase enzyme, is responsible for the H3K9me seen at the TAS.

Secondly, if the HDAC1 and SU(VAR)3-9 proteins associated with the TAS are indeed necessary for negative regulation of the HeT-A elements that maintain telomere length in Drosophila, mutations in *Hdac1* and *Su(var)3-9* should result in an increase in HeT-A transcription. Although I have not measured HeT-A transcription directly, I have conducted preliminary experiments to determine the number of HeT-A elements and HeT-A element transcripts present in various *Hdac1* and *Su(var)3-9* mutant strains (Appendix Two). A dot-blot Southern analysis indicated that *Su(var)3-9*<sup>130</sup>, *Su(var)3-9*<sup>309</sup>, and *Su(var)3-9*<sup>l</sup> mutant fly strains carry significantly more HeT-A elements (Figure 4.7). These strains also contained significantly more HeT-A transcripts, but when the ratio of transcripts to templates was calculated, it became apparent that the ratio of transcripts to templates was no different than in wild-type Drosophila (Figure 4.8).
Figure 4.7. HeT-A elements present in the genomes of various mutant fly strains, compared to wild-type Drosophila.

A dot-blot Southern analysis of $Su(var)3-9$ and $Hdac1$ mutant flies indicated that $Su(var)3-9$ mutant genomes contained more HeT-A elements than did wild-type flies ($p < 0.05$). Flies with long telomeres (Gaiano strain) also had greater numbers of HeT-A elements in their genomes. Bars represent the number of HeT-A elements, expressed as a percentage of wild-type, with error bars representing standard deviations (n=4).
Figure 4.8. Ratio of HeT-A transcripts to HeT-A templates, relative to wild-type Drosophila.

Although *Su(var)3-9* mutant flies had greater numbers of HeT-A elements in their genomes, the ratio of HeT-A transcripts to HeT-A templates was not found to be significantly different from that of wild-type flies.
Although HeT-A transcription rates were not measured directly, these results imply that HeT-A transcription rates do not increase in \textit{Hdac1} and \textit{Su(var)3-9} mutant strains.

Interestingly, however, a similar analysis of TART elements in mutant fly strains indicated that \textit{Hdac1} and \textit{Su(var)3-9} mutants carry significantly fewer TART elements than wild-type Drosophila (Figure 4.9). Nevertheless, despite the lower number of TART elements, a greater number of TART transcripts were seen in these mutants, such that the ratio of TART transcripts to TART templates was significantly higher in \textit{Hdac1} and \textit{Su(var)3-9} mutant Drosophila than in wild-type Drosophila (Figure 4.10). This raised the possibility that HDAC1 and SU(VAR)3-9 may be involved in regulation of TART transcription, rather than HeT-A transcription.

Recall that TART elements are also believed to be involved in the process of telomere length maintenance in Drosophila, and that the POL reverse transcriptase protein produced by TART is thought to be necessary for transposition of the related but non-autonomous HeT-A elements that do not encode a POL protein (PARDUE and DEBARYSHE 2000; PARDUE and DEBARYSHE 2003; PARDUE \textit{et al.} 2005; RASHKOVA \textit{et al.} 2003; RASHKOVA \textit{et al.} 2002). Thus, regulation of TART elements may be at least as important to telomere length maintenance in Drosophila as regulation of HeT-A elements, and may in fact be a rate-limiting step in HeT-A transposition. If this were the case, increased transcription of TART elements could easily result in increased transposition of the HeT-A elements, without a corresponding increase in HeT-A transcription.
A dot-blot Southern analysis indicated that *Hdac1* and *Su(var)3-9* mutant genomes contained significantly fewer TART elements than wild-type flies ($p < 0.05$). Bars represent numbers of TART elements, expressed as a percentage of that seen in wild-type flies, with error bars representing standard deviations ($n=4$).
Figure 4.10. Ratio of TART (POL) transcripts to TART elements, relative to wild-type Drosophila.

With the exception of $Su(var)3-9^{O6A}$, the ratio of TART transcripts to TART templates was significantly higher in the genomes of $Su(var)3-9$ and $Hdac1$ mutant flies than in wild-type flies ($p < 0.05$)
Although I did not measure telomere length directly, most HeT-A elements are believed to reside at telomere ends (MASON et al. 2000; WALTER et al. 1995). The finding that Su(var)3-9 mutant strains have greater numbers of HeT-A elements in their genomes suggests that these mutants may also have longer telomeres. I have observed, for example, that the Gaiano strain which is known to have abnormally long telomeres (Siriaco et al. 2002), also carries greater numbers of HeT-A elements (Figure 4.7). A direct measurement of telomere length in Su(var)3-9 mutants would be helpful, and I have constructed a special reporter strain for the purpose of measuring both TPE and telomere length in a Su(var)3-9 mutant background (described in Appendix Three).

It should be remembered, however, that strains that have sustained mutations causing loss of telomere length control probably also accumulate second site mutations that counter this effect, lest telomeres lengthen indefinitely. Interestingly, I have observed that the Gaiano strain carries greater numbers of HeT-A elements in its genome, but also appears to have the lowest HeT-A transcript to template ratio of any of the mutant strains examined (Figure 4.8). I would suggest, therefore, that the Gaiano strain has accumulated second site mutations that cause down-regulation of transcription from the increased number of HeT-A elements to avoid runaway telomere elongation. Other telomere length-control mutations may have the same effect, and outcrossing should be included in any experimental strategy designed to measure telomere elongation in mutant flies. Outcrossing was used in Chapter Two, in part to minimize the effects of second site mutations on TPE. A similar regime should be used when analyzing telomere length-control mutations.
Finally, since the issue of telomere length control has often been linked to issues of longevity, genome stability, apoptosis, and cell senescence (Bischoff et al. 2005; Bischoff et al. 2006; Joeng et al. 2004; Raices et al. 2005; Stewart and Weinberg 2006), Hdac1 and Su(var)3-9 mutants should also be analyzed for effects on viability and longevity. I have conducted preliminary experiments in this regard, and found that these mutations do not result in an increase in either longevity or viability. Quite the opposite in fact, and both Hdac1 and Su(var)3-9 mutations were seen to cause reductions in viability, as measured by fecundity in females, and longevity. Hdac1 and Su(var)3-9 mutations were associated with significant decreases in fecundity in females (Figure 4.11; Appendix Four), and in the case of Hdac1 a lowered ratio of male to female offspring was also observed. Also, Hdac1 and Su(var)3-9 mutant flies do not live as long as their wild-type counterparts (Figure 4.12; Appendix Five), and significant reductions in the median lifespans of Hdac1 and Su(var)3-9 mutant flies were observed.

The reduction in lifespan associated with the Hdac1 mutation was particularly interesting given that other investigators have reported an increase in longevity associated with Hdac1 mutations (Chang and Min 2002; Rogina et al. 2002). The most likely reason for the discrepancy in results is that my experiments were carried out with mis-sense mutations, while the previous studies used null mutations and deficiencies. Our lab has already demonstrated that mis-sense mutations of HDAC1 have different effects on target genes than null mutations, and have suggested that mis-sense mutations tend to be antimorphic to the complexes they participate in, while null mutations are not
Figure 4.11. Viability of various mutant Drosophila, as measured by fecundity in females.

Bars represent the mean number of male and female offspring produced by females of the indicated genotype, expressed as a percentage of wild-type. Error bars represent standard deviations (n=50). Results indicated that, with the exception of the Su(var)3-9$^i$ allele, Su(var)3-9 and Hdac1 mutant flies produced significantly fewer offspring than did wild-type flies ( p < 0.05).
Figure 4.12. Longevity of various mutant Drosophila, relative to wild-type.

Longevity was measured in various mutant flies, and compared to the lifespan of wild-type flies. Bars represent median lifespan, with error bars representing 95% confidence intervals (n=300). Results indicated that *Hdac1* and *Su(var)3-9* mutant flies had significantly shorter lifespans than did wild-type flies.
(MOTTUS et al. 2000). Thus, the involvement of HDAC1 in telomere length maintenance, and/or longevity is interesting, and warrants further investigation, together with the other issues mentioned here.

While many of the experiments listed above did not give the predicted results, indicating that a re-evaluation of the models for telomere biology currently in use may be necessary, at least three observations about the nature of the Drosophila telomere can be made. First, the HDAC1, SU(VAR)3-9, and HP1c proteins are constituents of telomeric chromatin, at least at the 2L Drosophila telomere. Second, these proteins are associated primarily with the TAS region of the telomere, and not with the areas that surround it, and finally, normally active, euchromatic reporter genes take on a chromatin profile that is similar to the TAS region when inserted into the TAS region.

A fourth, and possibly more interesting observation was also made during the course of this work. Despite the presence of increased levels of HDAC1 in the TAS region, the histones associated with this area were highly acetylated. This observation was inconsistent with the idea of HDAC1 being a histone deacetylase, and raises the possibility that the function of this protein may be different in different areas of the nucleus, or that the other proteins in the area may counterbalance the histone deacetylating activity of HDAC1. All of these possibilities should be taken into consideration when future studies of telomere biology are planned.
IV REFERENCES


Appendix One: ChIP analysis of the unadulterated 2L telomere in a $Su(var)3-9^{O64}$ mutant background.

Overview:
Using ChIP DNA provided by P. Kalas, a ChIP analysis was carried out for the unadulterated 2L telomere in $Su(var)3-9^{O64}$ mutant flies.

Results:
Results indicated that, in the absence of the SU(VAR)3-9 protein, acetylation of H3K9 increases across the entire 2L telomere, including the TAS region, as illustrated in Figure 1.
A ChIP analysis, similar to that described in Chapter Three, was carried out on the unadulterated 2L telomere, using ChIP extracts from Su(var)3-9O6A mutant embryos (supplied by P.Kalas). In the complete absence of the SU(VAR)3-9 protein, acetylation of the entire 2L telomere increases relative to that seen in wild type flies (Chapter Three).
Appendix Two: Dot-blot Southern and northern analysis of HeT-A and TART elements in wild-type and mutant flies.

Overview:
The wild-type Drosophila genome is believed to contain 30 to 50 HeT-A elements, and five to 10 TART elements (MASON et al. 2000), which are located primarily at telomere ends, and are believed to be involved in telomere length maintenance. In Chapter Four of this thesis I suggest that the SU(VAR)3-9 and HDAC1 proteins may be involved in the negative regulation of HeT-A and TART transcription, and propose that mutations in the Su(var)3-9 and Hdac1 loci should cause deregulation of HeT-A or TART transcription, leading to either longer telomeres, and/or more HeT-A or TART elements being present in the genome. As a partial test of this hypothesis I estimated the number of HeT-A and TART elements, as well as the number of HeT-A and TART element transcripts present in various mutant fly genomes, using a dot-blot Southern and a dot-blot northern analysis.

The number of HeT-A and TART elements, as well as the number of HeT-A and TART element transcripts was determined for various mutant strains, and compared to wild-type flies. A ratio of HeT-A elements to HeT-A transcripts, and TART elements to TART transcripts was then determined as an indirect measurement of HeT-A and TART transcriptional regulation, with the assumption that loss of negative regulation should lead to a higher transcript to template ratio. The HeT-A element produces only one transcript (GAG), while the TART element produces two transcripts (GAG and POL). Both GAG and POL transcripts were evaluated for the TART elements. The Actin 42A gene is present in only one copy per genome, and was used as an internal control for normalization of hybridization signals.

Four Su(var)3-9 mutations were analyzed, including Su(var)3-9^{O6A}, Su(var)3-9^{330}, Su(var)3-9^{360}, and Su(var)3-9^{I}, all of which suppressed TPE (Chapter Two). The Hdac1 mutant Hdac1^{326}, which strongly suppressed TPE, was also analyzed. The Su(z)25 mutation, and the Su(var)2-55 (HP1a) and Su(var)2-1 mutations were also analyzed as positive and negative controls for suppression of TPE, respectively. The Gaiano strain, which is known to have abnormally long telomeres, composed primarily of HeT-A elements (SIRIACO et al. 2002), was included as a positive control for both longer telomeres and increased numbers of HeT-A elements. With the exception of Su(var)3-9^{O6A}, the Su(var)3-9 and Hdac1 mutants are homozygous non-viable, and are kept in a w⁻; TM3 genetic background. Thus, w⁻ and w⁻; TM3 strains were also tested to control for the genetic background in which the Su(var)3-9 and Hdac1 mutations are maintained.

Materials and Methods:
Slot-blot analysis of HeT-A and TART elements.
Both DNA and RNA were extracted from the same flies, using the TRIzol™ Reagent (Invitrogen™ 15596-026), according to the manufacturer’s instructions, to ensure a more accurate comparison of DNA templates to transcripts. RNA and DNA samples were
extracted simultaneously from 50 male flies, aged three weeks, in replicates of four. RNA and DNA samples were then transferred to HybondTM-N+ membranes using a Mini-PROTEAN™ II dot-blot manifold apparatus (Bio-Rad™ 170-4017), using standard transfer protocols (AUSUBEL 1999a). Southern (AUSUBEL 1999b) and Northern (AUSUBEL 1999a) hybridization protocols were then carried out according to standard protocols. Hybridization signals were quantified using a Molecular Probes™ fluorescent screen, and the Storm™ Imager and ImageQuant™ software. In all cases, membranes were probed, stripped and re-probed in the following order: TART (GAG), TART (POL), HeT-A, and Actin 42A.

For generation of Southern and Northern probes, HeT-A and TART element DNA sequences were kindly provided by M. L. Pardue (DANILEVSKAYA et al. 1999; PARDUE et al. 1996). PCR was used to amplify the HeT-A and TART element open reading frames, and single stranded antisense probes were generated using PCR. HeT-A and TART probes were confirmed to hybridize to a 6 kb band, in the case of the HeT-A probe, and a series bands approximately 9 kb in length, in the case of the TART probes, as described by (DANILEVSKAYA et al. 1999).

The primers used to generate the Actin 42A fragments and probes were the same as those listed in Chapter Three. Primers used to generate the HeT-A and TART PCR fragments and probes were as follows:

HeT-A element: for both Northern and Southern blots, the 3’UTR primers listed in Chapter Three were used, as the entire HeT-A element is known to be transcribed (DANILEVSKAYA et al. 1999).

TART (GAG):
TOrf15: 5’-CTC  TTT  TCC  GCC  AAA  ACA  CAC  ATT
TOrf13: 5’-ATC  CTT  CGA  TAC  CCG  TTTC  CTT  TC

TART (POL):
TOrf25: 5’-CTG  GAA  ACC  AAA  AGA  GGC  CCC  AAA  TGG
TOrf23: 5’-CCT  CTA  GTC  TCA  GCA  GGG  TGG  GGA  GTT

Results:
Dot-blot Southern analysis (Figure 1) indicated that the Su(var)3-9 and Hdac1 mutants carried greater numbers of HeT-A elements in their genomes than did wild-type flies (Figure 2). This was also seen in the Gaiano strain, which is known to have longer telomeres, comprised by longer arrays of HeT-A elements. This result suggested that certain Su(var)3-9 and Hdac1 mutants may have longer telomeres than normal, although telomere length was not measured directly. (A special fly strain was constructed to facilitate direct measurement of telomere length in Su(var)3-9O6A flies, and is described in Appendix Eight.)
In contrast to the increased number of HeT-A elements seen in Su(var)3-9 and Hdac1 mutant flies, the number of TART elements was significantly reduced in all but the w; TM3, Su(var)2-1, and HP1a mutant strains (Figure 3). Dot-blot northern analysis (Figure 4) indicated increased numbers of HeT-A transcripts in Su(var)3-9330, Su(var)3-9309, and Su(var)3-9I mutant strains (Figure 5). Increased numbers of TART (GAG) transcripts in Su(var)3-9330, Su(var)3-9309, and Su(var)3-9I mutant strains (Figure 6), and increased numbers of TART (POL) transcripts in Su(var)3-9330, Su(var)3-9309, Su(var)3-9I, and Hdac1326 mutant strains (Figure 7) were also seen.

Although increases in the number of HeT-A transcripts were seen in three of the four Su(var)3-9 mutants, when the number of HeT-A transcripts was divided by the number of HeT-A templates (Figure 8) a ratio of approximately one or less was seen in all cases. Although transcription rates were not measured directly, this result suggested that HeT-A transcription rates were not elevated in Su(var)3-9 and Hdac1 mutant strains. Interestingly, the lowest HeT-A transcript to template ratio was seen in the Gaiano strain, suggesting that flies with longer telomeric arrays of HeT-A elements may down-regulate HeT-A transcription as a method of preventing runaway telomere elongation.

In contrast to the transcript to template ratios obtained for HeT-A elements, transcript to template ratios for TART elements were significantly higher in mutant fly strains. This was true of both the TART GAG transcript and the TART POL transcript. Su(var)3-9330, Su(var)3-9309, and Su(var)3-9I mutant flies all showed increased transcript to template ratios for the TART GAG transcript (Figure 9). These same mutants, as well as the Hdac1326 mutation, also showed an increased transcript to template ratio for the TART POL transcript (Figure 10).

These findings suggest that the SU(VAR)3-9 and HDAC1 proteins may be involved in the negative regulation of TART, rather than HeT-A elements. However, since the POL protein supplied by the TART element is believed to be essential for HeT-A element transposition, these results are still consistent with the hypothesis that the SU(VAR)3-9 and HDAC1 proteins may be involved in the negative regulation of telomere length maintenance in Drosophila.
Figure 1. Dot-blot Southern analysis of HeT-A and TART elements in mutant and wild-type flies.

DNA was extracted from 50 male flies in quadruplicate, and transferred to a Hybond\textsuperscript{TM}-N+ membrane. Radiolabelled probes specific for the TART and HeT-A elements, as well as the Actin 42A gene were then hybridized to the membrane according to standard protocols. The Actin 42A gene is present in only one copy per genome, and was used as an internal control for normalization. HeT-A and TART element signals were then normalized to the Actin 42A signal, and compared to wild-type.
Dot-blot Southern analysis was used to determine the number of HeT-A elements present in various mutant fly strains. Bars represent the number of HeT-A elements present in various mutant fly strains expressed as a percentage of wild-type. Error bars represent standard deviations (n=4). *Su(var)3-9* and *Hdac1* mutants carried significantly more HeT-A elements than wild-type (*p < 0.05*), as did the Gaiano strain, which has abnormally long telomeres.

**Figure 2. Number of HeT-A elements present in various mutant strains, relative to wild-type flies.**
Figure 3. Number of TART elements present in various mutant fly strains, relative to wild-type flies.

Dot-blot Southern analysis was used to quantify the number of TART elements present in various mutant fly strains. Bars represent the number of TART elements present in various mutant fly strains, expressed as a percentage of wild-type. Error bars represent standard deviations (n=4). With the exception of the \textit{w}; \textit{TM3} and \textit{HP1a} mutant strains, all mutant strains carried significantly fewer TART elements than wild-type ( \( p < 0.05 \)).
Figure 4. Dot-blot northern analysis of HeT-A and TART transcripts from mutant and wild-type flies.

RNA was extracted from 50 male flies in triplicate, and transferred to a Hybond™-N+ membrane. The membrane was then hybridized to radiolabelled probes specific for the HeT-A GAG transcript, the TART GAG and POL transcripts, and the Actin 42A transcript.
Figure 5. Number of HeT-A transcripts present in various mutant strains, relative to wild-type.

Dot-blot northern analysis was used to determine the number of HeT-A transcripts present in various mutant fly strains. Bars represent the number of HeT-A transcripts, expressed as a percentage of wild-type, with error bars representing the standard deviation (n=3). The Su(var)3-9<sup>326</sup>, Su(var)3-9<sup>309</sup>, and Su(var)3-9<sup>1</sup> mutant strains showed a significant increase (p < 0.05) in the number of HeT-A transcripts present, relative to wild-type.
Figure 6. Number of TART (GAG) transcripts present in various mutant strains, relative to wild-type.

Dot-blot northern analysis was used to determine the number of TART (GAG) transcripts present in various mutant fly strains. Bars represent the number of TART GAG transcripts as a percentage of wild-type, with error bars representing the standard deviation (n=3). Significantly greater numbers of TART GAG transcripts were found in the $Su(var)3-9^{O6A}$, $Su(var)3-9^{330}$, and $Su(var)3-9^{309}$ mutant strains ($p < 0.05$).
Figure 7. Number of TART (POL) transcripts present in various mutant strains, relative to wild-type.

Dot-blot northern analysis was used to determine the relative number of TART (POL) transcripts present in various mutant fly strains. Bars represent the number of TART (POL) transcripts as a percentage of wild-type, with error bars representing standard deviations (n=3). Significantly larger numbers of TART (POL) transcripts were seen in Su(var)3-9^{306}, Su(var)3-9^{330}, Su(var)3-9^{309}, Su(var)3-9^{1}, Su(var)2-1^{326}, HP1a, Su(z)^{25}, and Long telomere mutant flies (p < 0.05).
Figure 8. Ratio of HeT-A transcripts to HeT-A elements in various mutant fly strains, relative to wild-type.

The ratio of HeT-A transcripts to HeT-A elements was determined for wild-type flies by dividing the HeT-A northern signal by the HeT-A Southern signal. This ratio was then defined as a ratio of one, and the ratios obtained from mutant fly strains were then compared to the wild-type ratio. None of the mutant strains were found to have HeT-A transcript to HeT-A template ratios that deviated significantly from wild-type.
The ratio of TART (GAG) transcripts to TART elements was determined for wild-type flies by dividing the TART (GAG) northern signal by the TART element Southern signal. This ratio was then defined as a ratio of one, and the ratios of the various mutant strains were then compared to it. The $\text{Su(var)3-9}^{330}$, $\text{Su(var)3-9}^{309}$, and $\text{Su(var)3-9}^{I}$ mutant strains showed transcript to template ratios for the GAG open reading frame that were significantly greater than wild-type ($p < 0.05$).
Figure 10. Ratio of TART (POL) transcripts to TART elements in various mutant strains, relative to wild-type.

The ratio of TART (POL) transcripts to TART elements was determined for wild-type flies by dividing the TART (POL) northern signal by the TART element Southern signal. This ratio was then defined as a ratio of one, and the ratios of the various mutant strains were then compared to it. The $\text{Su(var)3-9}^{06A}$, $\text{Su(var)3-9}^{330}$, $\text{Su(var)3-9}^{309}$, $\text{Su(var)3-9}^{1}$, $\text{Hdac1}^{326}$, $\text{Su(var)2-1}$, $\text{HP1a}$, $\text{Su(z)}^{2}$ and Long telomere mutant strains showed transcript to template ratios for the POL open reading frame that were significantly greater than wild-type ($p < 0.05$).
Appendix Three: Construction and proposed use of fly stocks homozygous for the reporter gene and the Su(var)3-9^{O64} mutation in a w' genetic background, for direct measurement of telomere length.

Overview:
A special stock of flies (designated 1A1) was constructed which carries two copies of the telomeric reporter gene, one in each of the 2L telomeres (refer to Chapters Two and Three), and is also homozygous for the Su(var)3-9^{O64} and w' mutant alleles. The w' mutation is a null deletion, such that, in the 1A1 stock, the only resident copy of the w gene is the TPE reporter gene. This stock was created for use with three types of experiments: 1) to facilitate easy measurement of telomere length in both wild-type and Su(var)3-9^{O64} mutant flies. 2) To facilitate a ChiP analysis of the reporter gene in a Su(var)3-9^{O64} mutant background, and 3) to facilitate easy measurement of mini-white transcription levels from the reporter gene in both a wild type and a Su(var)3-9^{O64} mutant background. The stock was constructed as illustrated in Figure 1.

1) Measurement of telomeres.
The reporter gene (WALLRATH and ELGIN 1995) is inserted into the 2L TAS region. The reporter gene contains a unique Srf1 (‘eight cutter’) restriction site, which cuts the reporter gene into two, roughly equal parts. Thus, if genomic DNA is extracted from flies, cut with Srf1, and separated with pulse field gel electrophoresis (designed to separate large fragments of DNA), the resulting fragments can be subjected to Southern blotting, and probed with the reporter gene sequence. The reporter gene sequence will hybridize to the telomere end (distal side of the Srf1 cut), which will be of a variable length, depending on the length of the telomere, as well as to the remainder of the chromosome (proximal side of the Srf1 cut) will be of a constant size, and serve as an internal control. Thus, the relative length of the telomere can be measured by measuring the distance between these two bands.

This procedure can be carried out for flies carrying zero, one, or two Su(var)3-9^{O64} mutant alleles, and either one or two copies of the reporter gene, using the following crossing schemes:

**Two reporter genes:**
w'; reporter/reporter; +/- (+ use stock directly)
w'; reporter/reporter; 3-9/3-9 (use stock directly)

w'; reporter/reporter; 3-9/3-9 X w'; reporter/reporter; +/- = w'; reporter/reporter; 3-9/+ 

**One reporter gene:**
w'; +/-; +/- X w'; reporter/reporter; +/- = w'; reporter/++; +/-
w'; reporter/reporter; 3-9/3-9 X w'; +/-; +/- = w'; reporter/++; 3-9/+ 
w'; reporter/reporter; 3-9/3-9 X w'; +/-; w'; +/-; 3-9/3-9 = w'; reporter/++; 3-9/3-9
2) **ChIP analysis of the reporter gene in a** $Su(var)3-9^{O64}$ **mutant background.**
The ChIP analysis, described in Chapter Three, can be repeated for the reporter gene in
the presence of either zero, one, or two mutant alleles, using the same crossing scheme
outlined above.

3) **Transcriptional analysis of the reporter gene in** $Su(var)3-9^{O64}$ **mutant backgrounds.**
Southern and Northern blot analysis can be carried out, as described in Chapter Four,
using the $w$ gene as a probe, using the crossing schemes outlined above.

4) **Eye pigment analysis of single, or double reporter genes in mutant backgrounds.**
Eye pigment assays can be carried out, as described in Chapter Two, using either one or
two reporter genes, in various mutant backgrounds, including both homozygous or
heterozygous $Su(var)3-9^{O64}$ mutations, as well as $Su(var)3-9^{O64}/Hdac1$ and other trans-
heterozygotes, as shown in Figures 2 through 4.
Figure 1. Construction of stocks homozygous for both the telomeric reporter gene, and the Su(var)3-9O6A mutation.

1) Males carrying double balancer chromosomes, marked with the CyO and Tft phenotypes for the second chromosome, and the TM3 and Gl phenotypes for the third chromosome, were crossed with females homozygous for the reporter gene, and males with pale yellow eyes, and tufted stubble bristles were selected in the offspring. 2) Males homozygous for the Su(var)3-9O6A mutation were crossed to females carrying the balancer chromosomes, and curly winged glued eyed females were selected. 3) The two selected phenotypes were crossed, and curly winged, stubble bristled progeny were selected and intercrossed in step 4. Progeny with orange eyes, and lacking curly wings and stubble bristles were selected from the progeny of this cross, and used to make the stock. Males of this stock were then crossed to the wmd4 stock, and the appearance of male offspring with red (suppressed) eyes confirmed the presence of Su(var)3-9O6A. A similar technique was used to construct a stocky carrying the dmHDAC1326 mutation, except that the final stock carried Hdac1326 balanced over TM3, since Hdac1326 is not homozygous viable.
Figure 2. Mutation of $Su(var)3-9$ suppresses TPE in flies homozygous for the reporter gene, but not in an additive or synergistic manner.

Flies carrying the eye pigment reporter gene in both 2L telomeres have eye pigment levels approximately 40% of wild-type due to TPE. Mutation of one copy of the $Su(var)3-9$ gene suppresses TPE, with heterozygous $Su(var)3-9^{O6A}$ flies having significantly more eye pigment than wild-type flies carrying TAS-embedded reporter genes (p < 0.05). Mutation of both copies, however, does not increase suppression of TPE, and flies homozygous for the $Su(var)3-9^{O6A}$ mutation do not have eye pigment levels that are significantly different from flies heterozygous for the mutation.
Figure 3. Mutation of *Su(var)*3-9 suppresses TPE in flies hemizygous for the reporter gene in an additive manner.

Repression of a hemizygous TAS-embedded reporter gene is much stronger than repression of homozygous TAS-embedded reporter genes on a pro-rated basis. Two TAS-embedded reporter genes have eye pigment levels approximately 40% of wild-type (Figure 2), while a single TAS-embedded reporter gene gives eye pigment levels close to the background level for the eye pigment assay (bar 2). Repression of the hemizygous reporter gene can be partially alleviated by introducing a *Su(var)*3-9 mutation into the genome, and eye pigment levels increase (bar 3). Mutation of both copies of the *Su(var)*3-9 gene increase eye pigment levels to about twice that of a single mutation (bar 4; *p* < 0.001). Note, *Su(z)*2*5* (bar 1) and *Su(var)*2-1 (bar 5) are included as positive and negative controls for suppression of TPE, respectively.
Figure 4. *Su(var)3-9* and *Hdac1* trans-heterozygotes show additive suppression of TPE, but not synergistic suppression of TPE.

Flies carrying two copies of the *Su(var)3-9*O6A mutation have eye pigment levels higher than those carrying a single *Su(var)3-9*O6A mutation (bars 2A and 2B). Trans-heterozygotes carrying both the *Su(var)3-9*O6A and *Su(var)3-9*O6A alleles have eye pigment levels higher than in flies carrying either mutation alone (bars 3A, 3B and 2A). Trans-heterozygotes that are mutant for both *Su(var)3-9* and *Hdac1* also have eye pigment levels greater than flies carrying only single mutations to either gene (bars 4A, 4B and 2A). However, the effects of having single mutations to both *Su(var)3-9* and *Hdac1* appear to be additive, rather than synergistic, since flies that are trans-heterozygous for both a single *Su(var)3-9* mutation and a single *Hdac1* mutation do not have eye pigment levels that are higher than flies homozygous for two *Su(var)3-9*O6A mutations (bars 4B and 2B).
Appendix Four: Fertility of various mutant flies, measured as fecundity in females.

Overview:
Since telomere maintenance is believed by many to be related to genome stability, gametogenesis, and other aspects of biology, I determined the relative fertility of various mutant fly strains by measuring fecundity in female flies. The number of male and female progeny produced by females carrying various mutations was determined. The ratio of male to female offspring was also determined.

Materials and Methods:
Fecundity of females was measured by mating ten virgin females (aged one week) with ten males in standard vials, containing standard sucrose/cornmeal media, in quadruplicate. Flies were allowed to lay eggs in vials for two days, and then transferred to fresh vials three times, in order to avoid overcrowding of the offspring. The number of male and female offspring were counted and tabulated.

Males and virgin females were collected from these mating experiments, and used in the longevity test (Appendix Six). Thirty males and thirty virgin females were transferred into vials, in quadruplicate, for a total of 120 flies, of each sex, in all. Flies were then transferred to fresh vials every two days, and the number of dead flies was counted and tabulated. Survival curves were then plotted using SoftMax Pro™ software, specifically designed for plotting longevity and survival curves.

Results:
With the exception of Su(var)3-9O6A, Su(var)3-9 and Hdac1 mutants are not homozygous viable, and are maintained in a w; TM3 genetic background. Hence, w and w; TM3 were included as controls, as were the Su(z)25 strain, which strongly suppressed TPE, and the Gaiano strain, which is known to have abnormally long telomeres (Siriaco et al.2002).

Results (Figures 1 and 2) indicated that, with the exception of the Su(var)3-9I strain, all Su(var)3-9 and Hdac1 mutant females tested produced significantly fewer male and female offspring ( p < 0.001). Sex ratios of offspring were also determined. Only in the case of Hdac1326 was the ratio of male to female offspring significantly less than one ( p < 0.001), indicating that this mutation may effect gametogenesis, or other processes that determine the sex of offspring.
Figure 1. Average number of offspring produced by various mutant females.

Bars represent the average number of offspring produced per mutant female. Error bars represent standard deviations. Three different Su(var)3-9 mutants are shown, as well as one mutant strain of Hdac1. Su(var)3-9 and Hdac1 mutants are not homozygous viable, and are maintained in a w; TM3 mutant background. w and w; TM3 were included as controls for the genetic background. The Su(z)25 strain, which strongly suppresses TPE, and the Gaiano strain, which has abnormally long telomeres, are also shown. Results indicated that Su(var)3-9^{O6A}, Su(var)3-9^{330}, Su(var)3-9^{309}, and Hdac1^{326} mutant strains produced significantly fewer offspring than wild-type flies (p < 0.001), while the other mutant strains did not. Results from the Su(z)25 strain were of marginal significance.
Figure 2. Average number of offspring per female, expressed as a percentage of wild-type.

Bars indicate the average number of male and female offspring produced by various mutant female flies, when compared to wild-type. Error bars represent the standard deviation. Only in the case of $Hdac^{362}$ was the ratio of male to female offspring significantly less than one ($p < 0.05$).
Appendix Five: Determination of median lifespan and longevity for various mutant flies.

Overview:
Since telomere length and telomere maintenance are believed by many to be related to either genomic stability, or longevity, I determined the median lifespan for various mutant flies. The results presented in Appendix Four suggest that certain Su(var)3-9 and Hdac1 mutant flies have greater numbers of HeT-A elements in their genomes, and since most HeT-A elements are believed to reside at the telomeres, this infers that these mutant strains may have longer telomeres. Since telomere length is believed by many to be related to lifespan, I measured the median the lifespans of various mutant fly strains, including Su(var)3-9 and Hdac1 mutant flies.

Materials and Methods:
Fecundity of females (Appendix Five) was measured by mating ten virgin females (aged one week) with ten males in standard vials, containing standard sucrose/cornmeal media, in quadruplicate. Flies were allowed to lay eggs in vials for two days, and then transferred to fresh vials three times, in order to avoid overcrowding of the offspring. The number of male and female offspring were counted and tabulated.

Males and virgin females were collected from these mating experiments, and used in the longevity test. Thirty males and thirty virgin females were transferred into vials, in quadruplicate, for a total of 120 flies, of each sex, in all. Flies were then transferred to fresh vials every two days, and the number of dead flies was counted and tabulated. Survival curves were then plotted using SoftMax Pro™ software, specifically designed for plotting longevity and survival curves.

Results:
The median lifespan of various mutant fly strains was measured and expressed as a percentage of wild-type. The median lifespan of flies with abnormally long telomeres (Gaiano strain) was not found to be significantly different from that of wild-type flies. All other mutant strains showed significantly shorter median lifespans than wild-type (p < 0.001). Su(var)3-9 and Hdac1 mutant flies are kept in a w; mutant/TM3 mutant background, and w and w; +/-TM3 median lifespans were determined to estimate the effects of the genetic background on the median lifespan of Su(var)3-9 and Hdac1 mutants. All Su(var)3-9 and Hdac1 mutant strains had a significantly shorter median lifespan than the w and w; +/-TM3 strains (p < 0.05). Thus, while the results presented in Appendix Four suggest that Su(var)3-9 and Hdac1 mutant fly strains may have longer telomeres, it is highly unlikely that longevity is increased in either the Su(var)3-9 or Hdac1 mutant fly strains.
Figure 1. Median lifespan of various mutant flies, expressed as a percentage of wild-type.

The median lifespan of various mutant fly strains is expressed as a percentage of wild-type. Error bars represent standard deviations. The median lifespan of flies with abnormally long telomeres (Gaiano strain) is not significantly different from that of wild-type flies. All other mutant strains show significantly shorter median lifespans than wild-type (p < 0.001). Su(var)3-9 and Hdac1 mutant flies are kept in a w-; mutant/TM3 mutant background, and w- and w-; +/TM3 median lifespans were determined to estimate the effects of the genetic background on the median lifespan of Su(var)3-9 and Hdac1 mutants. All Su(var)3-9 and Hdac1 mutant strains had a significantly shorter median lifespan than the w- and w-; +/TM3 strains (p < 0.05).